UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Agronomy, Food, Natural Resources, Animal and Environment

(DAFNAE)

PhD COURSE OF CROP SCIENCE

CYCLE: XXXV

Development of superior yeast strains for the production of bioethanol from multiple waste streams: a combination of classical and modern genetic approaches

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An expert is a person who has made all the mistakes that can be made in a very narrow field

- Neils Bohr

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Abstract

Climate change is demanding a reduction in the usage of fossil fuels and shifts to cleaner and greener alternatives. The abundance of agricultural waste streams suggests that this material could be considered a renewable resource for biofuel production. Amongst all agricultural products, rice is one of the most largely grown crops, and more than 4.8 % of the total production goes to waste at different stages from farm to fork. This study was intended to review all the rice waste streams including starchy and lignocellulosic residues to convert them biotechnologically into liquid (bioethanol, biobutanol, biodiesel), and gaseous (biogas, biohydrogen) biofuels, through the use of pure or mixed microbial cultures. Importantly, the global accessibility of each rice byproduct like rice husk, rice straw, broken rice, discolored rice, and unripe rice, has also been investigated. One of the focuses of the study was to identify and establish the potential of rice waste as a 'fuel farm' with detailed attention given to bioethanol. The physical, chemical, enzymatic, or microbial pretreatments were studied in depth as their role in making carbon available for enzymatic hydrolysis and fermentation plays a key role. Although much research has already been done, further investigation is still required for the upscale of the process for industrial commercialization. Moreover, future process integrations will open the platform to biorefinery schemes where rice waste streams can be processed into multiple biofuels and other added-value products, towards the full exploitation of the feedstocks and the economic and environmental sustainability of the overall process.

Considering this scenario, unripe rice, the least explored rice mill byproduct, was employed for the integrated biofuel production approach. 1 L scale fermentation trials were carried out for the first time using unripe rice as a substrate. A comparison of enzymatic hydrolysis between recently developed recombinant Consolidated Bioprocessing (CBP) strains was performed and the most efficient recombinant yeast *S. cerevisiae* ER T12.7 showed outstanding performances with hydrolysis similar to those of the commercial enzymatic cocktail STARGEN^{TM} 002. Another CBP strain, *S. cerevisiae* ER

T12 showed great hydrolysis yields. Similar results were observed during the fermentation experiment where ER T12.7 showed the highest ethanol yield from 20% w/v unripe rice. In a biorefinery context, the spent CBP fermentation slurries from ER T12.7 and ER T12 were then distilled to remove ethanol, and further exploited for biogas production, both yielding 372 and 370 mL CH_4/g VS, respectively. With this integrated approach, maximum carbon was utilized to produce liquid and gaseous biofuel which helped to make its production commercially viable.

While unripe rice is an agricultural byproduct, other wastes are generated on daily basis, such as domestic residues, that could be considered as another source of carbon with the potential to be transformed into biofuel. Therefore, for the first time, the organic fraction of municipal solid waste (OFMSW) was employed for biofuel production using highly efficient amylolytic CBP yeast. The OFMSW sample (separated at source) was hand segregated to obtain the compositional structure of different fractions. Attention was also given to seasonal variation, mainly for bread and pasta fraction, and winter conditions were simulated during the fermentation of OFMSW to ethanol by the efficient amylolytic CBP strain ER T12.7, which was then compared to the non-amylolytic parental strain ER V1. As expected, ER V1 could hardly produce any ethanol from available free sugars while a high titer of ethanol was produced by ER T12.7. As the concentration of starch increased, higher productivity was achieved by ER T12.7. The location of the rice mill here considered which is in the proximity of the OFMSW management site encouraged to co-ferment discolored rice, an underutilized rice mill byproduct, along with OFMSW. Co-fermentation of these feedstocks originated through different waste streams, enhanced the starch content and eventually the ethanol productivity up to 0.69 g/L/h after 96 h. HPLC analysis revealed the presence of VFAs which might affect the fermentation. Hence, the need to develop an inhibitor-tolerant yeast strain was emphasized.

In order to obtain inhibitor-tolerant yeast, a non-GMO approach was chosen taking advantage of yeast classical genetics. The focus of the study was to prove if variation generated during a cross-over

event of meiosis in yeast can bring about phenotypic variation to produce new industrially important strains. Three yeast from different locations and origins were sporulated. Monosporal colonies (MSC) were obtained by developing a new ascospores separation protocol and about 100 colonies from each parental strain were tested for their growth kinetics in the absence and presence of a specific stressing agent, sequentially, to funnel down numbers using statistical tests. Finally, 6 strains from each parental strain were tested in the presence of different concentrations of a specific stressing agent, and Fourier-transform infrared (FTIR) spectroscopy was performed to obtain a metabolic profile of each MSC and parental strain. It was evident that a significant variation was produced among MSCs of each parental strain, indicating that the classical genetics can in the future be exploited to obtain a natural variety of yeast strains with industrial traits.

After studying all these underutilized rice waste, it is clear that rice can efficiently be converted into ethanol. Furthermore, secondary biofuel can be produced from spent fermentation broth under an integrated approach to develop an effective biorefinery process. Combination of rice waste with domestic or industrial waste can enhance the process dynamics and possibly, ethanol yield. However, the efficiency of the entire process depends mainly upon the strain of the organism to be employed. More specifically, what is needed as key step to control the economy of the process, is an organism producing starch and cellulose hydrolyzing enzymes and, simultaneously, capable of fermenting those sugars generated under CBP. All this will presumably represent a sound contribution to the ecological transition that foresees a gradual step from fossil fuels to biofuels, also helping to wastes eco-disposal, thus safeguarding the environment we live in.

Riassunto

I cambiamenti climatici oggi in atto suggeriscono il progressivo abbandono dei combustibili fossili a favore di alternative più pulite ed eco-compatibili. I rifiuti/residui di origine agro-industriale rappresentano un potenziale substrato di partenza per la produzione di biocombustibili. In particolare, il presente studio prende in considerazione il riso, una delle coltivazioni agricole più diffuse, che in vari punti della filiera, dal pieno campo fino al prodotto alimentare finito, produce una notevole quantità di materiale di scarto che si aggira intorno al 4.8% del totale. L'indagine si propone di studiare i vari streams che comprendono scarti sia cellulosici che amilacei, quali "lolla di riso", "paglia di riso", "riso rotto", "riso macchiato", "grana verde", "farinaccio di riso", da trasformare biotecnologicamente in carburanti liquidi (bioetanolo, biobutanolo, biodiesel) e gassosi (biogas, bioidrogeno) per il tramite di specifiche colture microbiche pure o miste. Oltre alla disponibilità e all'accessibilità dei vari sottoprodotti, si è dedicata particolare attenzione al bioetanolo come principale prodotto ottenibile. Sono stati anche presi in esame i possibili trattamenti chimici, fisici, enzimatici o microbici da applicare al materiale di partenza al fine di rendere più disponibile la componente carboniosa all'idrolisi enzimatica e alla successiva fermentazione, per passare poi agli studi di up-scaling e marketing. Inoltre, studi di integrazione di processi consentiranno la realizzazione di una vera e propria bioraffineria nella quale dai residui del riso sarà possibile ottenere biocarburanti diversi e altri prodotti a valora aggiunto, con l'obiettivo di raggiungere la piena sostenibilità dell'intero processo.

Per l'approccio integrato della produzione di biocarburanti è stato utilizzato come substrato riso di scarto immaturo chiamato "grana verde", in fermentatore da 1L per la prima volta. Il confronto tra l'idrolisi enzimatica a carico di due lieviti ricombinanti (CBP) di recente realizzazione ha consentito di selezionare il ceppo S. cerevisiae ER T12.7 che ha mostrato capacità idrolitiche simili a quelle ottenute tramite l'uso del cocktail enzimatico STARGENTM 002. Il medesimo ceppo ha inoltre fornito la migliore performance anche in termini di etanolo ottenuto dallo stesso substrato con concentrazione

ottimale di 20% w/v. In successive sperimentazioni mirate alla bioraffineria, il residuo esausto di fermentazione CBP a carico dei due ceppi ricombinanti veniva distillato per rimuovere l'etanolo e quindi utilizzato come substrato per la produzione di biogas, che alla fine veniva liberato in ragione di 372 (ER T12.7) e 370 (ERE T12) mL CH_4/g VS. Questo approccio integrato, adottato per la prima volta ha consentito di utilizzare la massima quantità di carbonio disponibile nel substrato per la produzione simultanea di un biocarburante liquidi e uno gassoso, aumentando così le possibilità future di commercializzazione.

A fianco del riso immaturo "grana verde", prodotto secondario dell'agricoltura, i rifiuti domestici quotidiani possono rappresentare un'altra fonte di carbonio potenzialmente trasformabile in biocarburante. Per la prima volta la frazione organica dei rifiuti solidi urbani (OFMSW) è stata impiegata per la produzione di biocarburanti tramite l'uso di lieviti amilolitici CBP altamente efficienti. Il materiale è stato separato manualmente per indagare sulla composizione delle diverse frazioni. È stata verificata la variabilità in termini stagionali, soprattutto sul contenuto in pane e pasta, e simulate le condizioni invernali durante la fermentazione degli OFMSW ad etanolo confrontando il miglior lievito CBP selezionato ER T12.7 con il ceppo parentale non-amilolitico ER V1. Come ci si attendeva il ceppo parentale non ha prodotto etanolo mentre il ceppo ricombinante ne ha prodotto con alta efficienza, aumentando la produzione all'aumentare dell'amido presente.

La presenza di un impianto molitorio in prossimità del sito di raccolta degli OFMSW considerato in questo studio ha incoraggiato la sperimentazione di co-fermentazioni anche con riso macchiato, un altro prodotto secondario della macinatura del riso attualmente sottoutilizzato. La co-fermentazione di questi materiali misti ha consentito anche di aumentare la produttività in etanolo fino a 0.69 g/L/h dopo 96 ore. Le analisi HPLC hanno mostrato la presenza di VFAs che potrebbero influenzare negativamente la fermentazione, evidenziando perciò la necessità di sviluppare lieviti tolleranti a tali inibitori.

Al fine di ottenere ceppi tolleranti si è partiti da un "non-GMO" seguendo le linee della genetica classica dei lieviti. In pratica, l'obiettivo di questo studio era quello di stabilire se variazioni fenotipiche verificatesi a seguito di eventi di crossing-over meiotici nei lieviti producessero ceppi nuovi e di maggiore interesse industriale. Tre ceppi di lievito di diversa origine venivano indotti alla sporulazione e colonie di monosporulanti (MSC) ottenute grazie allo sviluppo di un nuovo protocollo di separazione delle ascospore. Circa 100 colonie per ciascun ceppo parentale venivano saggiate, in sequenza, per le loro cinetiche di accrescimento sia in presenza che in assenza di specifici agenti di stress. Alla fine, sono stati selezionati sei ceppi per ciascun parentale, saggiati poi a diverse concentrazioni dello specifico agente di stress e al termine, mediante FTIR, sono stati ottenuti i profili metabolici di ciascun ceppo. È così risultato evidente che tra le MSCs di ciascun ceppo parentale si sono prodotte variazioni significative e che la genetica classica potrà essere in futuro utilizzata per sviluppare ceppi di lievito interessanti dal punto di vista industriale.

Lo studio di questi sottoutilizzati prodotti di scarto della lavorazione del riso ha dimostrato alla fine che questo materiale può essere efficacemente convertito in etanolo e che il residuo esausto di fermentazione può anch'esso essere utilizzato, secondo un approccio integrato, per ottenere un secondo biocarburante ponendo così le basi per una vera e propria bio-raffineria. La combinazione dei sottoprodotti del riso con i rifiuti solidi urbani o industriali potrà verosimilmente migliorare la dinamica del processo ed eventualmente anche le rese produttive in etanolo. L'efficienza dell'intero processo dipende dal ceppo microbico CBP che dovrà essere equipaggiato con enzimi cellulosolitici e amilolitici, nonché con capacità fermentative a partire dagli zuccheri generati.

Tutto ciò potrà rappresentare un buon contributo alla transizione ecologica che prevede un progressivo passaggio dai combustibili fossili ai biocarburanti, contribuendo nel contempo allo smaltimento eco-compatibile dei rifiuti e preservando in ultima analisi l'ambiente in cui viviamo.

State of the art

1 Bioethanol: an alternative liquid fuel

The demand for goods, which is rising with the rapid growth of the world population, needs a similar increase in available energy for its production. Moreover, transportation is one of the most important aspects to suitably support this progress as it fulfills those demands from source to destination. In Europe, 95% of energy consumption for transportation is based on oil-derived fuels (European Environmental Agency, 2020). This sector is not only responsible for 60% of global oil consumption, but also contributes 21% of total CO_2 emissions to the environment. Regionally, the highest growth in oil consumption was recorded in the USA (1.5×10^6 barrels/day), China (1.3×10^6 b/d) and the European Union (5.7×10^5 b/d) (*bp Statistical Review of World Energy*, 2022). It is estimated that reserves of fossil fuels will be exhausted completely between 2069 and 2088 (Ishika et al., 2017).

The higher greenhouse gas (GHG) emission is directly proportional to the higher use of fossil fuels. The release is expected to rise by 50% in the coming years as compared to 2011, with projections that atmospheric concentrations of GHG may rise to 685 parts per million (ppm) in 2050. Being those ascribed to the group of GHG, higher atmospheric concentration will result in an increased global average temperature of 3° to 6 °C. The strategies for controlling global average climate change within 2 °C, as pledged in Cancún Agreements at United Nations in 2010, were not sufficient ("OECD environmental outlook to 2050: the consequences of inaction," 2012). This shortcoming was well answered in the Paris Agreement of 2015. Wherein, 196 countries were legally bound to reduce GHG release to control global average temperature rise within 2°C, preferably 1.5°C, compared to pre-industrial levels (United Nations, 2015).

In addition to climate change, national energy security has become essential for every oil-importing country. The highest oil is produced in the Middle East and North American region (bp Statistical Review of World Energy, 2022). It was evident in the first and second quarters of 2022 that energy dependency on a group of countries responsible for deciding oil prices and production can globally affect economies. In the summer 2021, Eurozone economies plotted 17.4% inflation in the energy sector (Popkostova, 2022). This creates the necessity of searching for alternative fuels that are not of fossil origin and more environmentally friendly.

Amongst alternatives for fossil fuels, the so-called "biofuels" are destined to become a key solution for future requirements. By definition, a biofuel is a fuel made from plants or other biological materials (Ruan et al., 2019) Agricultural biomass can definitely stand out as a budding resource for the production of biofuels. Importantly, the use of biofuels is the best strategy to mitigate pollution as they cause a

significant decrease in nitrogen oxides (NO_X), particulate matter (PM), and sulfur oxides (SO_X). Brazil, as an example, makes up 25% of its transportation using biofuel, which is the largest in the world, and further aims to make it 32% by 2030. This approach is aimed to achieve a reduction in the carbon intensity, a measurement of amount of CO2 released per unit of power generated (Xiao et al., 2020). due to transport fuel by 10% and avoid the release 620 million tons of CO_2 (equivalent) by 2030 (IEA Bioenergy, 2021). This should control pollution and also arrest (or at least reduce) global climate change. Moreover, socioeconomic analyses showed that the development of the bioethanol industry in emerging countries (e.g. Zambia) can potentially increase economic growth without adversely affecting national food security (Hartley et al., 2019). Furthermore, as biomass is a cheap and abundant resource for biofuel production, it also helps to strengthen national energy security and reduces dependence on oil-producing countries.

Many vehicles running on the streets use traditional internal combustion engines (ICE) which need liquid fuel. Hence, bioethanol is the most suitable biofuel which can cater to existing as well as upcoming vehicles. Brazil is now planning for flex fuel vehicles (FFV) which can use E100, i.e. 100% bioethanol as fuel (IEA Bioenergy, 2021).

Emission studies showed up to 70% reduction of GHG when ethanol was blended with traditional gasoline (Saini et al., 2010). When multiple gasoline samples were collected from petrol pumps of each country and analysed for ethanol content, blending as per regional blending policies (Table 1.1), no consistency in blending was observed, except for Brazil and India (Table 1.1). Gasoline causes the release of 10-45 ppm of sulfur that could be reduced upon higher ethanol blending (Abel et al., 2021). Moreover, ethanol has high oxygen content, hence providing a greater lean burning ratio in ICE than gasoline and it burns completely, thus reducing the formation of carbon monoxide (CO) and other volatile organic compounds (Panahi et al., 2019). On the other hand, the use of blended fuels increases the emission of reactive aldehydes, like acetaldehyde and formaldehyde as well as ketones, thus producing great environmental concern. Nevertheless, the solution to these problems lies in esterification, since esters such as isobutyl acetate, n-butyl acetate, and methyl acetate, increase the octane number and reduce the emissions of aldehydes and ketones (Dabbagh et al., 2013).

Table 1.1: Fuel blending programs adopted by some countries (Abel et al., 2021; Khatiwada et al., 2016)

Country	Feedstock	Percentage of Ethanol in gasoline[b]	Remarks[c]
Brazil	Sugarcane	27	FFV in Brazil are engineered to operate on E100 as well as E85
China	Corn	10	There was no consistency of blending in all the samples tested. Some of them show no blending
Japan	Sugarcane[a], corn[a]	2.5	3% of the blending is allowed. Negligible or no ethanol was observed in many samples
India	Sugarcane	10	E20 blending is beginning in April 2023 with full implementation by 2025
Mexico	un	0.4	Banned blending in some bigger cities which are largest gasoline markets
South Korea	un	un	No ethanol is produced in the country. The feasibility studies for blending of ethanol are under review.
Canada	Corn, wheat	6.6	Nearly half of ethanol is imported from the United States
Indonesia	Sugarcane	-	Despite blending mandates, no ethanol detected in gasoline samples

FFV- flex fuel vehicle, [a] - Ethanol obtained (imported) from the mentioned feedstocks, [b] - Average, [c] - Based on multiple samples collected and analysed from each country, un- Unknown

Ethanol has some characteristics which provide a better edge than traditional gasoline. It offers broader flammability, higher octane number, greater flame speeds, and heats of vaporization as a consequence it provides a greater compression ratio, a shorter burn time, and a greater lean burn in ICE, when compared to gasoline (Table 1.2). Octane number measures indicate anti-knock properties of fuels, namely the susceptibility of fuel to explode due to premature burning in ICE reduces as the octane number increases. However, ethanol has only 65% of the energy density of gasoline, lesser vapor pressure and flame luminosity. Lesser vapor pressure makes 'cold starts' difficult, but blending ethanol with gasoline could help solving this problem (Panahi et al., 2019).

Table 1.2: Comparison of fuel properties of gasoline and ethanol (Panahi et al., 2019)

Parameters	**Ethanol**	**Gasoline**
Boiling Point (°C)	78.37	100-400
Composition, weight %C	52.2	85-88
Density (kg/m^3)	789	719-760
Ignition temperature (°C)	365	247-280
Thermal expansion coefficient (K-1)	$11.2x10^{-4}$	$9x10^{-4}$
Viscosity (m^2/s)	$14x10^{-7}$	$5x10^{-7}$
Flammability limits (vol %)		
lower	4.3	1.4
higher	19	7.6
Calorific Value (MJ/kg)	29.7	46.7
Air-fuel ratio (kg/kg)	9	14.7
Heat of vaporization (MJ/kg)	0.92	0.36
Research octane No.	108.6	90-98

Considering its characteristics, ethanol, can be easily produced by fermentation processes, it is highly miscible with fossil gasoline and hence it is a good blending chemical. Since 2008, the global production of fuel alcohol has increased tremendously. USA is the world leader in fuel ethanol production, and in 2021 maintained its position by producing 55% (56,781 million liters) of the world's fuel ethanol, while Brazil is ranked second with 27%. Table 1.3 indicates the variations in fuel ethanol production from 2008 to 2021. Most of the countries increased fuel ethanol production (+77% for Thailand in 2021) except China, which showed a 17% decrease, probably related to the emergence of COVID-19 pandemic in this country. Overall, world fuel ethanol production has increased 33% in 2021 as compared to 2008 (Table 1.3), indicating a tangible worldwide acceptance of fuel ethanol.

Table 1.3: Comparison of global fuel ethanol production from 2008 to 2021 (Flach et al., 2022; RFA 2022, 2022)

Country	2008	2021	Increase
	(million liters)		(%)
USA	34,068	56,781	40
Brazil	24,500	28,390	14
China	3,800	3,255	-17
European Union	2,777	4,921	44
of which			
France	1,000	1,201	9
Germany	568	747	19
Spain	317	487	35
Sweden	78	na	
Italy	60	na	
India	1,900	3,104	39
Canada	900	1,665	46
Thailand	340	1,476	77
Other	886	3,785	77
Total	**71,194**	**1,05,812**	**33**

na- numbers not available

The fermentation process is very well known since centuries for different alcoholic beverages. The process exploits microorganisms as the catalysts to obtain alcohol from grapes and malt from grains. The same approach of fermentation can be applied to produce fuel ethanol. Microorganisms can be considered potent and flexible cell factories, competent in metabolizing raw materials and producing high-value substances at the industrial level.

Since microorganisms are characterized by a huge diversity in terms of phenotypic characters, the selection of the microorganisms most suitable for the desired process is the bottleneck in the fermentation process. While yeast is the most suitable and hence industrially acceptable microorganism for bioethanol production, the selection of an inexpensive yet easily fermentable substrate is another obstruction.

2 Feedstocks for bioethanol production

Bioethanol, also referred to as fuel ethanol can be obtained from a variety of different feedstocks ranging from simple sugars to complex biopolymers like cellulose. Depending upon the feedstock used for its production, fuel ethanol can be categorized into first- or second-generation bioethanol. First-generation bioethanol refers to the ethanol produced from sugars or starchy feedstocks (i.e. sugar cane molasses, beet, corn, wheat etc.), while second-generation bioethanol is obtained from cellulosic biomass as substrate.

2.1 Sugars: a simple source of bioethanol production

Sugarcane is the main feedstock for bioethanol production. 90% of the total sugar of the crop is sucrose (Dhaliwal et al., 2011). Cane juice and molasses (by-products of the sugar industry) are the sources of simple sugars which can be easily converted into ethanol. In Brazil, only 21% of the ethanol is produced using cane molasses while 79% comes from fresh cane juice (Wilkie et al., 2000). Although molasses is a byproduct, it contains a total sugar concentration of about 50%, which makes it the most important non-food raw material for bioethanol production (Wu et al., 2020). India is the second-highest sugar producer and mainly utilizes sugarcane molasses as a feedstock for ethanol production. In addition, beet molasses is another possible option as a substrate for ethanol fermentation (Haq et al., 2016). Beet molasses was tested for scale up assays at 100 L ethanol fermentation achieving 85% ethanol yield (Beigbeder et al., 2021).

Sweet sorghum is considered to be an alternative to sugarcane as its stems have as much sugar as sugarcane. Also, sweet sorghum has some additional benefits over sugarcane like its drought and water logging resistance and saline–alkaline tolerance (Ratnavathi et al., 2010).

As discussed in section 1, the yeast, *Saccharomyces cerevisiae* is the most trusted and industrially accepted microorganism owing to its capability to hydrolyze sucrose, the main sugar in sugarcane, into glucose and fructose, two easily assimilable hexoses. Furthermore, it produces ethanol with stoichiometric yields and has a tolerance to a wide variety of inhibitors as well as elevated osmotic pressure. Aeration plays a key role in growth and ethanol production by *S. cerevisiae*, indeed, although it can grow under anaerobic conditions, microaerophilic conditions are needed for the synthesis of fatty acids and sterols increasing the resistance of the yeast to the ethanol that it produces. Biochemically, sugars are converted to ethanol by yeasts in oxygen-limited conditions hence known as fermentation.

Based on present-day knowledge of pentose metabolism, several metabolic engineering strategies have been explored in laboratory strains of *S. cerevisiae* to ascertain their effect on the fermentation of xylose and arabinose. It was observed that the *S. cerevisiae* do not ferment xylose at economically feasible rates. Hence, respiration deficient strain of *S. cerevisiae* was engineered by deleting COX15 gene. The strain can ferment xylose aerobically to produce ethanol (Lee et al., 2021). Among the yeasts *Pichia stipites* showed production of ethanol from xylose, present in lignocellulosic hydrolysate of plywood, bagasse and bamboo (Lin et al., 2016).

Schizosaccharomyces pombe and *S. cerevisiae* Fm17 are the yeast offering added advantages such as tolerance to high osmotic pressure exerted by salt and sugar, respectively, along with higher solid content (Bullock, 2002; Favaro et al., 2013). A fermentation process using a wild strain of this yeast under high osmotic pressure has been patented (Carrascosa, 2006).

Many attempts were made to obtain ethanol from bacteria. The most promising microorganism was found to be *Zymomonas mobilis*, giving higher ethanol yields (up to 97% of the theoretical maximum). However, the variety of fermentable substrates for *Z. mobilis* was found narrow and the bacterium could utilize only glucose, fructose, and lactose (Claassen et al., 1999). During the use of sucrose or sugarcane syrup, the formation of the polysaccharide levan (made up of fructose units) and sorbitol was observed. This increases the viscosity of fermentation broth and decreases the efficiency of the conversion of sucrose into ethanol (Lee and Huang 2000).

Escherichia coli KO11 after multiple genetic modifications could convert glucose and xylose to ethanol at yields 103 to 106% of theoretical (Dien et al., 2003). Unfortunately, although bacteria gave higher yields, they can't tolerate increased ethanol concentration and osmotic pressure. Hence, yeast is preferred over bacteria.

2.2 Starch: a complex carbon source for ethanol production

Starch is the major reservoir of energy for plants. In plants, starch can represent 20 to 70 % of the dry weight and it's the most important source of energy for them (Table 1.4). Starchy crops are widely available across the world in different forms like cereals (60-80 % starch content), legumes (25-50 %), tubers, and roots (60-90 %). These starchy feedstocks can be used for the production of ethanol because of the simplicity of conversion, storage competency for a long period, and high ethanol yield. However, the ethanol yield varies upon the bioavailability of starch to hydrolyzing enzymes, which depends upon its structure, botanical origin, and crop hybrid. Moreover, starch content should not be considered as the benchmark for ethanol production per hectare as a higher ethanol yield of up to 5 kiloliters per hectare (kL/ha) was achieved using tuber crop of sweet potato with starch content of 14-28% (Table 1.4). On contrary, sorghum which possesses higher starch content of 68-71% has a potential ethanol yield of 1.1 kL/ha. The ratio of linear amylose (α-1→4 glyosidic linkages) and highly branched amylopectin (α-1→4 and α-1→6 glyosidic linkages) defines the structure and affects ethanol yield for a given starchy substrate. USA and Europe exploit cereals such as corn, wheat, or barley for ethanol production, while tropical countries rely on tubers (e.g. cassava). In plants, starch can represent 20 to 70 % of the dry weight and it's the most important source of energy for them (Table 1.4).

Table 1.4: Starch content and potential ethanol yields of selected crops, adapted from (Burešová et al., 2010; Zabed et al., 2017)

Crops	Starch content (%)	Potential ethanol yield (kL/ha)	Crop type
Barley	63-69 [a]	uk	Cereal
Oat	66 [a]	uk	Cereal
Triticale	62-71 [a]	uk	Cereal
Sweet Potato	14-28 [b]	2-5	Tuber
Cassava	35 [b]	5	Tuber
Sorghum	68-71 [a]	1.1	Cereal
Jerusalem artichoke	15 [b]	2-6	Tuber
Potato	73 [a]	1.5	Tuber
Wheat	65-76	1-2	Cereal
Yam	20-40 [b]	5	Tuber
Corn	70-72 [a]	4	Cereal
Rice	88 [a]	uk	Cereal

[a]- dry weight, [b]- wet weight, kL/ha- kiloliter/hectare, uk- unknown

Rice is one of the most important crops in the world and 1000 teragrams (Tg) of rice were produced globally in 2018. Rice contains around 88 % (dry w/w) starch (Table 1.4) which makes it suitable as starchy feedstock for the production of ethanol, even because out of this huge production around 5% of grains goes to waste (Gupte et al., 2022). Rice starch wastes mostly include rice bran (RB), broken rice (BR), unripe rice (UR), and discolored rice (DR). The detailed availability and bioethanol production from each starchy rice waste is given in Table 1.5. A total of more than 40 Tg of bioethanol could be produced from available rice starchy waste feedstock (Table 1.5)

Table 1.5: Bioethanol potential of rice starchy feedstock. Adapted from (Gupte et al., 2022)

Rice starchy feedstock	Starch (% dry weight)	Bioethanol potential (Tg)
BR	77.7	16.0
DR	84.6	2.9
RB	29.6	11.5
UR	68.6	9.9

BR-broken rice, DR-discolored rice, RB- rice bran, UR- Unripe rice

Corn is another cereal with high starch content (Table 1.4) that ranges around 70-72 % (dry w/w). For instance, ethanol production in the USA is entirely dependent upon corn. Starch is extracted from corn and then enzymatically hydrolyzed to produce glucose which in turn is fermented into ethanol. Before enzymatic hydrolysis and fermentation to ethanol, corn is treated either by dry or wet milling. Dry milling involves less capital investment and results in higher ethanol production, while wet milling

allows the separation of different added-value components, making it more capital-intensive. Owing to this basic difference, the production of ethanol by dry milling maximizes capital return per gallon of ethanol than wet milling (67% vs 33%) (Bothast et al., 2005).

The capital-intensive wet milling process involves the separation of starch, fiber, gluten, and germ. Starch is converted into ethanol while residual fractions are sold separately. In the dry milling process, the clean corn is milled to obtain fine particles and mixed with water to form a slurry. The slurry is then cooked at 85°C during the gelatinization step, and pH is adjusted to 6.0. In the next step of liquefaction, amylolytic enzymes such as α-amylase are added to reduce polymerization thus contributing to reduce the viscosity of the reaction mixture. Cooking of the mash is continued at 110-150°C in a pressurized vessel for an hour. During the saccharification step, the mash is cooled down to 60-70°C, and the pH adjusted to 4.5. Glucoamylase enzymes are then added to break oligo-saccharides into glucose. The resulting glucose syrup is then used for ethanol fermentation while the remaining un-fractionated grains called Distillers Dried Grain and Soluble (DDGS) can be utilized as animal feed (Cardona et al., 2007; Lennartsson et al., 2014).

Wheat is one of the most demanded cereals which contains about 65-76% starch (Table 1.4). It can be processed similarly to corn to obtain ethanol, but efforts are still ongoing to optimize the fermentation conditions and costs of the process. For example, Wang et al. (1999) optimized the temperature and specific gravity of the fermentation process (Wang et al. 1999), while Soni et al. (2003) found optimum conditions of enzymatic starch hydrolysis of wheat bran by a solid-state fermentation process. Using a metagenomics approach, Rajabi et al. (2022) isolated an endo-1,4-xylanase gene (*xyn-2*) from camel rumen encoding for an enzyme hydrolyzing 100% of wheat bran. During simultaneous saccharification and fermentation (SSF), the product of *xyn-2* gene along with *Bacillus subtilis* AP gave significantly higher yields (Rajabi et al., 2022).

2.3 Lignocellulose: an abundant feedstock for ethanol production

The most abundant biomasses available on Earth are wood, tall grasses, forestry, and starchy-cellulosic crop residues. These materials create a huge variety of substrates suitable for bioethanol production. Conventionally, starchy substrates obtained from plants were used as important feedstocks for bioethanol production, but they represent a small fraction of the entire plant biomass which is mainly cellulosic. Hence, cellulosic energy crops can produce more biomass per hectare on land which is not used primarily for agricultural purposes. Second-generation biomass doesn't require any special land

mass and is largely available in the form of agro-forestry and agro-industrial waste. At the same time, the agro-forestry residues can create a major problem for the farmers. As an example, if the interval between two farming cycles is too short, farmers do not get sufficient time to take care of the farm residues and eventually end up burning those residues in farmland, thus creating air pollution (Gadde et al., 2009; He et al., 2016). GHG production can be arrested if the same farm residues could be converted successfully into bioethanol, providing farmers with additional income by selling this farm waste as a commodity.

Many lignocellulosic substrates have already been evaluated for the production of bioethanol. Generally, they can be categorized into six main groups: crop residues (cane bagasse, corn stover, wheat straw and bran, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp), hardwood (aspen, poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (switchgrass, reed canary grass, coastal Bermudagrass, timothy grass), and Municipal Solid Wastes (MSW). The compositions of the most important materials are reported in Table 1.6

Table 1.6: Common feedstocks for bioethanol production and their cellulose, hemicellulose and lignin content modified from (Chen et al., 2007; McKendry, 2002; Sun et al., 2002).

Lignocellulosic material	Feedstock Category	Cellulose	Hemicellulose	Lignin
Hardwood stems	Hardwood	40-50	24-40	18-25
Softwood stems	Softwood	45-50	25-35	25-35
Nutshells	Crop residues	25-30	25-30	30-40
Corn cobs	Crop residues	45	35	15
Wheat straw	Crop residues	33-40	20-25	15-20
Wheat bran	Crop residues	10-12	25-35	2-6
Rice straw	Crop residues	40	18	5-7
Cotton seed hairs	Crop residues	80-95	5-20	-
Grasses	Herbaceous biomass	25-40	35-50	10-30
Switchgrass	Herbaceous biomass	30-50	10-40	5-20
Coastal Bermuda grass	Herbaceous biomass	25	35	6-7
Newspaper	Cellulose waste	40-55	25-40	18-30
Paper	Cellulose waste	85-99	0	0-15
Waste paper from chemical pulps	Cellulose waste	60-70	10-20	5-10

The process design depends upon feedstock used as a substrate for bioethanol production. The process complexity intensifies as feedstock changes from simple sugars to complex biomaterials such as lignocellulose. Accordingly, simple sugar fermentation turns into a multi-stage process when feedstock changes to lignocellulose which demands for more process integration and increases the cost of

production. However, new research in this field is the key to reduce the cost of production by fine tuning the process integration and thus increasing the competitiveness of bioethanol. Especially in the case of lignocellulosic materials, the main limiting factor is the complexity of feedstock because cellulose is surrounded by hemicellulose and lignin (Figure 1). Thus, pretreatments are needed to break down lignin and obtain free cellulose and hemicellulose.

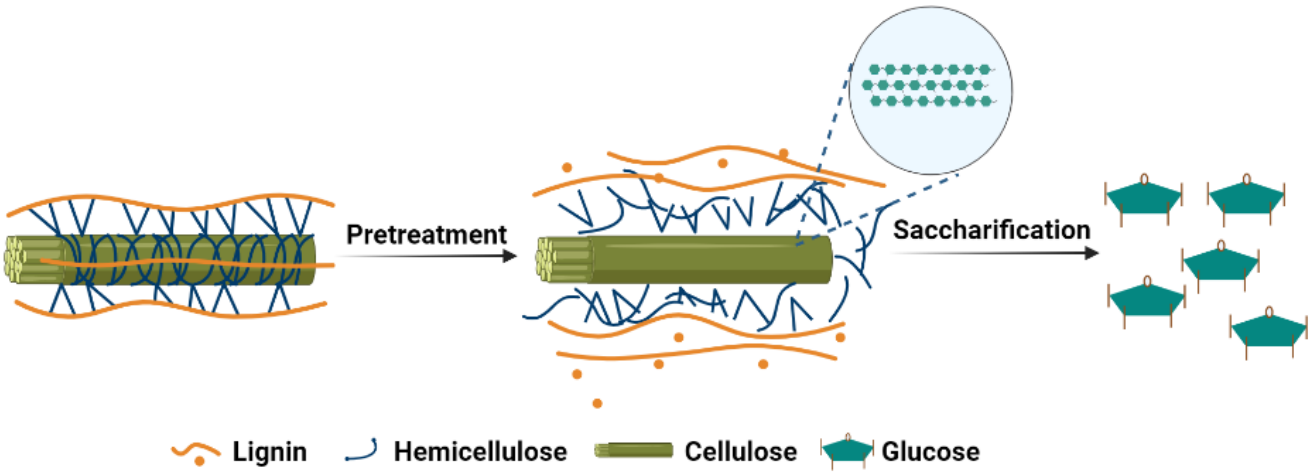

Figure 1: Structure of lignocellulose available in nature and the steps involved in obtaining fermentable glucose

3 Pretreatment of lignocellulosic biomass

The main challenge in the utilization of lignocellulosic feedstock is the pretreatment of biomass. This is one of the most important cost-determining steps for the conversion of biomass into biofuel. As shown in Figure 1, lignocellulose is a complex matrix and pretreatment helps to reduce the degree of its crystallinity. It also helps to increase the fractionation of amorphous cellulose, the most suitable form for successive enzymatic depolymerization. Cellulose hydrolysis is highly influenced by the available surface area. Ideal pretreatment presents several key properties (Yang et al., 2007):

- Low need for chemicals
- Use of less toxic and easily disposable chemicals
- Little or no loss of cellulose
- Adaptability to a wide range of biomass
- Minimum production of toxic compounds
- Recovery of metabolizable carbon
- and valorization of byproducts (lignin and its monomers)

There are three basic types of pretreatments: physical, chemical and enzymatic/microbial pretreatments. The choice of a single or a combination of various pretreatments depends upon the physico-chemical characteristic of the biomass under investigation.

3.1 Physical Pretreatments

Physical pretreatment mainly targets the reduction of crystallinity of cellulose and of particle size of biomass providing maximum surface area for enzymatic hydrolysis, Chipping, grinding and milling are the most common pretreatments. However, microwave and ultrasound methods are also used for some biomasses (Zhang et al. 2018). Generally, physical pretreatments are combined with chemical or microbial/enzymatic pretreatments. Grinding and milling are highly effective, although they are more energy-inefficient and require higher capital costs (Behera et al., 2014; Hendriks et al., 2009). Pyrolysis has also been considered as a physical pretreatment method as cellulose easily decomposes when it is exposed to elevated temperatures (Drapcho et al., 2020).

3.2 Chemical Pretreatments

Chemicals such as acids, alkalis, organic solvents and ionic liquids have been reported to break lignocellulosic biomass efficiently. Different concentrations of acid and alkali are used according to the structure of biomass. Moreover, a combination of both was found useful when applied in a stepwise manner (Kaur et al., 2017).

3.2.1 Acid pretreatments

Acid pretreatments have proven highly efficient in disrupting lignocellulosic matrix, attacking mostly on glycosidic bonds, leading to the transformation of polysaccharides into oligomeric and monomeric sugars (Hoon-Jung et al., 2015). Although the most affected fraction of biomass is hemicellulose, acids also act partially on cellulose and lignin, thus releasing carbohydrate oligomers (Jönsson et al., 2016). Even so, researchers are continuously working on this pretreatment which has been identified as the process that has the potential towards commercialization (He et al. 2014). Although acid pretreatment is effective for de-polymerization on hemicellulose and cellulose, many inhibitory compounds are produced from the decomposition of sugar and lignin, such as aldehydes, ketones and phenolic acids that are the main drawback of this process (Solarte-Toro et al., 2019)

The main process parameters studied in acid pretreatment are solids loading, acid concentration, temperature and residence time. Fine-tuning these parameters, the responses are measured in terms of the solids recovery, sugars yield, hemicellulose conversion, glucose, xylose and furfural concentration

for a wide variety of raw materials (López-Linares et al., 2013; Triana et al., 2011; Yoon et al., 2015). For pretreatment, concentrated and diluted acids were found to be useful. Diluted acids are preferred over concentrated ones, as the corrosive effect on pretreatment vessels and equipment is reduced. Also, lower amounts of inhibitors are produced during the process of hemicellulose hydrolysis (Wyman, 1996). inorganic acids such as sulfuric acid, but also nitric, hydrochloric, and phosphoric acids are largely employed at high temperature (180°C) for a limited time or at lower temperatures (120°C) for increased periods (30 to 90 min) (Mosier et al., 2005)

3.2.2 Alkali pretreatment

Alkali pretreatment acts mostly on lignin counterpart but also increases digestibility of cellulose by causing swelling of the structure and reducing the degree of polymerization and crystallinity. Alkali used for this pretreatment are sodium, potassium, calcium and ammonium hydroxides (Carvalheiro et al., 2008). As alkali destruct the bond between lignin and other polymers, the effectiveness of this process depends upon the lignin content of biomass. Alkali pretreatments are more effective on agricultural residues but have a limited effect on lignin-rich biomasses like softwood and hardwood (Kumar et al., 2009). Although, a combination of alkali with organic solvents like ethanol or methanol allows the solubilization of lignin (organosolv process), the cost of the process is too high, especially in view of commercial plants (Lynd, et al1999).

3.3 Biological pretreatment

Biological pre-treatment refers to the exploitation of fungal species capable of degrading lignocellulosic material. White-rot fungi, belonging to division Basidiomycota are capable of producing extracellular ligninolytic enzymes like laccase, manganese peroxidase, and lignin peroxidase. These fungal species are more tolerant to toxic compounds generated during the lignocellulosic breakdown, hence becoming potential candidates for pretreatment of lignocellulose (Cianchetta et al., 2014; Ellouze, 2016). Many white-rot fungi, including *Phanerochaete chrysosporium*, were observed to be predominantly selective in their substrate (Kumar et al., 2009; Sun et al., 2002). They primarily target lignin and hemicellulose, while leaving cellulose almost intact.

However, while this pre-treatment offers many significant advantages, such as low capital investment, no chemicals involvement and lesser energy requirement, the process is too slow for an efficient industrial applicability, due to a higher residence time of 10-14 days. Industrial application of this pretreatment would then require large space for operation and important efforts for careful and controlled growth conditions (Behera et al., 2014).

4 Inhibitors: effect, mechanism and detoxification

Pre-treatments help in increasing the bioavailability of lignocellulosic substrates to hydrolytic enzymes and microbes by breaking down the physical barrier that makes biomass recalcitrant to enzymatic hydrolysis. This allows a higher recovery of sugars in the following steps of bioethanol production process. Generally, harsh conditions are adopted for efficient pre-treatments which result in the formation of derivative byproducts which show inhibitory effects on microbial metabolism or on the efficiency of hydrolytic enzymes used during the saccharification step (Jönsson et al., 2016). The type and amount of inhibitors released during pretreatment is subjected to the chemical characteristics of each different substrate and the specific pre-treatment conditions used during the process. Inhibitors concentrations increase in case of pre-treatment procedures that involve recycling of process water, due to accumulation over a prolonged time.

Many pretreatments alter the structure of cellulose, without degrading it. Unlike hemicellulose, which is typically solubilized and partially degraded to oligomers of various lengths, lignin is normally broken down, but still, the majority remains intact. Most of the inhibitors are byproducts and derivatives formed by the degradation of lignin and sugars released from hemicellulose. Briefly, these molecules can be categorized into three major groups: furans, weak acids, and phenolic compounds (Palmqvist et al., 2000a).

4.1 Effect on microbial metabolism

4.1.1 Furans

Furfural and HMF, are the product of the dehydration of pentose and hexose sugars under acidic conditions, respectively (Jönsson et al., 2013). The formation of these inhibitors is at the expense of fermentable sugars which in turn reduce the final ethanol yield. Moreover, furfural and HMF are directly responsible for disturbing microbial metabolism. Furfural, a product of the Maillard reaction, acts on enzymes in glycolysis. Specifically, dehydrogenases are sensitive to furfural, which might be the reason for the inhibition of growth and ethanol production (Banerjee et al., 1981). Under the fermentative condition, *S. cerevisiae* yeast can survive to these inhibitors by altering furfural to less toxic furfuryl alcohol (Horváth et al., 2003) and HMF to 2,5-bishydroxymethylfuran (M. J. Taherzadeh et al., 2000), although the latter occurs at a lower rate than furfural conversion. However, furan aldehydes i.e. furfural can demonstrate inhibitory effects on the fermenting yeast at concentration as low as 0.1 mM (Larsson et al., 2000).

4.1.2 Weak acids

Hydrolysate of lignocellulosic biomass shows a large variety of weak acids out of which acetic, levulinic and formic acids are more prominent. Production of the weak acid depends upon the type of pretreatment. During severe pretreatments, HMF degrades into levulinic acid, while formic acid generates from furfural, in the same way. Acetic acid, on the other hand, is not a degradation product but it is released from the acetyl group of hemicellulose (Jönsson et al., 2016).

These weak acids inhibit cell growth by disturbing pH balance of cytosol. Undissociated weak acids enter into the cell cytoplasm and get dissociated so producing a proton inside the cell, and reducing the cytoplasmic pH as a consequence, ultimately resulting in cell death. Since the cells cope with this challenge by ATP-driven proton efflux pump (Stratford et al., 2013), the flux of energy toward cell biomass production is reduced.

4.1.3 Phenolics

Phenolics belong to the lignin monomers and their derivatives. During acidic pretreatments, multiplicity of phenolic compound is generated. Due to the great diversity among chemical species and low concentrations of these compounds, the identification and quantification of each compound are difficult. Although the mechanism of action is still unclear, few of phenolic compounds are known for producing reactive oxygen species (ROS).

Feltcher et al. (2019) reported that ferulic acid and 4-hydroxybenzoic acid induce cytoplasmic ROS in yeast, while coniferyl aldehyde-induced ROS confined partially to the mitochondria and, to a smaller extent, to the endoplasmic reticulum. In yeast Glucose-6-phosphate dehydrogenase enzyme (Zwf1) catalyzes the rate-limiting step of the pentose phosphate pathway. This is required for decreasing the accumulation of coniferyl aldehyde-induced ROS, possibly through the sequestering of Zwf1 to sites of ROS accumulation (Fletcher et al., 2019). In general, phenolics are accountable for the reduction of membrane integrity and the consequent permeabilization, as well as the change in protein-to-lipid ratio (Palmqvist et al., 2000b). *S. cerevisiae* can tolerate low concentrations of phenolics by modifying them into less harmful compounds (Larsson et al., 2000).

Despite the lower concentrations of phenolic compounds in hydrolysate, their ability to affect yeast growth is much higher. Consequently, fine-tuning the pretreatment method to produce lesser phenolics is essential. Although the production of phenolics depends upon intrinsic characteristics of feedstock, no general pretreatments can be designed for all the feedstocks.

4.2 Detoxification of pretreated lignocellulose

One possibility to avoid the formation of a significant concentration of inhibitors would be the selection of less recalcitrant feedstocks clubbed with mild pretreatment processes. However, mild pretreatment is not always suitable and lowering sugar yield just to get lower inhibitors during pretreatment step, is unacceptable as it is not economically sustainable. (Jönsson et al., 2013). Moreover, second generation bioethanol production should be designed to exploit a vast variety of available feedstocks as the chemical composition of feedstock varies often with environmental factors.

Washing the water-insoluble pretreated material is the easiest and most economical countermeasure to get rid of soluble inhibitors. However, this would result in the loss of massive amounts of sugars, as well as the need of processing large amounts of toxic wastewater. A number of detoxification processes has been designed to increase efficiency of hydrolysis of feedstocks, while minimizing the intrinsic costs incurred by the addition of one more processing step (Moreno et al., 2015).

Detoxification methods, also known as conditioning, can be sub-divided into three groups: chemical, physical, and biological.

4.2.1 Chemical detoxification

Acids are known to increase the solubilisation of hemicellulose and the digestibility of cellulose. This pretreatment reduces the pH to the level that fermenting organisms can't tolerate. The pH of hydrolysate in this case has to be raised to a range suitable for the growth of fermenting yeast (Pienkos et al., 2009). As explained in section 4.1.2, lowered pH arrests cellular functions, and by this mechanism, yeast can remain dormant (Lucena et al., 2020). Although the mechanism is not fully understood, an increment in pH results in reduced inhibiting material in the hydrolysate.

Activated charcoal is a well-studied adsorbent material which is at the basis of low-cost detoxification methods. Kamal et al. (2011) reported that when sago trunk hydrolysate was treated with 2.5% activated charcoal, reductions of 53% and 78% of furfural and phenolic compounds were observed with a retention time of 60 minutes (Kamal et al., 2011). The same adsorption strategy can also be performed using ion-exchange resins, as reported by De Arruda et al. (2011). Cation and anion exchange resins are used for the study with a hydrolysate to resin ratio of 1:2 v/v resulting in the removal of 93% phenolics and 64.9% of acetic acid (de Arruda et al., 2011). The use of trialkylamine is another low-cost and highly efficient detoxification strategy requiring low temperatures and the use of less energy, hence allowing lower costs. The extraction with phase composition of trialkylamine:n-octanol:kerosene

(30:50:20) could remove around 73% acetic acid, 46% of 5-HMF and 100% of furfural from corn stover prehydrolysate (Zhu et al., 2011).

4.2.2 Physical detoxification

Nanofiltration (NF), an industrially used membrane separation technology, helps to separate desired molecular weight particles with the help of ceramic nanofilters, just designed to provide a molecular weight cut off range. Nguyen et al. utilized NF and reverse osmosis (RO) with low molecular weight cut-off (150-400 g/mol) for their capacity to separate C5 and C6 sugars from acetic acid, furfural, 5-HMF and vanillin in a model solution (Nguyen et al., 2015).

Vacuum membrane distillation (VMD) is another membrane separation technology which is getting popularity because of its cost-effective inhibitor removal. This technology works on the principle of liquid-vapor-based equilibrium which controls the selectivity of the process and enables the removal of volatile compounds. Chen et al. (2013) studied VMD for the removal of two components from lignocellulosic hydrolysate i.e. furfural and acetic acid. More than 98% of furfural was removed by VMD with optimized conditions, while acetic acid removal was quite lower as compared to furfural (Chen et al. 2013).

4.2.3 Biological detoxification

The biological detoxification is receiving increasing interest owing to its environmental safety as no chemicals are involved, lesser byproduct formation and lower energy needs (Parawira et al., 2011). Biological detoxification is performed in two ways 1. by the use of microbial enzymes, 2. By the use of bioagents (bacteria/fungi/protozoa), which convert toxic compounds into less toxic forms by changing their structure (Morozova et al., 2016). As an example, ferulic acid, one of the inhibitors generated during lignin pretreatment, was converted into less toxic vanillic acid by the actinomycete *Amycolatopsis* sp. (Pérez-Rodríguez et al., 2016). The enzymes used in this process include laccase and different peroxidases. Different bacteria are known to utilize organic acids while yeast *Issatchenkia occidentalis* CCTCC M 206097 could consume phenolic components and furfural (Fonseca et al., 2011).

Zhang et al. (2013) performed detoxification using isolated *Enterobacter sp.* FDS8 which degraded furfural and HMF with a loss of sugar below 5%. Interestingly, in this study, cellular biomass was recycled 5 times without loss of detoxification efficiency (Zhang et al. 2013).

Lee et al. (2012) reported for the first time the novel laccase enzyme from the yeast *Yarrowia lipolytica,* which showed efficiency in degrading phenolic and non-phenolic compounds higher than any other previously reported enzyme from bacteria or fungi (Lee et al., 2012).

5 Hydrolysis of Cellulose

The pretreatment processes are designed in order to degrade hemicellulose and lignin with the possible formation of monomeric inhibitors. Although pretreatments break down cellulose partially, they largely make cellulose bioavailable for hydrolyzing cellulase enzymes like endoglucanase and β-glucosidase. Cellulases are important since highly fermenting yeasts can't utilize cellulose as a carbon source owing to its complexity. Hence, cellulose has to be first hydrolyzed into monomeric sugar to obtain ethanol (Olofsson et al., 2008). The easier method to hydrolyze cellulose is by acid hydrolysis. Historically, inorganic acids like hydrochloric and sulfuric acids have been utilized. In case of sulfuric acid, the hydrolysis reaction is carried out at high temperatures ranging from 150°C to 190°C. On the contrary, lower temperatures are sufficient to complete the reaction using hydrochloric acid (Rinaldi and Schüth, 2009).

Harris (1985) obtained a 50% theoretical yield of fermentable sugars when the hydrolysis was performed in two steps. High purity of glucose was achieved when hemicellulose was hydrolyzed before cellulose at 170 and 190 °C, respectively (Harris, 1985). Unfortunately, one of the main problems with using strong acids and high temperatures, is that they react with glucose and transform it into 5-hydroxymethyl furfural (HMF) and other undesired products.

While acid hydrolysis operates at high temperatures and harsh conditions, enzymatic hydrolysis offers milder conditions that prevent corrosive damage to industrial installations and work at lower temperature without harsh chemicals. Furthermore, the specificity of enzymes allows to obtain highly pure fermentable sugars and high yield without generating inhibitors. However, several disadvantages cannot be neglected, one of which is the longer hydrolysis time (days, compared to minutes in acid hydrolysis); in addition, the increasing concentration of the released sugars in hydrolysate can cause the inhibition of cellulases (Olofsson et al., 2008; Taherzadeh et al., 2007). Finally, despite enzymes recycling being possible with the purpose of cost reduction, solid lignin residuals hamper enzyme recovery, as lignin absorbs part of the introduced enzymes.

Being the most promising technology, enzymatic hydrolysis is considered for further discussion and it will be referred to as enzymatic saccharification.

The group of enzymes collectively referred as "cellulases" includes three proteins each of which has high specific activity on β-1,4-glycosidic bonds that exist within the structure of cellulose.

- Endoglucanases,
- Exoglucanases (or cellobiohydrolases) and
- β-glucosidases.

Endoglucanases act on amorphous and low-crystallinity regions of cellulose, which are more exposed as a consequence of pretreatments. Endoglucanases decrease the degree of polymerization by randomly cleaving β-1,4-glycosidic linkages within cellulose chains, as a result of which shorter oligomers with reducing ends are generated. Cellobiohydrolases attack the reducing ends and release cellobiose units, which are then cleaved into glucose by β-glucosidases (Lynd et al., 2002; Taherzadeh et al., 2007).

Another site for cellobiohydrolase activity is the microcrystalline cellulose. For this purpose, a combination of enzyme pairs of cellobiohydrolases is used in industrial applications. These cellobiohydrolases are selected because of their specific preference for reducing (CBHI) and non-reducing (CBHII) ends of microcrystalline cellulose (Lynd et al., 2002). Several fungal species were scrutinized for the ability to sustain the production of cellulases, including *Trichoderma reesei*, *A. niger*, *Penicillium chrysosporium*, *Humicola insolens*, out of which *T. reesei* dominates this industry by producing endoglucanases, cellobiohydrolases and β-glucosidases of industrial grade. Moreover, β-glucosidases from *A. niger* are also employed, as they show more tolerance to high concentrations of glucose in the hydrolysate without getting inhibited (Lynd et al., 2002).

6 Fermentation of biomass hydrolysates and process integration

Although bioethanol is promising as liquid biofuel, commercialization of the most sustainable, second-generation bioethanol would be feasible only if the cost of the entire process, from the procurement of waste feedstock to the attainment of the final product, will be sustainable (Giakoumis et al., 2013). This would be possible by (i) decreasing the number of steps involved in the process (Figure 2), i.e. by converging them together in a single reaction unit, (ii) reducing the use of additives such as commercial enzymes, (iii) shortening the reaction time. To make bioethanol production industrially and economically feasible, the following strategies were designed:

1. Separate Hydrolysis and Fermentation (SHF)

2. Simultaneous Saccharification and Fermentation (SSF)

3. Consolidated Bioprocessing (CBP)

SHF is a largely studied and industrially employed strategy. It involves different vessels for hydrolysis and fermentation (Figure 2), hence, it is not economically feasible. So, SHF is not discussed in this thesis. Much of the work is being done on the other two strategies that reduce the capital costs by integrating different steps involved in process.

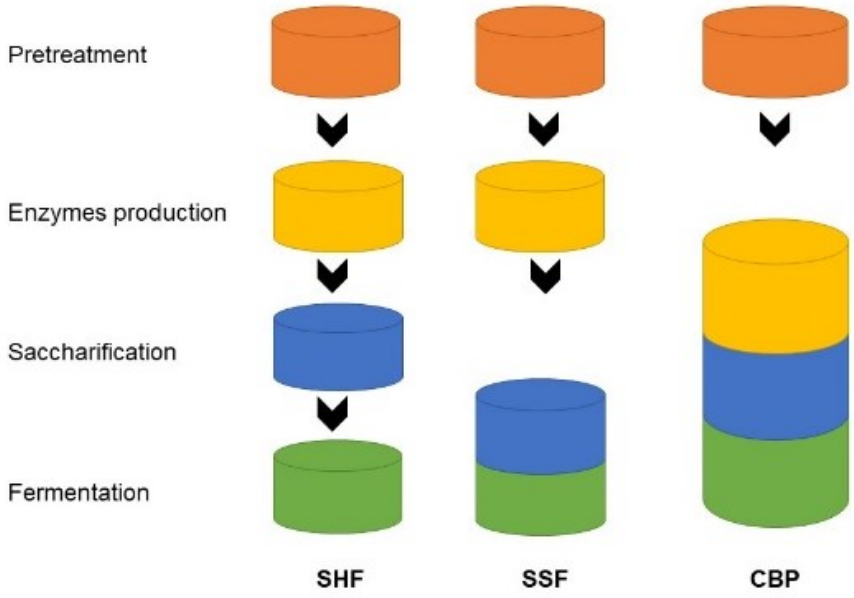

Figure 2: Graphical representation of strategies involved in ethanol production.

6.1 Simultaneous Saccharification and Fermentation (SSF)

In SSF, the same reactor is used for both saccharification and fermentation. The objective is to reduce both the capital costs and the possibility of contamination of the cell suspension during transferring processes (Figure 2). The two steps are occurring simultaneously which reduces the process time. In addition, the selected enzymes work at room temperature, so reducing heating and cooling costs (Figure 3). The problem of the saccharification step is the inhibition of enzyme activity due to product formation, also discussed in Section 4.2.3. As discussed in Section 4.2.3, one of the problem of the saccharification step is the inhibition of enzyme activity due to glucose formation, In SSF, as the released sugar is instantly utilized by fermenting organisms, enzyme activity is maintained for a longer time (Olofsson et al., 2008). For these reasons, SSF technology is receiving much attention from industrial partners. Generally, substrate loading is a key parameter in the SSF setting because the highest substrate loadings, the higher ethanol titers can be expected.

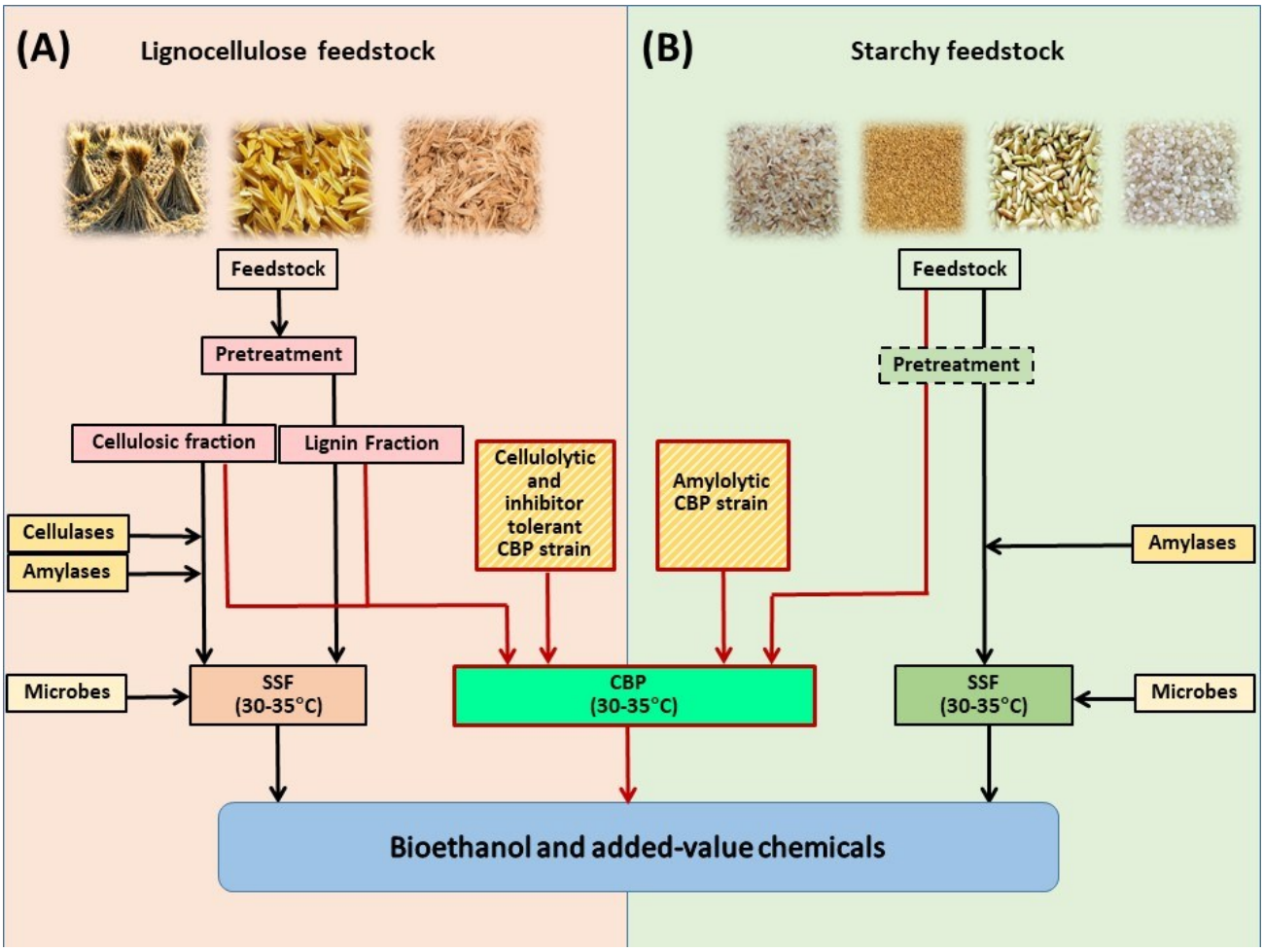

Figure 3: Comparison between SSF and CBP of starchy and lignocellulosic feedstocks (Gupte et al., 2022)

However, in reality, in SSF process, it is necessary to attain optimal rheological properties which lower the final product concentration (Sánchez et al., 2008). Also, it is difficult to obtain optimum cellulase activity at room temperature and hence, high dosages of cellulase are required. Since the addition of enzyme is the cost-determining step in this process, therefore it is necessary to optimize the cellulose dosage. The use of surfactants was proposed to fulfil this aim. The addition of non-ionic surfactants such as Tween-20 to steam exploded wood, increased ethanol yield by 8% when the cellulase concentration was reduced to 50% (from 44 Filter paper Cellulase Unit (FPU) to 22 FPU/g cellulose) (Alkasrawi et al., 2003). On the other hand, saccharification of rice hulls showed a marginal increase of 3.5% when 2.5 g/L of Tween 20 was used (Saha et al., 2005). However, it is postulated that the surfactants reduce the adsorption of cellulase to the lignin, thus increasing the concentration of active enzyme units in the fermentation media.

Different substrates were exploited for the production of ethanol using SSF strategy. Oil palm fruit bunch was used by Sukhang et al. (2020) producing 34.39 g/L alcohol (Sukhang et al., 2020) while 31 g/L of ethanol was produced from sugarcane leaves (Hari Krishna et al., 1998). Rice straw and rice husk were utilized for ethanol fermentation using *Mucor heimalis* (Khaleghian et al. 2015) and *E. coli*, respectively. A higher temperature of 40 °C was explored for the fermentation of paper industry waste using *Kluyveromyces marxianus* (Kádár et al., 2004)

6.2 Consolidated BioProcessing (CBP) of starchy and lignocellulosic feedstocks

Lignocellulosic biomass is the only conceivable renewable feedstock for the sustainable production of biofuels. The main scientific impairment to this extensive resource is the absence of low-cost technologies to overcome the recalcitrance of the lignocellulosic matrix (Lynd et al., 2002). New technologies of direct conversion of cellulose to ethanol using zirconium, platinum, and ruthenium nanoparticles-based catalysts have been developed. These nanoparticles break C-C and C-O bonds in glucose subunits and convert them into C2 products with comparable ethanol yields (Anggoro et al., 2021). Unfortunately, the cost of this novel process is high which makes it difficult to adapt to industry.

As discussed above, in ethanol production, four biological steps are involved in the transformation of lignocellulose to ethanol: production of a saccharolytic enzyme (cellulases and hemicellulases), hydrolysis of the polysaccharides present in pre-treated biomass, fermentation of C6 sugars, and fermentation of C5 sugars (Van Zyl et al., 2007). The saccharification and fermentation steps have been combined in the SSF of C6.

The eventual objective would be a one-step “consolidated” bioprocessing (CBP) of lignocellulose to bioethanol, where all four of these events occur in one fermentation vessel and are supported by a single microorganism (pure culture) or by a microbial consortium (mixed culture) able to ferment pretreated biomass without the addition of any enzymes (Figure 4). CBP is getting higher recognition as a potential innovation for low-cost biomass processing. Overall, a mature CBP process could reduce capital cost by two-fold and process cost by four-fold (Lynd et al., 2006).

Studies indicated that the wild-type cellulolytic microorganisms provide confirming indications that CBP is achievable. When cellulose hydrolysis of growing cultures of *Clostridium thermocellum* was compared with that of purified enzymes obtained from the same organism, it was observed that *C. thermocellum* exhibited 4-fold higher hydrolysis rates than that of purified enzymes (generally used in the SSF approach). This increase has been attributed to “enzyme-microbe synergy” that requires metabolically active cellulolytic microbes (Lu et al., 2006).

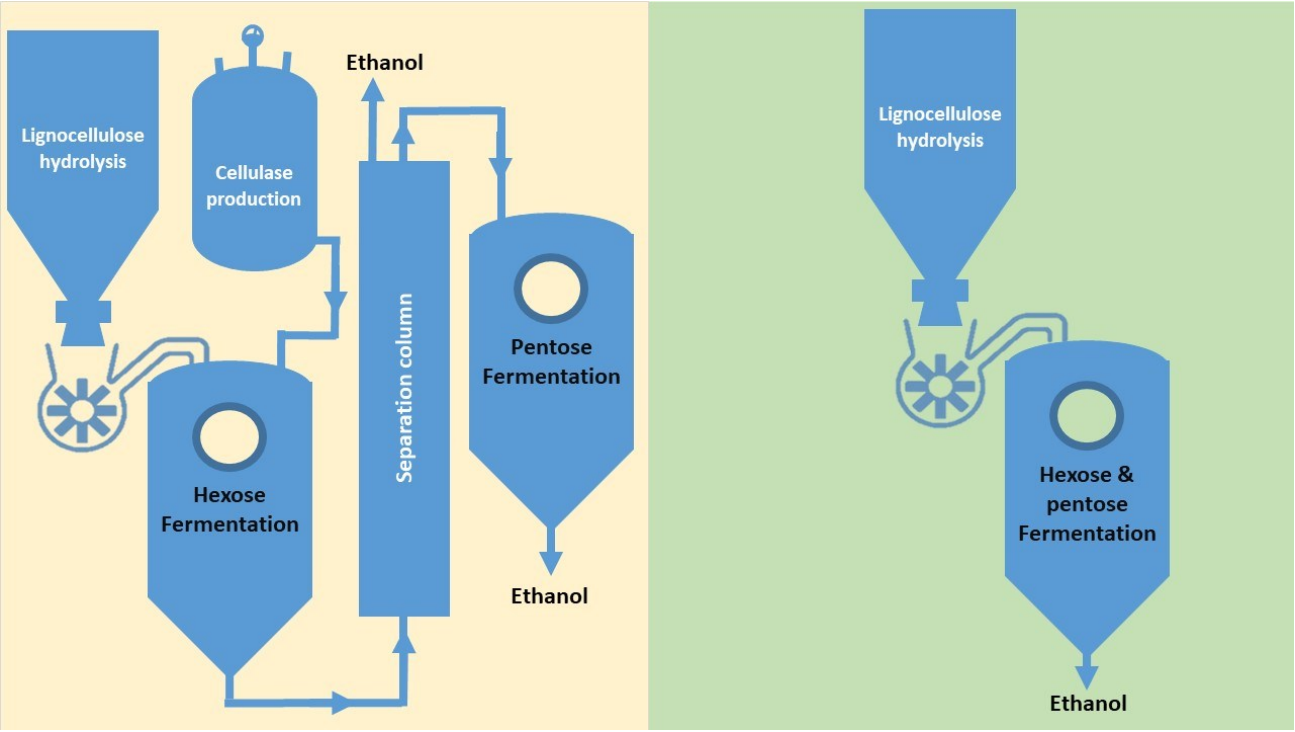

Figure 4: Comparison of process complexities in SSF and CBP strategies for the production of second-generation ethanol.

None of the wild-type microorganisms exhibits all the features necessary for CBP. Several microbes, both bacteria, and fungi, possess only some of the desirable characteristics and can be divided into two groups: (1) native cellulolytic microbes which possess superior saccharolytic capabilities but are unable to efficiently produce any of the desired product, and (2) microbes that naturally give higher product titers, but lack saccharolytic systems, which need to be genetically engineered.

Many native cellulolytic microorganisms are anaerobic bacteria which show highly efficient and complex saccharolytic systems and include mesophilic and thermophilic *Clostridium* species (Demain et al., 2005; Lynd et al., 2005). Additionally, there are fungi that naturally produce a large repertoire of saccharolytic enzymes, e.g. *Fusarium oxysporum* (Panagiotou et al., 2005; Singh et al., 1991).

However, although the anaerobic bacteria produce a pool of fermentation products, the titer of ethanol remains low. On the other hand, filamentous fungi are slow cellulose degraders and also offer low ethanol yields. Hence, there is a need for an organism that can produce a desired product but also possess cellulolytic or amylolytic genes. This can be achieved by genetically modifying an organism that can express the recombinant heterogeneous cellulolytic enzymes

Cellulolytic microorganisms into which recombinant saccharolytic systems have been engineered include the bacteria *Z. mobilis, E. coli* and *Klebsiella oxytoca*, the yeast *S. cerevisiae,* and the xylose-fermenting yeasts *Pachysolen tannophilus*, *Pichia stipitis* and *Candida shehatae*.

Many combinations of genes like *Saccharomycopsis fibuligera* β-glucosidase (Sf-*BGLI*), *T. reesei* endoglucanase (Tr-m*EGII*) and *Talaromyces emersonii* cellobiohydrolase (Te-*CBHI*) have been expressed in yeast and only partial hydrolysis of lignocellulose was observed (Davison et al., 2019). Substantial developments in relation to recombinant enzyme expression support the enormous potential for *S. cerevisiae* as a CBP host (Lynd et al., 2005; van Zyl et al., 2007). However, the expression of all these heterogeneous genes in a particular ratio while keeping the cellular ethanol production pathway unharmed, is the major challenge. A pertinent question on the metabolic burden, asking if *S. cerevisiae* maintains normal cellular activities while producing recombinant protein, was clarified by Favaro et al. (Favaro et al., 2019).

However, important challenges are (i) the level of expression of each gene, (ii) the number of different genes to be expressed in a defined ratio, and (iii) the stress related to their expression. The main factors that could impose avoidable stress on the host cell are:

1. the sequestering of transcription factors at strong promoters (e.g. *PGK1, ENO1, TEF1* etc.) used for heterologous gene expression;
2. an unfavorable codon bias on the translation of heterologous protein (can be overcome by the use of codon-optimized synthetic genes);
3. the improper folding of recombinant proteins.

Hence, the appropriate strategy would not be the singular overexpression of all the desirable genes to ensure an efficient CBP yeast with the required enzymatic activities, but attention should also be given to the post-transcriptional modification of the enzymes, their activities and their production at the right concentration (Wang et al., 2018).

7 Development of cellulolytic *S. cerevisiae* strains

Several approaches have been used for the overexpression of multiple genes in industrial *S. cerevisiae* strains. YEp (yeast episomal plasmid) vectors, which are helpful to maintain a high copy number and YRp (yeast replicating plasmid) vectors, which contain Autonomous Replicating Sequence (ARS), have been very useful in validating the proof of concept in laboratory strains of *S. cerevisiae* (Den Haan et al., 2007; La Grange et al., 2001; Van Rooyen et al., 2005). However, these vectors are generally mitotically unstable i.e. they may be lost during budding, while selective pressure is required

for YEp, which means the use of a defined medium, not applicable in industrial scenarios (Romanos et al., 1992).

The ideal route taken for industrial strains has been the exploitation of integrative YIp (yeast integrating plasmid) vectors that assist direct integration of foreign expression cassettes into a target site on the yeast genome or recycling dominant selectable markers for multiple integrations. Even though, these strategies provide stable expression from the yeast genome and are applicable to industrial strains, the key disadvantage could be low expression levels.

Different approaches have been pursued in order to obtain the benefits of overexpression from multi-copy plasmids with the stability of chromosomal integration. This is also applicable to industrial strains when dominant selectable markers are used. These comprise the use of repetitive chromosomal DNA sequences such as rDNA and δ-sequences (Lee et al., 1997). Nearly 140-200 copies of rDNA exist in the haploid yeast genome; however, rDNA is sited in the nucleolus, which may affect the accessibility to RNA polymerase II transcription. Also, the size of pMIRY (multiple integrations into ribosomal DNA in yeast) vectors could define the mitotic stability of these multiple integrations (Lopes et al., 1996).

A more strategic methodology would be essential to design a yeast that provides the proper enzyme activities, yet maintaining the capability to perform well under industrial conditions. Such a strategy will perhaps start by building on a well-validated industrial yeast platform that co-metabolizes hexoses and pentoses, and then finding the right combination as well as the level of expression for saccharolytic enzymes (Van Zyl et al., 2007).

This methodology will use repeated metabolic engineering and flux analysis, selection and mutagenesis strategies, and strain breeding to allow the microorganism to go beyond rate-limiting barriers towards the development of an efficient CBP yeast. Examples of such approaches in the past have been performed to augment xylose fermentation in laboratory and industrial strains (Kuyper et al., 2005; Wahlbom et al., 2003).

8 Development of amylolytic CBP *S. cerevisiae* strains

S. cerevisiae has been engineered to express amylolytic genes obtained from specific microbes. These genes can be expressed in two ways of: the codified enzymes can be secreted in the medium or remain anchored on the cell surface of yeast. When the enzymes are anchored on the cell wall, yeast can be recycled for successive fermentations, and thus can be considered as true biocatalysts., The risk of contamination during fermentation is therefore reduced, as glucose is generated near the cell wall and is immediately utilized by the yeast. Anchorage of the enzymes on the cell surface has some disadvantages.

In fact, the cells need to be in close proximity to the substrate to efficiently hydrolyze it. Hence, the reaching of cells near the substrate plays a pivotal role during fermentation, while secreted enzymes move freely in the fermentation medium.

Novel whole-cell biocatalysts are developed by anchoring the glucoamylase gene obtained from *R. oryzae* on the surface of fermenting yeast. Thus, this genetically modified *S. cerevisiae* could grow on soluble starch during aerobic cultivation (Murai et al., 1999; Murai et al., 1998; Ueda et al., 2000). The strain was in turn improved by the integration of the *Bacillus stearothermophilus* α-amylase. An ethanol concentration of 60 g/L was achieved in 100 h fed-batch soluble starch fermentation (Shigechi et al., 2002). Noteworthy, a very high cell load was supplemented to achieve this yield (about 30 g/L dw cells). However, this yeast could not metabolize raw corn starch (Shigechi et al., 2004).

Two α-amylase genes from *Lipomyces kononenkoae* were incorporated into the *S. cerevisiae* genome (Eksteen et al., 2003). 6.1 g/L ethanol was obtained by this recombinant strain after 156 hours of fermentation, when 2% starch in the medium was used, and the ethanol yield of 0.38 g/g was achieved. The same strain was improved by incorporating *sfg1* glucoamylase from *S. fibuligera* and *lka1* α-amylase from *L. kononenkoae* into the yeast genome for increased secretion. Up to 21 g/L ethanol was obtained after 120 h of fermentation when soluble starch was used. The volumetric productivity of 0.175 g/L/h and yield of 0.40 g/g was achieved (Knox et al., 2004).

9 Genomic approaches to obtain inhibitor-tolerant *S. cerevisiae* strains

Detoxification of hydrolysate is not always economically feasible, even because, as discussed above, to reduce the cost of fuel ethanol it is essential to eliminate as many steps as possible. The production of superior yeast strains which can tolerate higher concentration of inhibitors, and possessing CBP characteristics, could be the solution (Cagnin et al., 2019). Inhibitor tolerance is the function of genetic diversity amongst different species. This genetic diversity can be obtained naturally or artificially by human interference (Figure 5). Although both approaches are equally promising, obtaining superior yeast strains which are suitable for industrial purposes is time consuming and speculative. While creating artificial diversity needs a detailed study of genetic regulation.

As demonstrated in Figure 5, when artificial approaches were adopted, inhibitor tolerance was achieved by the overexpression of homologous or heterologous genes, encoding properties which provides resistance to specific inhibitors (Petersson et al., 2006). It was observed that the tolerance to furfural was conferred by pentose phosphate pathway (Gorsich et al., 2006). The gene encoding phenylacrylic acid decarboxylase, *PAD1*, was overexpressed in fermenting yeast *S. cerevisiae* and tested

in presence of cinnamic and ferulic acid. Higher conversion of cinnamic and ferulic acid was observed in aerobic conditions. Also, ethanol productivity was improved by 50 to 100% (De Beer et al., 2001).

Another approach providing promising results is sexual breeding. Jansen *et al.* (2018) bred inhibitor tolerant strain with temperature tolerant strain using spore-to-spore hybridization which resulted in a temperature and inhibitor-tolerant strain having ethanol yield similar to the parental strain (Jansen et al., 2018). Apart from this, evolutionary engineering and mutagenesis approaches are also pursued to get superior yeast strains (Ruchala et al., 2020; Tomás-Pejó et al., 2014).

Classical genetics is another tool to obtain inhibitor-tolerant industrial strains (Jansen et al., 2018). Multiple tactics were developed, aimed to provide appropriate yeast strains for precise industrial goals. A deceivingly simple, yet very potent way is to exploit the natural biodiversity by choosing a strain that performs best in a specific industrial process. Recent genomics studies specify that the natural fungal biodiversity is enormous and mostly uncharted, with the existing industrial strains only representing a small portion of the natural biodiversity (Boekhout et al., 2022; Liti et al., 2009; Wang et al., 2012). This suggests that, perhaps, nature harbors manifold, as yet unknown species and strains that may result superior for certain industrial fermentations. Although, many of these strains may not be suitable for direct industrial operation, they may possess some relevant characteristics for industrial processes. Some techniques could help in transferring these properties to industrial strains, thereby creating novel yeasts with extra advantageous features (Figure 5).

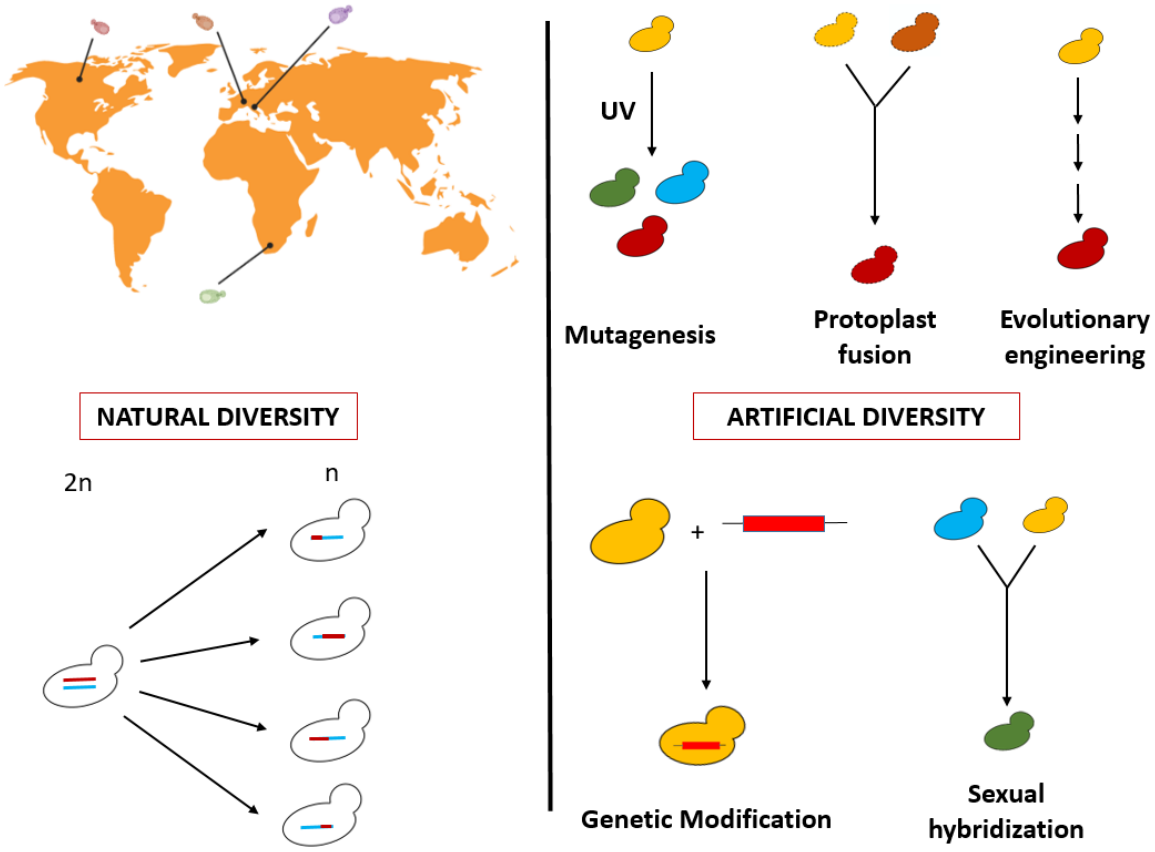

Figure 5: Different strategies to obtain superior yeast strains for second-generation bioethanol production (adapted from Steensels et al., 2014).

Many industrial *S. cerevisiae* strains have a much more complex genetic structure in comparison to laboratory strains, often carefully bred and selected for sexual reproduction, optimal growth, and easy handling under lab conditions (Jansen et al., 2018; Mortimer et al., 1986). On the other hand, industrial strains often demonstrate polyploidy, poor sporulation efficiency, unstable mating types, etc. Furthermore, the latest full-genome sequencing and large-scale phenotyping experiments emphasize that these laboratory strains are not representative of the wide variety of industrial strains available (Borneman et al., 2011; Liti et al., 2009).

Natural diversity can also be observed by sexual reproduction (where the genomes of two parents are clubbed and shuffled), changes in the DNA makeup such as point mutations (i.e. changes in single nucleotides), and InDels (i.e. insertion or deletion events observed in relatively short pieces of DNA), variations in ploidy level (wherein the number of chromosomes can be higher or lower than normal), by the use of transposons (mobile genetic elements that can cause mutations by insertion in the genome), genetic recombination (where portions of the genome are re-organized; it can act on both homologous

and nonhomologous loci), or gaining of exogenic pieces of DNA by horizontal gene transfer (Steensels et al., 2014).

Crossover events take place during the anaphase II of meiosis and generate variations in progenies. The results yeast can be categorized as a natural, non-genetically modified strain (Figure 5). If the yeast is homothallic i.e. meiosis progeny (spore) is capable of getting self-diploid and possesses the capability to undergo meiosis (Herskowitz, 1988), the variations increase and the resulting strains can be checked for the desired characteristic and mated with industrial strain for increased ethanol yield. A part of this study is focused on exploiting the variations generated during meiosis and the relevance of the variations to provide desired characteristics necessary for industrial usage.

Aims of Ph.D. thesis

Biofuels are considered renewable energy sources because they are produced from materials that can be replenished, such as plant matter and waste materials. While first-generation biofuels are produced from food crops, such as corn, wheat, and soybeans, second-generation biofuels are obtained from non-food feedstocks, such as agricultural waste and forestry waste. Moreover, these types of feedstocks do not compete with food production and their conversion could help to reduce wastes that end up in landfills. With these perspectives, this PhD thesis is aimed to identify and evaluate feedstocks alternative to those currently available with a focus on agricultural byproducts as well as domestic waste, converting them into biofuels in a biorefinery approach.

As reported in the Introduction, a major problem while utilizing wastes is the generation of inhibitors that are produced during various processing steps such as pretreatment, a crucial step that makes complex carbon bioavailable for enzymes. Hence, the aim is extended to obtain a superior yeast strain that could tolerate the inhibitors and produce high ethanol titers. The focus is given to proving 'classical genetics' as a 'tool' to obtain superior yeast strains using a non-genetically modified (GMO) approach. Genetically modified yeasts were considered when complex carbon like starch substrates were assessed.

To achieve these goals following paths were pursued:

1. Rice milling industry byproducts were deeply studied to identify possible substrates suitable for the production of a spectrum of biofuels. Unripe rice, the least exploited starchy residue from the rice milling industry, was selected as feedstock to be converted into liquid and gaseous biofuels in an integrated biorefinery approach by recently developed efficient amylolytic CBP strains of *S. cerevisiae*. The spent fermentation slurry from CBP fermentation was then employed to obtain methane by anaerobic digestion.
2. The starch-rich organic fraction of municipal solid waste (OFMSW) was also investigated as feedstock for bioethanol production by amylolytic recombinant CBP strains of *S.cerevisiae.* The presence of rice milling industry near domestic waste collection plants encouraged to ferment discolored rice, a rarely used rice mill waste, together with OFMSW.
3. A protocol utilizing classical genetic methodologies was developed to produce variation amongst yeast strains. This can then be applied to obtain inhibitor tolerant superior yeast strains for bioethanol production. New variability was generated by sporulation and ascospores separation experiments, obtaining a variety of *S. cerevisiae* strains that were then investigated for tolerance to specific inhibitors by a metabolomic approach.

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Identification and study of alternative feedstock for biofuel production

Review Article published in Renewable and Sustainable Energy Reviews

Rice waste streams as a promising source of biofuels: feedstocks, biotechnologies and future perspectives

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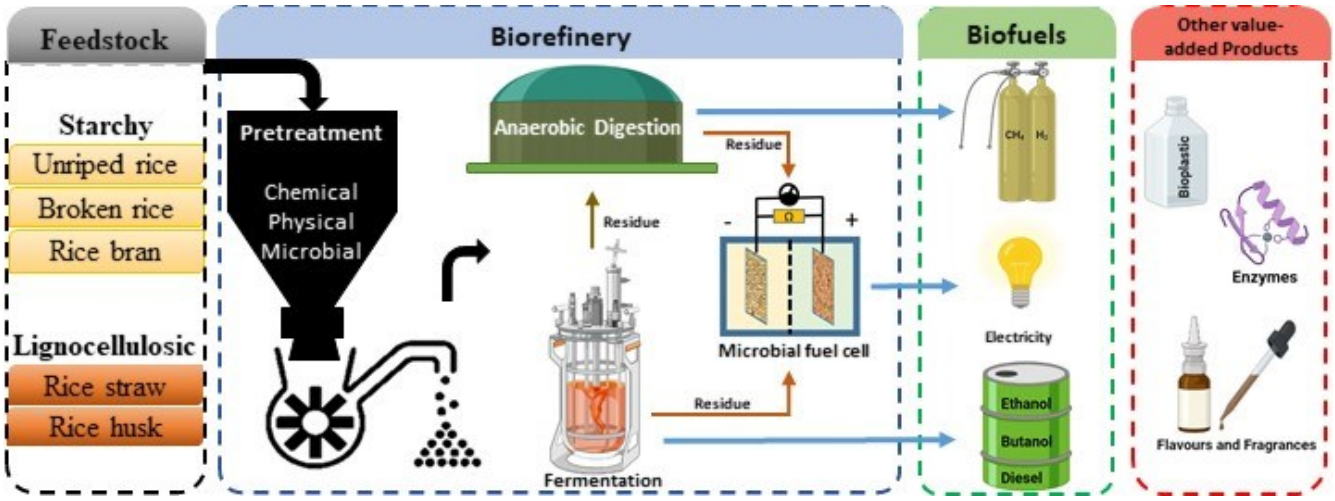
Published in 'Renewable and sustainable energy reviews' Journal, doi: 10.1016/j.rser.2022.112673

Graphical Abstract



Abstract

Increased environmental concern over climate change due to higher oil usage has made human being to shift to cleaner and greener alternatives. The utilization of abundant agricultural waste streams as renewable feedstock for biofuels production can be a pivotal strategy. Among others, rice is one of the most largely grown crops, and more than 4.8 % of the total production goes to waste. Although previous reviews are related to biofuels obtained from some rice waste, most of those are focused on lignocellulosic rice residues with much attention to thermo-chemical processes. The present paper, instead, reviews for the first time the biotechnological approaches to convert all rice wastes, like rice husk, rice straw, broken rice, discolored rice, unripe rice, into liquid (bioethanol, biobutanol, biodiesel), and gaseous (biogas, biohydrogen) biofuels through the use of pure or mixed microbial cultures. The global availability of each rice byproduct has been also investigated and the potential of rice waste as a 'fuel farm' has been estimated for bioethanol. The physical, chemical, enzymatic, or microbial pretreatments, which play a key role in making carbon available for hydrolysis and fermentation, are here discussed and evaluated. Despite the great promise of technologies so far developed, further research is still required for their upscale and industrial commercialization. Moreover, future process integrations will open the landscape to biorefinery schemes where rice waste streams can be processed into biofuels and other added-value products, towards the full exploitation of the feedstocks and the economic and environmental sustainability of the overall process.

Keywords

Rice biowaste, Bioethanol, Biogas, Microbial fuel cells, Biorefinery, Microbial cell factories

Abbreviations

ABE- Acetone Butanol Ethanol

ADSS- Anaerobically digested sewage sludge

AFEX- ammonia fiber expansion

AFP- Acid fungal protease

AFP- Acid fungal protease

AS- Anaerobic sludge

AS- Anaerobic sludge

ASS-Activated Sewage Sludge

ASSP- Anaerobic sludge from sediment of pond

ATCC- American type culture collection

BR- Broken rice

BTU- British thermal unit

C/N-Carbon to nitrogen ratio

CBP- Consolidated bioprocessing

CD- Cow dung

CDSM- Cellulose degrading soil microflora

CDTD- Combinative dispersion thermochemical disintegration

CDTD- Combinative dispersion thermochemical disintegration

CDW- Cell dry weight

CO- Carbon monoxide

CRF- Cow rumen fluid

dAT- Deacetylation acid pretreatment

DDGS - Distillers' dried grains with solubles

DM- Dairy manure

DR- Discolored rice

DRB- De-oiled rice bran

DS- Digested sludge

FAME- Fatty acid methyl ester

FW- Food waste

GHG- Green house gases

GL- Gigalitres

GMO- Genetically modified organism

GSHE- Granular starch hydrolyzing enzyme

GSHE- Granular starch hydrolyzing enzyme

HC- Hydrodynamic cavitation

HRT- Hydraulic retension time

HTB- Hybrid thermochemical-biological

IEA- International Energy Agency

LCA- Life cycle Assessment

Mg- Megagram

MFC- Microbial fuel cell

MTCC- Microbial type culture collection

MWTPS- Municipal wastewater treatment plant sludge

NMMO- N-methyl morpholine N-oxide

NMR- Nuclear magnetic resonance

NO_X- Nitrogen oxide

nr- not reported

OLR- Organic loading rate

PB- Pond bottom

PEM- Proton echchange membrane

PFS- Paddy field soil

PM- Particulate matter

RB- Rice bran

RBDW- Rice Bran De-oiled wastewater

RH- Rice husk

RRC- Rice residues from canteen

RS- Rice straw

RWW-Rice washing water

S/I- Substrate to inoculum ratio

SHF- Simultaneous hydrolysis and fermentation

SHS- Slaughterhouse Sludge

SM- Swine manure

SO_X- Sulfur Oxides

SRSH- Synthetic rice straw

SS- Sewage sludge

SSF- Separate saccharification and fermentation

SWOT- Strength, Weakness, Opportunity, Threat

Tg- Teragram

TS- Total solids

UR- Unripe rice

VFA- Volatile fatty acids

VOC- Volatile organic compounds

VS- volatile solids

[BMIM][OAc]- 1-butyl-3-methylimidazolium acetate

1. Introduction

Considering the highest economic growth, the energy consumption in 2050 is expected to increase by almost 70 % [1], with an overall energy demand rising to almost 680 quadrillion BTU by 2030 [2]. Up to 85 % of this demand will be fulfilled by fossil fuels, thus continuing to contribute to environmental pollution by the release of greenhouse gases (GHG) in the atmosphere (50 % higher than in 2011) [3]. These are carbon monoxide (CO), nitrogen oxides (NO_X), particulate matter (PM), sulfur oxides (SO_X), methane, and volatile organic compounds (VOC) [4]. To overcome the continuous increase of energy demand, alternative solutions for cleaner and more environmentally friendly fuels than the available fossil ones, are needed [5].

Among various alternatives, biomass can unambiguously play a pivotal role as a source of renewable energy, with great potential in the production of biofuels for heat, electricity and transportation [6–9]. Moreover, biofuels show a significant reduction in NO_X, PM, and SO_X [4], thus helping to mitigate climate change. Within this scenario, the most promising biofuels are represented by bioethanol, biomethane, biobutanol, biohydrogen [10] from waste

Lignocellulose is by far the main component of farm residues like bagasse, straw, husks, brans and it is the most abundantly available raw material on the Earth. It contains an aromatic polymer (lignin) and 80% of polymeric carbohydrates (cellulose, hemicellulose) [11], suitable for the production of biofuels. Moreover, since 140 x 10^3 teragram) of agricultural waste biomass is generated every year worldwide, the improper management of such organic material could lead to pollution [12]. For instance, the excess of biomass burned in the open [13] results in an important loss of resources potentially available for fuel production. In fact, the yearly generated lignocellulosic biomass is theoretically equivalent to 50 x 10^3 Tg of oil [14]. Some surveys have been developed and published on the evaluation and characterization of agro-food residues for biofuels production [15–20] and, among a number of different starchy and lignocellulosic residues, rice waste biomass has been indicated as one of the most abundant and promising feedstocks [2]. Globally, rice is the second most-produced food grain after wheat and generates around 972 Tg of waste per year. Noteworthy, the processing of rice crop results in a unique combination of lignocellulosic and starchy waste streams largely available at the proximity of the milling site. As such, the complex variety of composition is one of the challenges to be still faced at the industrial level towards the full exploitation of all rice byproducts into biofuels. Biofuels from rice waste can indeed be obtained by thermochemical as well as biotechnological pathways. Furthermore, the combination of the thermochemical and biotechnological processes can be a hybrid platform which catches the advantages while alleviating the weaknesses of the standalone thermochemical and

biotechnological pathways [21]. Thermochemical processes can indeed overcome the recalcitrance of rice biomass and thus eliminates the complex pretreatment step and the need for costly saccharification processes. On the contrary, the high instability of the thermochemical outputs, mostly in the case of pyrolysis, and their usually high toxicity can be finely alleviated by specific biotechnological approaches to produce high value products [22]. Despite these promising perspectives, hybrid thermochemical-biological (HTB) processes from rice waste streams have not yet been reported and can be an outstanding future research area.

Many thermochemical efficient technologies to process rice waste, like pyrolysis [23,24], gasification [25], combustion [26] etc. are available, whereas the biotechnological routes received less attention and the present review is focused on the latest biotechnological approaches devoted to biofuels production. Table 1 provides the timeline and important findings of previous reviews which are mostly related to biofuels obtained from the rice waste mostly using the biotechnological route.

Table 1: Reviews on rice waste to biofuels: main findings and timeline

Year	Main Topic	Reference
2010	Pretreatment of rice straw for the production of ethanol.	[27]
2010	RH as a cellulosic feedstock to meet one-fifth of world energy demand.	[28]
2012	Physico-chemical characteristics and pretreatment techniques, thermochemical as well as biochemical technologies available to convert rice straw and husk into energy.	[2]
2012	Effect on socio-economics of Thailand due to the conversion of rice straw into ethanol and power	[29]
2016	Novel techniques to convert rice mill effluent into energy and value-added products with curbing pollution caused due to effluent.	[30]
2018	The efficiency of the solid-state digestion method over liquid anaerobic digestion for lignocellulosic rice husk.	[31]
2019	Insights on pretreatment, co-culture approaches, consolidated bioprocessing, and strain improvements for biobutanol tolerance using rice straw.	[32]
2020	Utilization of rice husk and straw for energy generation, environmental adsorbents, construction materials, and speciality products.	[33]
2020	Socio-economic effects, LCA and SWOT analysis of biofuel production from rice straw.	[34]
2022	Biotechnological routes to convert all rice waste streams into biofuels	This article

Many papers described details of lignocellulosic rice waste with much attention to physico-chemical processes for the production of biofuels [2,30]. Other reviews dealt with LCA [34] and socio-economic effects [29,34] of producing biofuels from specific rice byproducts, with great emphasis mainly on rice straw. The present paper, instead, reviews for the first time the biotechnological approaches to convert all rice wastes, as rice husk (RH), rice straw (RS), broken rice (BR), discolored rice (DR), unripe rice (UR), into liquid and gaseous biofuels using pure or mixed microbial cultures. Moreover, the global availability of each rice waste has been investigated and their bioethanol potential has been calculated.

As such, this manuscript is specifically devoted to researchers with expertise and interests in biotechnology and process engineering, as well as to agronomists and industries seeking potential and more promising valorization routes of the widely available rice by-products. Furthermore, this review can be of interest also for governmental agencies and institutions which can be supported in the implementation of tax incentives and commercial carbon credits related to bioeconomy approaches dealing with rice residues.

2. Rice waste biomass: global availability and composition

Oryza sativa and *O. glaberrima* are the two domesticated rice species that originated in Asia and Africa. Although both can grow in dry land and deep water, *O. sativa* has a higher yield and milling quality and thus is cropped in at least 112 countries [2] with approximately 90 % of production in Asia [35]. Rice is a staple food for about half of the world's population and thus it is one of the most important crops with a worldwide production of almost 1000 Tg in 2018 [35]. Around 88 % of cropped rice goes for human consumption and 2.6 % for animal feed, thus, due to the global economic and population growth, rice demand is likely to remain robust in the next decades. Although eating preferences of Asian countries are shifting to a diet including food other than rice, the rice industry should remain significant for a long time and thus the availability of rice waste high.

Besides food, feed and seed, more than 4.8 % of total rice grains go to waste [36]. For instance, in North America, 12 % of produced rice is wasted and in Asia around 22 Tg of dry rice are discharged. Rice agricultural waste include crop and agro-industrial residues: crop residues are parts of the plant remaining on the field after harvesting and include RS, while agro-industrial residues are generated by cleaning and milling processes and comprise RH, removed from the seed during milling, rice bran (RB) and BR, DR and UR.

The disposal of high quantities of RS can cause multiple hazards to environment and ecosystem. Traditionally it was used as animal fodder [37], fertilizers [38], hatching, manures, burnt [13,39] or is incorporated into the soil or used as mulch potentially harbouring rice diseases [33,40]. Freshly generated rice straw burning in open field is commonly adopted by the farmers all over the world but especially among the Asian countries accounting for more than 70% of the world’s rice production [41,42]. This approach causes the release of pollutants and greenhouse gasses into the atmosphere, thus contributing to increasing the global warming [43] and adversely affecting the environment and human health [44–46]. In addition, Carlson *et al.* [46] demonstrated that the highest concentrations of the greenhouse gas emissions zones are in the major rice-growing areas of Asia as a result of open field burning of the waste

straw. Indeed, the management of rice wastes is a critical issue also taking into consideration the volume generated in the world [47]. In this perspective, many countries such as those of European Union, restricted field burning and adopted the "waste-to-resource" approach [42].

In recent years, along with traditional utilization, ways RS is also exploited for the production of biofuels, biochar, compost, mushroom, fuel-briquette, fuel-pellets, and paper etc. [39,48]. In this perspective, RS and RH were proposed as construction materials or adsorbents of heavy metals or for the production of energy and fuels [2]. The exploitation of these agro-industrial residues could give an income to farmers but poses logistic problems: in fact, while RH and RB are easily available at rice mills, RS needs the activation of short local chains because must be collected from the fields [29]. RB, obtained from the milling industry, is already used by the food industry for functional food due to its ability to inhibit colonization of *Salmonella* in the gastrointestinal tract [49] or as a source of oil with beneficial properties. RH, which is the seed cover obtained during grain processing (40 % wet weight), is cheap and can be easily collected and utilized for different purposes such as a component of polymeric composite resins or as an energy source [33]. RH is abundant, constitutes 20–22 % of total rice biomass and it is presently disposed by rice mill industry as a waste. RH contains SiO_2 large amounts ranging from 8.7 to 12.1 %, with a medium average 10.6 % [50]. Silica-rich RH could be converted to biofuels (e.g., bio-oil, vapors) and biochars simultaneously via thermochemical processes such as pyrolysis or gasification. Although proposed for other low-value products [51], it is mainly underutilized, on-site burned or landfilled, leading again to serious environmental problems [52].

The most desirable eco-friendly alternative is the conversion of this material by biological methods, alone or coupled with the above mentioned ones, which are already available in a large part. Indeed, the chemical composition of biomasses from rice cropping and industry includes both lignocellulosic and starch-rich residues (Table 2). RS and RH mainly contain hemicellulose and cellulose but low amounts of starch, while broken, discolored and unripe rice, contains large quantities of starch. Both lignocellulosic and starchy rice residues could be used as feedstock for the biochemical conversion into biofuels after their hydrolyses into fermentable sugars. The potential of bioethanol, here chosen as representative of other biofuels, was theoretically assessed considering the bioethanol attainable from the fermentation of starch, cellulose, and hemicellulose after pretreatments and/or enzymatic saccharification as previously recommended [17–19] considering both conversion coefficients as well as experimentally obtained values. The polymers' hydrolysis yields for starch, cellulose, and hemicellulose were supposed to be 91%, 81% and 96% respectively, the stoichiometric ethanol yields of monosaccharides were

estimated 92.5% for glucose and 86% for xylose with an ethanol recovery yield after distillation of 99.5%.

Lignocellulosic waste streams are the most abundant with up to 836 Tg. RS, the crop residue available on the field when the product is harvested (approximately 22 % wet weight), accounts worldwide for 685 Tg, with a potential ethanol of nearly 194 Tg. RH, which is the seed cover obtained as an agro-industrial waste during grain processing (40 % wet weight) can be converted into up to 41 Tg ethanol (Table 2). Starch-rich residual biomasses from rice could be also utilized for fuels production (Table 2). Their starch levels vary from 29 to 80 % of dry matter with a high content of proteins which were shown to support nitrogen requirements of microbial strains involved in their fermentation [53]. Broken rice (BR) is a promising feedstock with an availability of up to 45 Tg an ethanol potential of 16 Tg. RB, UR and DS are also largely available, with significant ethanol applications [54–57].

Overall, rice cropping results in the generation of large amounts of biomasses that are underutilized. This led the scientific community to identify potential uses, such as the generation of energy and fuels, that will be implemented only if specific legislation and taxation will make them more attractive and economically convenient.

Table 2: Average composition and availability of rice waste

Waste	Average composition (% dry matter)						World biomass availability (Tg)	Bioethanol potential (Tg)	References
	Starch	Cellulose	Hemicellulose	Lignin	Protein	Ash			
Lignocellulosic material									
Rice Husk	6.9	40.1	20.6	22.3	3.4	18.2	151.1	41.4	[2,31,54,58–60]
Rice Straw	11.8	34.3	25.1	18.6	1.3	15.0	685.0	193.7	[2,27,31,58,61–66]
Starchy material									
Broken Rice	77.7	0.2	0.5	-	8.3	0.5	45.3	16.0	[54]
Discolored Rice	84.6	0.1	0.9	-	8.0	0.5	7.5	2.9	[54]
Rice Bran	29.6	6.9	15.7	4.1	14.5	8.0	52.9	11.5	[61,67]
Unriped Rice	68.6	1.8	3.7	-	9.9	1.5	30.2	9.9	[54]

Lignocellulosic rice byproducts (Rice husk, Rice straw) and starchy waste streams (Broken rice, Discolored rice, Rice bran, Unripe rice), Yearly ethanol potential (Tg) from each feedstock has been calculated as previously described [19] considering both the availability and average composition.

3. Pretreatment of rice biomass

Pretreatment of rice waste streams is one of the most important and cost determining steps for their conversion into biofuels. This is necessary for the separation of lignin and hemicellulose, to reduce the crystallinity of cellulose and to increase the accessibility of hydrolytic enzymes [68]. Pretreatments should meet the following criteria: 1. obtain high efficiency of sugars formation either by the chemical, physical or enzymatic way [69]; 2. reduce loss of carbohydrates; 3. reduce inhibitory byproducts formation; 4. be cost-effective [70]. In principle, the treatment of lignocellulosic feedstocks is more complex than the processing of starch-rich substrates. Many efficient pretreatments of lignocellulosic and starchy rice byproducts have been recently developed to optimize the production of various biofuels and added valued compounds. Table 3 reports a selection of the most used physical, enzymatic and chemical methods.

Considering RS as raw material, a number of attempts have been reported to improve the efficiency of the enzymatic hydrolysis. For instance, a novel lime-pretreatment process was proposed without solid-liquid-separation. In the same vessel, xylan, starch and sucrose are present together and inhibitory effects on saccharification and fermentation were found to be not significant [62]. When the same pretreatment was applied on RH, no generation of detectable furfural and hydroxymethyl furfural was also observed [71]. Castro *et al.* focused on deacetylation of RS using alkali which resulted in a reduced concentration of inhibitors in pretreated hydrolysate [72]. NaOH combined with urea helped to increase the availability of cellulose and hemicellulose by effectively disrupting the structure of RS and increased maximum hydrogen production by over 160 % than control [73]. Zhu *et al.* combined microwaves along with NaOH to reduce reaction time and enzyme loading. This combination yielded around 5 % more ethanol than only alkali pretreatment [74]. Two-step pretreatment process consisting of aqueous ammonia and sulfuric acid helped in selective removal of lignin and hemicellulose respectively [75]. Teghammar *et al.* used N-methyl morpholine N-oxide (NMMO) for pretreatment of RS which increased the methane production by seven times than that of untreated RS. Also, 98 % of the solvent used during pretreatment was recovered, making this pretreatment method environmentally friendly and economically feasible [76]. When the same method was adopted for bioethanol production and compared with 1-buthyl-3-methyl imidazolium acetate, NMMO was found to be more efficient in producing bioethanol [77].

Glycerol, a byproduct of the biodiesel industry, was used in two forms (i.e., acidified aqueous glycerol and glycerol carbonate) for pretreatment of RH. Results showed that glycerol carbonate showed better bioethanol production than acidic counterpart [78]. Saha *et al.* [71] treated milled RH with 1.5 %

NaOH at 121°C along with a cocktail of three commercial enzymes (i.e cellulase, b-glucosidase and hemicellulase), whereas Ebrahimi *et al.* [79] used ammonium carbonate to improve the ethanol yield from 10 to 47 % in the 72 h fermentation. This indicates that usage of alkali for pretreatment of RH is helpful to boost bioethanol production. Treating RH at 900°C produced ash that provided the economic and efficient source of proton exchange membrane (PEM) for the production of electricity [80].

When above mentioned and recent alkali, acid, or ammonia based pretreatment methods on lignocellulosic waste are compared, it was prominent that alkali is widely used for pretreatment of lignocellulosic rice waste and can be one of the most important future research topic. Alkali breaks down easter bonds between lignin, cellulose and hemicellulose with release of less furfural, 5-hydroxymethyl furfural and vanillin than acidic pretreatment. Some processes combine alkali and/or acids with thermal pretreatments to improve product yield. These practices are well established in the industry [81], due to low energy demand, low temperature, short reaction time, and easy to scale up. The major disadvantages of these combined pretreatments are the corrosion of the reactors due to the extreme pH, the formation of fermentation inhibitors (phenolic), and thus the increase in operational cost [82].

Special attention must be given to the development of pretreatment exploiting solvents fully biodegradable and, hopefully, recyclable at industrial settings, as it was demonstrated for NMMO pretreatment on RS [77]. NMMO has been used to reduce crystallinity of cellulose by breaking the intramolecular hydrogen bonds and van der Waals forces [76]. Moreover, NMMO does not act as enzymes inhibitor [83], thus enhancing the Saccharification of polysaccharides into monosaccharides. Although higher theoretical (>80%) yields were obtained for production of biomethane and bioethanol, utilization of recycled solvent and scale up studies must be improved.

Considering now starchy-rich rice waste pretreament, it is clear that such materials are usually more prone to pretreatment than the lignocellulosic one (Table 3). As such, efficient enzymatic pretreatment is enough to release glucose and a cluster of mostly commercial amylolytic blends were tested resulting in high saccharification yields. Overall, towards the efficient processing of rice by-products into biofuels, with the large varieties of pretreatment technologies available, an in-depth assessment should consider the economic trade-off associated with pretreatment handling and transportation costs.

Table 3: Selection of the most used and efficient physical, chemical and/or enzymatic pretreatment recently adopted for rice waste streams. For the sake of clarity, this table also consider enzymatic or microbial hydrolysis as a pretreatment.

Feedstock	Pretreatment			Product	References
	Physical	Chemical	Enzymatic or microbial		
Lignocellulosic materials					
RH	Wet air oxidation	-	-	Bioethanol	[60]
RH	Milling, Autoclaving	2 % H_2SO_4, 3 % NaOH	-	Bioethanol	[84]
RH	Milling	$(NH_4)_2CO_3$	Cellulase	Bioethanol	[79]
RH	Thermal	-	-	Electricity	[80]
RH	Milling	Acidified aqueous glycerol	Cellulase	Bioethanol	[78]
RH	Milling	Glycerol carbonate	Cellulase	Bioethanol	[78]
RH	Milling, Autoclaving	1.5 % NaOH	Cellulase, β-glucosidase, hemicellulase	Bioethanol	[71]
RS	-	3.5 % H_2SO_4	-	Biolipids	[85]
RS	Steam explosion	10 % NaOH	-	Glucose	[86]
RS	Thermal	2 % $Ca(OH)_2$	-	Biogas	[87]
RS	Extrusion	-	-	Biogas	[88]
RS	Extrusion	3 % H_2SO_4	-	Bioethanol	[89]
RS	Autoclaving	-	-	Biogas	[90]
RS	Ozone	aqueous ammonia	-	Biogas	[91]
RS	Gamma irradiation	1 % NaOH	-	Biogas	[92]
RS	Milling, Autoclaving	0.4 % NaOH	*Trametes hirsute*	Bioethanol	[93]
RS	Milling, Autoclaving	-	*Pleurotus ostreatus*	Biogas	[94]
RS	Autoclaving	-	*Pleurotus ostreatus* *Trichoderma reesei*	Biogas	[95]
RS	Milling, Autoclaving	2.5-3 % HCl	Cellulase	Biohydrogen, Bioethanol	[96]
RS	CDTD	-	-	Biohydrogen	[97]
Starchy materials					
BR	-	-	α-amylase, amyloglucosidase	Bioethanol	[98]
BR	-	-	AFP, GSHE	Bioethanol	[99–101]
BR	-	-	Hyper active α-amylase	Bioethanol	[57]
BR, DS, RB, UR	-	-	GSHE	Bioethanol	[53,54]

AFP- Acid fungal protease, GSHE- Granular starch hydrolyzing enzyme, CDTD- Combinative dispersion thermochemical disintegration.

4. Biofuels production from rice waste streams

The world energy demand along with the pollution due to overuse of fossil fuels and potential of rice biomass as alternative raw material were well described in section 1 and 2 respectively. To overcome the continuous increase of energy demand, cleaner and more environmentally friendly fuels can be obtained by thermochemical as well as biotechnological ways. There are many technologies like pyrolysis [23], gasification [25], combustion [26] etc. are available which can also be applied to rice biowaste streams; but, this review is focused on the biotechnological strategies and their updates for the production of biofuels from rice biowaste streams. Hence, only those technologies are discussed in detail in following sub-sections.

4.1 The key role of microorganisms as cell factories

In general, the microbial conversion of a waste into a product is an approach that is becoming increasingly popular as microorganisms can be considered powerful cell factories, capable of metabolizing raw materials and producing useful substances at the industrial level [102–104]. Moreover, microorganisms can be further improved by genetic as well as evolutionary engineering approaches to maximize the desired product(s) yields and productivities. In this perspective, microorganisms can play an essential role in the transition from fossil fuels to biofuels from rice waste streams. Essentially, after the optimization of the pretreatments, two approaches have been developed to converting pretreated rice products into biofuels, namely the utilization of microbial consortia or the use of single bacterial or yeast strains (Figure 1).

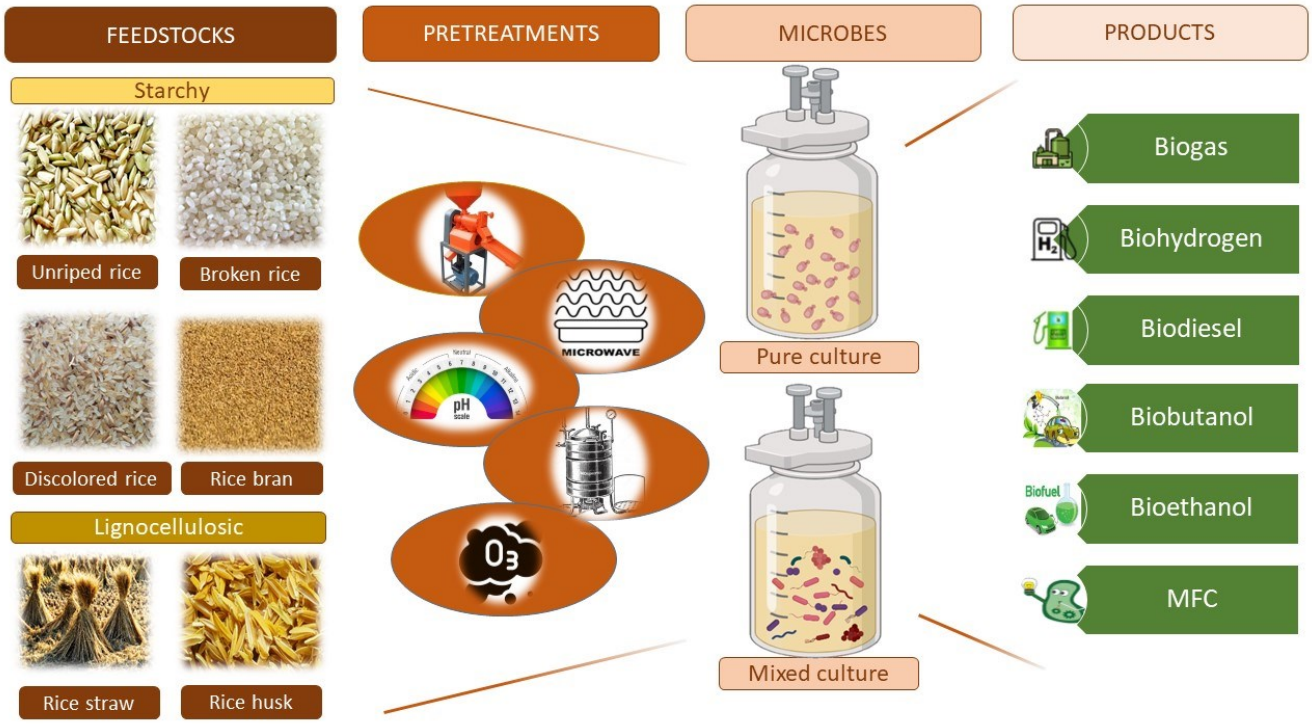


Figure 1: Biofuels production from different rice waste streams. Once subjected to a single or a combination of pretreatment(s), rice byproducts can be processed into different gaseous or liquid biofuels and electricity by using a pure or a mixed culture approach. MFC-Microbial fuel cell.

Mixed cultures are typically adopted for biohydrogen and biogas applications. The production of these biofuels provides that the process conditions select specific groups of microorganisms, naturally present in the inoculum or the feedstocks, acting sequentially to convert complex substrates into hydrogen or methane. Thus, the research is mostly focused on pretreatments optimization of the feedstocks as well as on the fine-tuning of process conditions aimed to select and facilitate the most efficient microbial populations. Pure cultures are mainly used to obtain bioethanol, biobutanol biodiesel and electricity. This approach considers the utilization of single strains and specific efforts were spent towards efficient biotechnological routes by exploiting properly selected and/or genetically modified bacterial and yeast strains.

In the next sections, biofuels applications (biogas and biohydrogen) dealing with mixed microbes will be firstly described. Pure cultures strategies to produce biodiesel, biobutanol and bioethanol will be then discussed.

4.2 Biogas

Anaerobic digestion is one of the proven technologies for converting organic waste into biogas. The generation of biogas, mainly a mixture of methane and carbon dioxide, is considered eco-friendly and contributes to the reduction of soil and water pollution [105], thus encouraging the circular economy [106]. The entire process of anaerobic digestion, from the recent biological innovations to downstream strategies to improve biogas production has been deeply reported by Tabatabaei *et al.* [107,108] who detailed and discussed the biological innovations and optimizations including upstream, mainstream, and downstream in biogas production from different feedstocks.

Methanogenesis is a complex process (Figure 2) that needs multiple reactions conducted by bacterial and archeal consortia under anaerobic conditions [106]. Insoluble organic compounds, mainly carbohydrates, proteins, and fats, are hydrolysed into soluble molecules, monosaccharides, amino acids, and fatty acids by extracellular enzymes synthesized by specific hydrolytic bacteria. Then, lactate, ethanol, propionate, butyrate, and higher volatile fatty acids (VFA) can accumulate and are converted to hydrogen by a specific microflora (Figure 2). In the following acetogenesis process, the acetate bacteria convert the acid phase products into acetic acid and hydrogen, used by methanogenic bacteria to produce methane [109,110] (Figure 2). Thus the syntrophic degradation of complex organic compounds to methane and carbon dioxide is a difficult process and requires the cooperation of diverse groups of microorganisms occurring in the natural environments and usually introduced in the industrial plants through specific inocula. Proportions of CO_2 and CH_4 in biogas are related to the degree of oxidation of carbon in the organic substrates [111]. Once biogas is generated, methane must be separated from carbon dioxide. As it is cost imposing process, methane yield in biogas is equally important.

The use of rice wastes to feed biogas plants has been proven feasible and sustainable, although anaerobic bacteria can hardly degrade lignocellulosic materials such as those contained in RS and RH (Table 2), due to the high C/N ratio, cellulose crystallinity, and great lignin content. The most important parameters controlling the efficiency and stability of anaerobic digestion are, among others, the chemical and physical characteristics of the substrate, the inocula and the feedstock to inoculum ratios, trace elements, C/N ratio, temperature, pH etc [111]. Operation temperature significantly influences reaction velocity. In general, the growth rate of microorganisms is best at mesophilic and thermophilic temperature ranges [112,113], killing most of the microbial pathogens [114] speeding up the digestion procedure, although the thermophilic system needs additional energy to sustain the higher temperature of the reactor [112]. Furthermore, at mesophilic temperatures, a wider range of microorganisms are

involved [115] and the entire process is more stable [116]. In addition, methanogenic bacteria are sensitive to thermophilic temperature and require time to adapt to higher temperature [117].

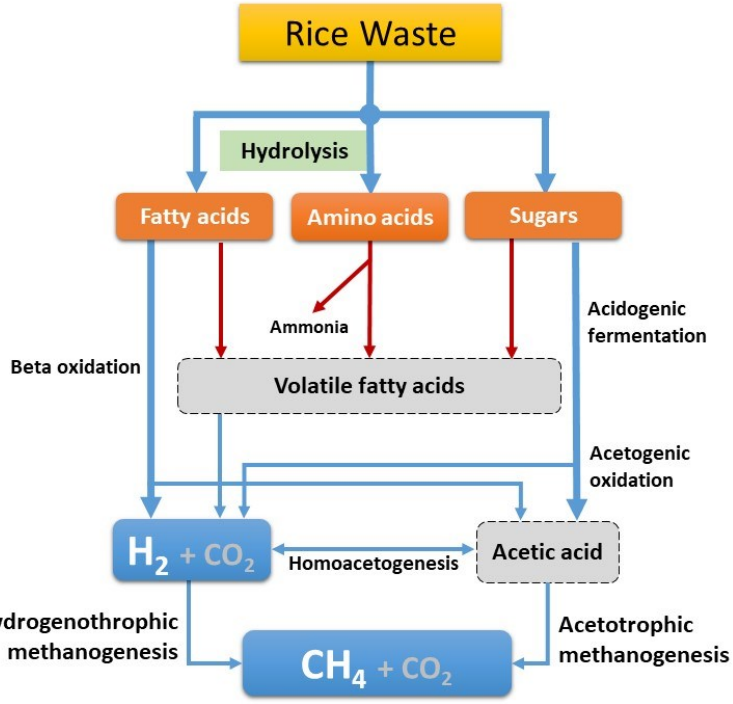


Figure 2: Steps of digestion of rice waste

C/N ratio is another key factor in anaerobic digestion [118] because an imbalance in C/N ratio could cause accumulation of VFAs or ammonia [119,120]. The recommended C/N ratio for hydrolysis is 16–45 and 20–30 for methanogenesis [121]. The C/N of RS reported in the scientific literature varies between 25 and 75 based on substrate origin and can be balanced by co-digesting with nitrogen-rich substrates such as pig urine, cow manure, and food waste [111]. Co-digestion of farm waste is the most applied method for increasing methane yields [122]. It is a treatment strategy in which several feedstocks are mixed [119] and thus it is a promising approach to balance the low C/N in the reactors. As an example, Ye et al. [65] suggested the co-digestion of RS with kitchen waste and pig manure as a promising approach to balance the low C/N ratio of lignocellulose biomass. Haider et al. [123] assessed the co-digestion of RH with food waste, using fresh cow dung as inoculum pointing out the substrate to inoculum ratio (S/I) as one of the key parameters.

As previously discussed, since the hydrolytic stage is usually considered the bottleneck mostly affecting the conversion rate of RS, many studies were focused on physical, chemical, and biological pretreatments, alone or in combination, aimed to improve hydrolysis (Table 4). Indeed, physical

pretreatments such as milling, extrusion, grinding, steam explosion and liquid hot water pretreatments, increase the accessibility of the substrates and reduce the degree of polymerisation and crystallinity of the cellulose. As an example, Chen *et al.* [88] evaluated the extrusion of RS compared to the milling. The authors demonstrated that the extrusion changed some physical properties of lignocellulose such as bulk density or porosity, thus enhancing the efficiency of bacterial cellulose and hemicellulose degradation. As a consequence, the digestion time of RS was shorter and methane yields increased. Biological pretreatment has great advantages because of fewer energy needs and does not generate toxic compounds [124]. Biological pretreatment mainly involves the use white, brown and soft rot fungi [125]. A biological approach treating RS with suspensions of *Pleurotus ostreatus* DSM 11191 and *Trichoderma reesei* QM9414 gave interesting outputs [95]. Although moisture content and incubation time affected the efficiency of the treatments, the fungal incubation significantly improved lignin removal as well as biogas and methane yields. In the work of Yan *et al.* [66], RS was firstly composted to facilitate the biodegradability of complex substrates and, then, treated in a solid-state anaerobic digester with anaerobic sludge as inoculum. After optimization of initial substrate concentration, temperature and C/N ratio, composted RS resulted to be more effectively degraded, thus increasing biogas yields.

Although biological pretreatments have undeniable advantages such as fewer energy requirements, specificity, or generation of fewer toxic compounds, they are expensive and need a long time and complex operating conditions [70]. Thus, to decrease operation time and enhance the biogas conversion efficiency of rice wastes, the utilization of acids or alkali, alone or in combination with physical pretreatments, is preferred. For example, Du *et al.* [87] reported that the alkaline thermal pretreatment of RS at mild temperature was more efficient than the hydrothermal in terms of lignocellulose decomposition and methane production. Kim and colleagues compared autoclaving the RS after the addition of H_2SO_4, with pretreatment with hot water and alkali [90]. However, although the highest lignocellulose decomposition was obtained by autoclaving after H_2SO_4 addition, the methane production potential was very low probably due to the inhibitory effect of the sulfate ion on methanogenesis, as reported previously [126]. The optimal process parameters for a combined synergistic pretreatment of RS with ammonia hydrochloride and ozone were also defined [91]. The combination of chemical and physical factors enhanced the enzymatic release of fermentable sugar and consequently biogas production.

Gu *et al.* [127] considered the role of inocula and found that digested manures (from dairy, swine and poultry) were more suitable than digested municipal, granular or paper mill sludges in increasing biogas production from RS. The effect of macro- and micro-nutrients on the performance of anaerobic digestion

of RS [128] and RH [129] was also studied. In small scale experiments, using cow rumen liquid and acclimated anaerobic sludge as inoculum, the supplementation with heavy metals, such as Ni^{2+}, Zn^{2+} and Cu^{2+}, improved biogas yield from RH [129], while methane production rate from RS was accelerated by optimizing phosphate levels (465 mg-P/L) [128]. The effect of organic loading rate (OLR) on the conversion of RS to biogas was explored in a 300 m^3 mixed bioreactor [130]. An increase in biogas was observed when OLR was below 2.00 kg $VS_{substrate}/m^3d$ while the maximum production rate was 323 m^3/t dry substrate. The monitoring of prokaryotic community structure in the plant during biogas production confirmed that the hydrogenotrophic and acetoclastic pathways are the most common in the digestion of lignocellulosic wastes to methane [131,132].

Overall, rice waste and, more specifically RS, have a great potential to generate biogas although it is necessary to adopt the appropriate pretreatments and inocula for the efficient utilization of the substrates and further research is needed to optimize mostly rice lignocellulosic substrates into biogas.

Table 4: Biogas production from rice wastes: main pretreatments, inocula and yields.

Feedstock	Pretreatments			Inoculum	Temperature	Biogas Yield[a]	Methane	Reference
	Physical	Chemical	Enzymatic or microbial		(°C)	mL/g VS	%	
RH	-	-	-	CRF	30	382	78	[129]
RH and FW	-	-	-	Acclimatized CD	37	584	-	[123]
RH and FW	Milling	-	-	AS and Pig manure	37	674	57	[65]
RS	-	Ozone, aqueous ammonia	Mixed Cellulases	DS	37	396	-	[91]
RS	Hydrothermal	Alkali	-	ADSS	37	411	49	[87]
RS	Milling	-	*Pleurotus ostreatus*	AS	37	353	73	[95]
RS	Autoclaving	Alkali or Acid	-	DS	35	932	-	[90]
RS	Milling	-	-	AS	37	227	-	[88]
RS	-	-	Composting	AS	35.6	447	-	[66]
RS	Milling	-	-	-	39	349	52	[130]
RS	Milling	-	*Pleurotus ostreatus* DSM 11191	AS	37	367	72	[95]
RS	Milling	-	*Trichoderma reesei* QM9414	AS	37	299	72	[95]
RS	Milling	-	-	DM	37	325	55	[127]
RS	Milling	-	-	Acclimatized AS	22 ± 2	340	77	[128]

[a] -Highest values of biogas reported (or calculated from available data) when available. FW- Food waste, DS- Digested sludge, ADSS- Anaerobically digested sewage sludge, AS- Anaerobic sludge, CD- Cow dung, CRF- Cow rumen fluid, DM- Dairy manure.

4.3 Biohydrogen

Biohydrogen can be obtained from carbohydrate-rich biomass by anaerobic (dark fermentation) and photoheterotrophic (light fermentation) microbes [133]. In recent years, biohydrogen has gained popularity as a clean fuel to reduce toxic gas releases. Like all other fuels, biohydrogen must be cost-effective as well. Though biohydrogen production can be performed by dark-, Photo- and combined (dark- and photo-), to the best of the author's knowledge, only the dark fermentation route was exploited to obtain hydrogen from rice waste streams. Baeyens *et al.* provided detailed insights of the different pathways adopted by bacteria for the production of biohydrogen [134]. Recent studies on combinative pretreatments of RS have to be considered as an emerging cost-effective, alternative energy technology [97]. The difference in composition of RS, RH, RB and cooked rice leftover waste requires a comparison between the effects of different temperatures on biohydrogen production potential, since for all rice biowaste, except for leftover cooked rice, a significant increase in biohydrogen yields was observed as the temperature increased [135]. Moreover, the concentration and particle size of the substrate were found to represent key parameters for determining the processing time. Similarly, hydrolysis time and concentration of additives were found to play an key role during the biohydrogen production from RS [96].

A further important aspect is concerning the nature and treatment of inocula, which are quite frequently obtained from anaerobic digestors. During anaerobic digestion, hydrogen is produced as an intermediate metabolite with hydrogen-producing and -consuming bacteria working together to obtain methane. To maximize hydrogen yield through dark fermentation, methanogens and hydrogen-consuming bacteria have to be inhibited. Several methods have been proposed to achieve this aim, including heat treatment, acidification, basification, freezing or dehydration [136–139]. Table 5 gives a summary of pretreatments of feedstocks, inocula and the corresponding biohydrogen yields. Along with biohydrogen yield, it is important to monitor the percentage of biohydrogen in the biogas, which ranged between 25-70 %.

Studies of heat treatment of inoculum were performed on activated sewage sludge and optimal results were obtained at 100^{o}C for 60 min [140]. However, at a C/N ratio of 25, the use of non-heat treated sewage sludge resulted in a biohydrogen production from RS higher than the yield obtained by heat-treated sewage sludge [141]. On the contrary, other studies suggest the importance of heat treatment of sludge in terms of the selection of hydrogen-producing microflora over methanogenic organisms. As an example, Chen and colleagues explored heat treatments of different sludges and cow dung compost used as inocula for untreated RS [142]. Maximum biohydrogen yields were obtained using heat-treated sludges from municipal waste treatment plants. Moreover, they demonstrated that the heat treatment enriched the inocula in both hydrolytic and fermentative bacteria [142]. This study further highlights the importance of heat treatment of sludge in

terms of the selection of hydrogen-producing microflora over methanogenic organisms. Unlike pre-treated mixed inocula, also single cultures approaches have been pursued to convert rice waste streams into hydrogen. Cellulolytic bacteria isolated from soil and observed that pure culture of *Clostridium butyricum* CGS5 gave efficient biohydrogen production using enzymatically hydrolysed RH as substrate [143]. A pure culture of *Clostridium acetobutylicum* YM1 was also adopted on an acid-treated starchy waste such as DRB (de-oiled RB) [144].

In concentrated acid-treated RS hydrolysate and wastewater from the food industry, the presence of *Clostridium pasteurianum* was found to support the production of biohydrogen using acetate and butyrate pathway. Also, a 1.5-fold increase in biohydrogen yield was observed with lower substrate utilization in a continuous system as compared to the batch reaction [145]. After confirming the increased biohydrogen production in a continuous system, Liu *et al.* [146] worked on the optimization of hydraulic retention time (HRT) of a continuously external circulating bioreactor, reporting that the highest hydrogen production rate was observed with an HRT of 4 h. The continuous production process also needs continuous organic loading. Therefore, studies on OLR optimization demonstrated that biohydrogen production from RS increased, reaching maximum biohydrogen production of 2.6 L per day when the range of OLR was between 7.1 and 21.4 g COD/L per day [147]. When rice waste is used as a substrate, OLR optimization and the augmentation with pure *clostridial* cultures, give a significant increment in biohydrogen production without any inoculum treatment thus making the process more economic. These studies demonstrate the necessity to develop more efficient microbes having the potential to produce higher biohydrogen yields.

Table 5. Biohydrogen production from rice wastes: main pretreatments, inocula and yields.

Feedstock	Pretreatment	Type of inoculum	Best inoculum treatment	T (°C)	Best H_2 Yield[a]	H_2[a] (%)	Reference
RH	Enzymatic	*Clostridium butyricum* CGS5	-	35	19.15 mmol/g reducing sugar	25	[143]
RS	Milling	ASS	100°C, 60min	35	14.67 mL/g VS	70	[140]
RS	-	SS	100°C, 15min	55	0.54 mmol /gVS added	42	[141]
		SS	-	55	0.74 mmol /g VS added	58	
RS	Milling	MWTPS	95°C, 40min	55	24.80 mL/g TS added	-	[142]
DRB	Acid	*Clostridium acetobutylicum* YM1	-	35	117.24 mL/g consumed sugars	-	[144]
RBDW	-	SHS	100°C, 60min	57	2.20 mol /mol substrate	42	[148]

[a] -Highest values of hydrogen yield or percentage are reported (or calculated from available data) when available. ASS-Activated Sewage Sludge; DRB-Deoiled rice bran; RBDW- Rice Bran De-oiled wastewater; SHS- Slaughterhouse Sludge; SS- Sewage sludge; MWTPS- Municipal wastewater treatment plant sludge

4.4 Biodiesel

Biodiesel refers to fatty acid methyl ester (FAME) produced through the transesterification of oils, mainly obtained from specific energy crops such as rapeseed, RB, sunflower, palm and soy, but even from animal fats or waste oils [149,150]. In addition, specific oleaginous microorganisms have been selected and proposed for the sustainable production of lipids as already elegantly reviewed [151,152]. Oleaginous yeast, bacteria, and microalgae are defined as microorganisms with an intracellular lipid content exceeding 20 % and reaching up to 70 %. Lipids accumulation usually starts when a nitrogen source is limiting but in the presence of an excess of carbon, which will be converted into triacylglycerols [153]. In the perspective of reducing biodiesel costs, residues from rice could be profitable substrates for microbial biomass and lipids production. For this purpose, rice starchy or lignocellulosic wastes have been assessed as feedstocks by few research groups. Since the employed microorganisms are generally lacking specific hydrolytic enzymes, again lignocellulose or starch hydrolysis was found to be necessary as well as the optimization of fermentation conditions. RS and rice food waste were mostly adopted so far as feedstocks for lipids production (Table 6).

Azad *et al.* [85] optimized pH values of a fermentation broth containing H_2SO_4-hydrolysated RS as a carbon source for *Lipomyces starkeyi,* and found that the yeast accumulated microbial lipids up to 36.14 % of cell dry weight (CDW). Diwan *et al*. [154] developed an effective H_2SO_4 based mild saccharification of RS and successfully employed the crude, non-detoxified hydrolysate for the growth of the yeast *Mortierella alpina* MTCC-6344 that accumulated lipids up to 40 % of CDW. A different approach was pursued by using the amylolytic oleaginous yeast *Sporidiobolus pararoseus* KX709872 [155]. This strain produces α-amylase and amyloglucosidase, and was used to directly convert canteen rice residues into biolipids in both flasks and stirred tank bioreactor without previous starch hydrolysis. After broth optimization, lipids reached 56.61 % of CDW. Moreover, the produced fatty acids contained high oleic content (60-62 %) similar to those of vegetable oil, indicating that these lipids could be a promising alternative to plant fats.

Another methodology was tested by exploiting *Cryptococcus curvatus* ATCC 20509 ability to accumulate lipids from RS. Firstly, RS was treated with NaOH and anaerobically digested using sewage as inoculum. Resulting VFAs were then used by *C. curvatus* ATCC 20509 as building blocks for the synthesis of lipids (up to 26 % CDW). The authors also assessed the techno-economical viability of their process, concluding that VFAs broth from anaerobic digestion of RS, compared to synthetic VFAs, appeared the most suitable carbon source for lipids production [153]. Microalgae have also been considered promising for biodiesel production due to their short cell cycle, ability to adapt to harsh

environments, and high oil content (up to 80 % CDW). Moreover, algae can be grown in fermentors without occupying cropped areas. Although algal biodiesel has still a price higher than conventional diesel which makes large-scale industrial applications not economically sustainable, attempts were made to reduce costs, such as using cheap carbon sources. For this purpose, Li *et al.* [156] used RS hydrolysate to support the fast-growing alga *Chlorella pyrenoidosa* MTCC-6344 which accumulated lipids up to 56.3 % CDW. The following *in situ* transesterification obtained promising results with 95 % biodiesel yield.

Table 6. Biolipids production from rice wastes: main pretreatments, microbes and yields.

Feedstock	Pretreatment			Microorganism	T	Lipids	Reference
	Physical	Chemical	Enzymatic/microbial		(°C)	(%CDW)	
RS	Microwave, Autoclaving	4.8 % NaOH, 1.5 % H_2SO_4	-	*Mortierella alpina* MTCC-6344	25	40	[154]
RS	-	1 % Trifluoracetate at 95°C	Cellulsae	*Chlorella pyrenoidosa* MTCC-6344	25	56	[156]
RS	Autoclaving	2 % NaOH	Synthesis of VFA by anaerobic digestion	*Cryptococcus curvatus* ATCC 20509	25	28	[153]
RS	Autoclaving	3.5 % H_2SO_4	-	*Lipomyces starkeyi*	30	36	[85]
RS	Gamma ray irradiation	1 % NaOH	Cellulase	*Chlorella protothecoides* strain 25	-	45	[92]
RRC	-	-	Gluco-amylase & α-amylase	*Sporidiobolus pararoseus* KX9872	22.4	57	[155]

RRC- Rice residues from canteen.

4.5 Biobutanol

Biobutanol is less popular among clean fuels although it represents a good alternative to fossil fuels, due to its unique features such as high energy content, improved heating value, and reduced corrosive action [157]. Moreover, it can be blended with gasoline with a proportion higher than ethanol. Butanol is largely used as an industrial intermediate, particularly for the manufacture of butyl acetate and other industrial chemicals, as a flavour in many food and beverage industries, or as an extractant for various manufactured chemicals and pharmaceuticals. Industrially, butanol is mainly produced via petrochemical synthesis (Oxo process) although biological synthesis is also possible and, for food safety reasons the butanol used in the food industry must be obtained only by microbial fermentation [158]. Biobutanol can be manufactured by the fermentation of glucose by anaerobic clostridia performing the acetone, butanol, ethanol (ABE) metabolism. The ABE catabolism involves a first acetogenic step generating acetic and butyric acids, CO_2, and hydrogen, and a second step (solventogenic) in which acetone, butanol, and ethanol are produced from the acids [159]. Butanol fermentation is much less efficient compared to ethanol fermentation. Therefore, great amounts of energy are necessary for product recovery from the diluted broth. This, together with the substrates cost, makes the entire process non-sustainable [160]. Thus, many efforts have been devoted to improve the efficiency of the process or decrease the costs of the raw material supporting microbial growth.

Rice wastes, especially RS, have a great potential to be efficiently used as a carbon source for butanol. Again, the use of such low-cost feedstock requires pretreatments, subsequent enzymatic hydrolysis to obtain fermentable sugars, and/or butanol-producing strains able to proficiently metabolize the released sugars, such as xylose together with glucose, into butanol (Table 7). The sulphuric or phosphoric acids or alkali pretreatments of RS are reported as cheap and effective, and thus have been extensively evaluated [32,159–164]. Once obtained, the sugars are utilized by specific clostridia to perform the ABE fermentation, with a yield of 2.0-18 g/L. Chen *et al*. [75] assessed a synthetic non-pretreated enzymatically hydrolysate from RS, under non-sterile conditions minimizing the contaminants interference by increasing the initial cell concentration of *C. sacchaperbutylacetonicum*. Such conditions ensured not only the biobutanol production in a non-sterile environment but demonstrated that the sterilization step of the agricultural wastes used as substrate can be avoided, thus reducing manufacturing cost.

While various research groups focused on the optimization of pretreatment and hydrolysis, others concentrated on fermentation modes. Parameters, such as initial pH, temperature, age and size of the inoculum, and the agitation rate, were optimized for the butanol production from pre-optimized RS

hydrolysate [165]. Gottumukkala and coworkers fine-tuned ABE fermentation parameters (i.e., pH, inoculum concentration and calcium carbonate concentration) resulting in enhanced biobutanol yields from a detoxified enzymatic hydrolysate of acid pretreated RS by *Clostridium sporogenes* BE01 [166]. Although not considered as efficient butanol producer in comparison with commercial strains such as *C. acetobutylicum*, *C. sporogenes* BE01 reached a maximum butanol concentration of 5.52 g/L in optimized conditions, one of the highest reported for this species. Moreover, this strain produced ethanol and butanol without acetone in the final mixture which is considered an advantage in the industrial bioconversion of biomass to alcoholic fuels [167]. To decrease the cost of the enzymes and increase sugar utilization and biobutanol production, Chi *et al.* [168] proposed a staged acidogenic/solventogenic fermentation process. In this study, alkaline-pretreated RS was firstly fermented by a microbial consortium of *Clostridium thermocellum* and *Clostridium thermobutyricum* to both hydrolyze lignocellulose and enrich the system with butyric acid. The resulting supernatant was used for ABE fermentation by *Clostridium beijerinckii* NCIMB8052. This strategy resulted in higher butanol production when compared to a conventional SHF (Separated Hydrolysis and Fermentation) process involving the use of commercial cellulases in the lignocellulosic hydrolysis step followed by the fermentation.

The development of a bioprocess for direct butanol production from cellulosic biomass was pursued by Wang et al.[169]. Although strains of clostridia have been reported to produce butanol from various substrates such as agricultural residues, none of them can directly convert cellulose into butanol. For this reason, the authors obtained butanol from filter paper developing a co-culture systems of the efficient butanol producer *Clostridium acetobutylicum* ATCC824 with a newly isolated *Clostridium celevecrescens* N3-2 strain or with a stable undefined consortium. Thus this strategy could be a simplified approach for direct conversion of cellulose to biobutanol and could be efficiently used also with other lignocellulosic substrates such as rice wastes.

Table 7. Biobutanol production from rice wastes: main pretreatments, microbes and yields.

Feedstock	Pretreatment			Microorganism	T	Biobutanol Yield[b]	Reference
	Physical	Chemical	Enzymatic/microbial		(°C)	(g/L)	
RS	Autoclaving	4 % H_2SO_4, Detoxification	Cellulase	*Clostridium. sporogenes* BE01	35	5.52	[166]
RS	Milling, Autoclaving	1 % H_2SO_4	-	*Clostridium acetobutylicum* NCIM 2337	37	13.50	[159]
RS	Temperature	1 % NaOH	Cellulase, *Clostridium thermocellulm* ATCC 27405, *Clostridium thermobutyricum* ATCC 49875	*Clostridium beijerinckii* NCIMB 8052	37[a] & 55[a]	15.90	[168]
DRB	Autoclaving	1 % H_2SO_4, Detoxification	-	*Clostridium acetobutylicum* YM1	30	6.87	[162]
DRB	Autoclaving	1 % HCl or H_2SO_4, Detoxification	Cellulase	*Clostridium saccharoperbutylacetonicum* N1-4	30	7.72	[161]
SRSH	-	-	-	*Clostridium saccharoperbutylacetonicum* N1-4	35	6.60	[170]

DRB- Deoilded rice bran, SRSH- Synthetic rice straw hydrolyzate. [a] -Temperature adopted for lignocellulosic hydrolysis by *Clostridium thermocellulm* ATCC 27405 and *Clostridium thermobutyricum* ATCC 49875, [b]- Best biobutanol yield.

4.6 Bioethanol

Although bioethanol is considered the most promising liquid biofuel potentially obtainable from rice waste streams (Table 2), its commercialization would be possible only if the cost of the entire process, from feedstock collection and treatment to the attainment of the final product, will be sustainable [171]. This would be possible by (i) firstly reducing the number of steps (Figure 3), i.e. by clubbing them together in a single vessel, (ii) by reducing as much as possible the use of extra reagents such as commercial enzymes, (iii) by shortening the processing time. In addition, fermentation efficiency represents another key factor directly linked to the available microorganisms used in the bioreactor. Further strategies are being applied in which organisms were genetically modified to produce enzymes for saccharification and fermentation, or consortia of different organisms or commercially available enzyme cocktails were used. In terms of fermentation effectiveness, *Saccharomyces cerevisiae* is the main candidate, even if several strains proved not capable of tolerating the inhibitors formed during pretreatments. Hence, detoxification of the resulting hydrolysates is needed or tolerant strains have to be developed [172,173]

This section reviews the following strategies available for the production of bioethanol from rice waste streams:

1. Separate Hydrolysis and Fermentation (SHF)
2. Simultaneous Saccharification and Fermentation (SSF)
3. Consolidated Bioprocessing(CBP)

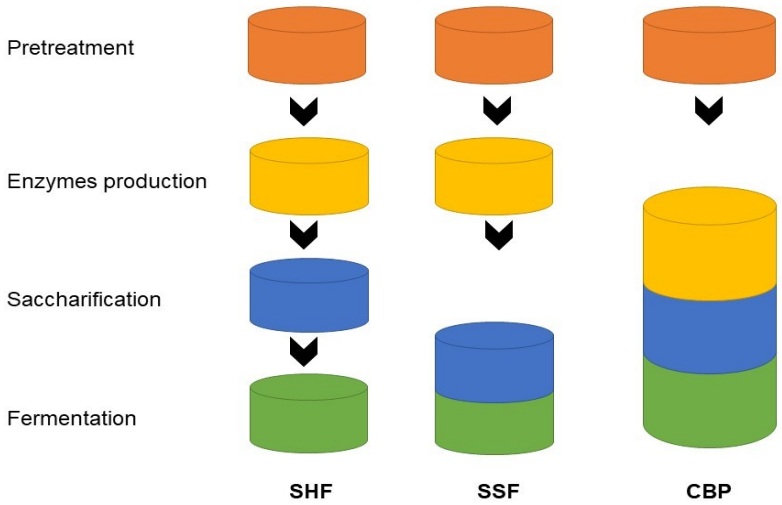


Figure 3: Graphical representation of process integration in SHF, SSF, CBP approaches to produce biofuels and other valuables products from biomass

4.6.1. SHF for Bioethanol

Through this method, enzymatic hydrolysis and fermentation are performed in sequence (Figure 2). Positive aspects are (i) the different optimal temperatures required by the two steps of the process can be optimized separately, (ii) the use of enzyme cocktails demands for different pHs, (iii) the whole design of the equipment, including stirring, can be organized independently [174–176]. Beyond several positive aspects, there are also some negative sides such as (i) this process requires considerable capital investments as more than one vessel must be involved, (ii) it is generally more time-consuming as the two steps are done separately, (iii) the increasing sugar concentration produced by cellulases activity leads to inhibit the enzyme action itself, (iv) in the pretreated biomass slurry several inhibitors are generally present, which may hinder the cellulases. These aspects will increase the final cost of the process [174–176].

Taken together, the above considerations gave rise to the limited number of SHF applications in the last decade, even if some interesting reports are available on several rice waste substrates (Table 8). For instance, some SHF approaches used enzymatic cocktails containing xylanase and pectinase on pretreated RS using ammonia fiber expansion (AFEX). The combination with *S. cerevisiae* in separate fermentation produced more than 175 g EtOH/kg treated RS. Interestingly, this ethanol yield was achieved even though pretreated biomass was not washed, detoxified, and added with supplemental nutrients. Fermentation of such hydrolysate with two *P. stipitis* strains also gave appreciable results in terms of g ethanol/L [177]. Abedinifar *et al.* [63] after investigating on optimal pH and temperature for commercial cellulase and β-glucosidase, reported that SHF could be efficiently adopted by using diluted acid pretreated RS. They also reported that the filamentous fungus *M. indicus* can perform at the same level as *S. cerevisiae* in terms of growth and ethanol yield. Moreover, filamentous fungus can convert pentoses into ethanol and produce chitosan, an interesting byproduct.

Saha *et al.* [178] worked with rice hull (RH) pretreated with alkaline peroxide and hydrolysed with a three enzyme cocktail containing cellulase, β-glucosidase and xylanase. This procedure resulted in a sugar yield of 90 %, without the release of any furfural and hydroxymethylfurfural into the medium, increasing up to 96 % by separately saccharifying the liquid and solid fractions. In that case, the fermentation step was performed using a recombinant strain of *E. coli* with noticeable ethanol production (Table 8). Biological pretreatments were proposed as promising alternatives to severe thermo-chemical applications on RS by the use of a white-rot fungus coupled to steam at 121°C [93]. The saccharification

efficiencies between the two approaches resulted to be very similar, but in the case of thermo-chemical strategies, the following *S. cerevisiae* fermentation resulted in low ethanol production, thus indicating the presence of inhibitory compounds within the hydrolysates that need to be detoxified.

When the complete process of fermentation is taken into consideration along with all the parameters involved (Table 8), detoxification of pretreated biomass resulted in a significant increase in bioethanol production.

The ethanol production from lime-pretreated and enzyme-hydrolysed RH was reported by Saha *et al.* [71]. These Authors used a recombinant *E. coli* FBR5 strain for both SHF and SSF and found that the total time to obtain the final product was shorter for SSF as saccharification and fermentation were simultaneous, while the SHF approach worked better in terms of fermentation time as saccharification was already done in the step before fermentation. However, one of the main benefits deriving by the use of lime could be the avoiding of inhibitors, completely absent in the resulting fermentation substrate. Unfortunately, the reported conversion yield seems to be still too low.

Table 8: Bioethanol production from rice waste streams using SHF technology.

Feedstock	Pretreatment			Organism	Fermentation time	Concentration[a]	Reference
	Physical	Chemical	Enzymatic/microbial		(h)	g/L	
RH	-	Alkali	Cellulase β-glucosidase Xylanase	*Escherichia coli* FBR5[c]	19	9.8	[71]
RH	-	Alkali peroxide	Cellulase β-glucosidase Xylanase	*Escherichia coli* FBR5[c]	24	8.2	[178]
RS	Milling, Autoclaving	Acid	Cellulase β-glucosidase	*Saccharomyces cerevisiae*	25	37	[63]
RS	-	Alkali	Cellulase β-glucosidase	*Clostridium acetobutilicum* NRRL B-591	80	2	[163]
RS	Ultrasound	Acid	*Trichoderma reesei*	*Saccharomyces cerevisiae*	168	11.0	[179]
RS	Milling, Autoclaving	-	Cellulase *Trametes hirsuta*	*Saccharomyces cerevisiae* LN	48	1.1	[93]
RS	Autoclaving	Alkali	Xylanase Pectinase Cellulase	*Saccharomyces cerevisiae* *424A(LNH- ST)*	144	37.0	[177]

[c] – GMO, [a]- Highest values of bioethanol are reported (or calculated from available data) when available

4.6.2. SSF for Bioethanol

As also reported in 4.6.1, the SHF has evolved and later compared to the SSF approach as an alternative procedure that is generally more effective [176,180]. In SSF, the same vessel is used for both saccharification and fermentation with the original objective to reduce both the equipment costs and the possible contamination of the cell suspension (Figure 3). The two steps are indeed occurring simultaneously and, as a further resulting advantage, the process time is reduced. In addition, the possibility to select enzymes usually working at room temperature can reduce or completely eliminate heating and cooling costs (Figure 4). Together with the removal of end-product inhibition of the saccharification process, these are the main reasons leading to devote more and more attention to SSF. Table 9 summarise the organisms, the conditions and the yield obtained by using SSF technology. Overall, substrate loading is a pivotal parameter in SSF setting, with the highest substrate loadings supporting the highest ethanol concentrations.

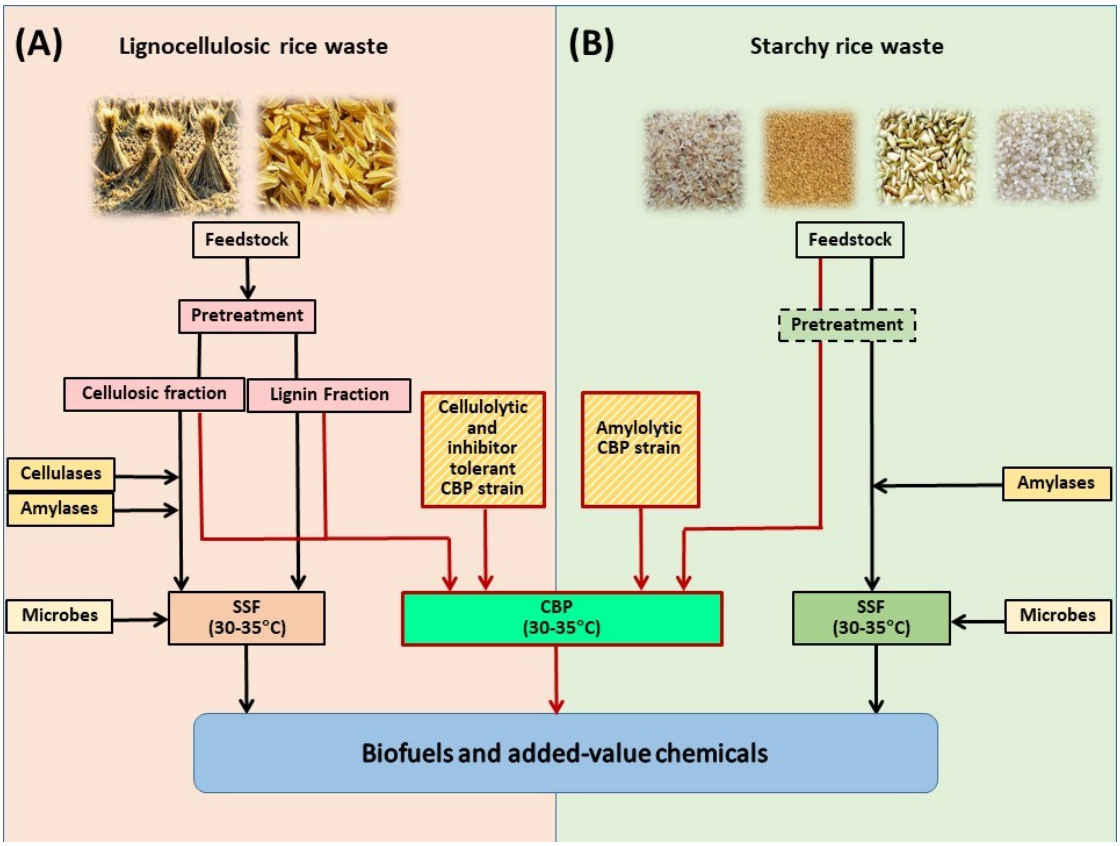


Figure 4: Detailed representation of the SSF and CBP approaches for the processing of A) lignocellulosic rice feedstocks and B) Starchy rice feedstocks for the production of biofuels and other added-value products.

Some studies indicated that inhibitor-free hydrolysates could be obtained from rice waste streams under specific conditions. For instance, Diwan *et al.* [154] optimized the hydrolysis process of RS by an experimental design with variable factors (duration, acid concentration, solid loading percentage, temperature) and found that the non-detoxified hydrolysate did not contain any furfural and hydroxymethylfurfural, thus supporting the growth and the metabolic activities of *M. alpina* much better than the detoxified hydrolysate (Table 6). Although the original objective of this work was the production of lipids, this hydrolysate could be efficiently used for alcoholic fermentation. Another efficient strategy to produce a sugar-rich hydrolysate that does not require a detoxification step, and hence simultaneously suitable as a fermentation medium, has been reported by Castro *et al.* [72] for RS processing, through SSF by *Kluyveromyces marxianus* NRRL Y-6860. In this case, a dilute acid pretreatment was preceded by biomass deacetylation, with the result to improve the recovery of both pentose and hexose sugars and the consequent ethanol production.

Another interesting attainment, carried out at 38°C for 48h, was described for RS by Poornejad *et al.* [77]. The ethanol production yield was improved if the straw was treated with NMMO and 1-butyl-3-methylimidazolium acetate ([BMIM][OAc]), respectively. The reduction of crystallinity by these two solvents was the main reason since glucan conversion yield increased from 28 % of the untreated straw to 96 and 100 %, respectively. Zhu *et al.* [74] optimised SSF to ethanol for RS pretreated with 1% NaOH or a combination of microwave and 1 % NaOH by using cellulases from *T. reesei* and *S. cerevisiae* YC-097 as fermenting yeast. They demonstrated that the microwave application improved the conventional alkali pretreatment. The reduction of high heating energy costs for liquefaction and saccharification was also proposed [181]. They used rice wine cake as feedstock for SSF without cooking and raw-starch-digesting enzyme prepared from *Rhizopus* sp. SSF conditions were optimized for *S. cerevisiae* in terms of incubation temperature, pH, fermentation time, and inoculum size. The effect of several additives such as nitrogen sources, surfactants and metal salts were also studied. The selected optimal SSF conditions resulted in ethanol production improvement within 90 hours of fermentation at 30°C.

A comparison between two filamentous fungi (*Rhizopus oryzae and M. indicus*) and a thermotolerant yeast strain of S. cerevisiae, was performed in terms of ethanol production in a SSF of RS [182]. The advantages of using the filamentous fungi are that they can grow at higher temperatures than *S. cerevisiae*, thus approaching the optimum for SSF process, and finally resulting in higher ethanol yield. By quantitative NMR screening methods, Wu et al. [183] investigated the different compositions of the pretreatment liquors deriving from RS and RH, and their consequences on SSF. High-pressure microwave processing was applied in combination with a range of severities, and among a number of

different compounds, they found that while fermentation inhibitors, such as hydroxymethylfurfural and furfural, were more present in husk liquor, formic acid was higher in straw liquor. The ethanol production from alkali-treated (NaOH) RS in a SSF process was reported by Oberoi et al. [184]. They used for the first time the recombinant *Pichia kudriavzevii* HOP-1 thermotolerant strain, producing ethanol at amounts comparable to those produced by *S. cerevisiae*. Further interesting investigations by coupling alkali pretreatment of RH with the use of zygomycetes fungi (*M. hiemalis*) for the production of ethanol, was performed [185]. The alkali pretreatment enables to increase the low ethanol yield generally obtainable (around 15 %) to more than 85 %, as a consequence of lignin removal and cellulose crystallinity decrease. On the other hand, the use of *M. hiemalis* resulted in ethanol yield higher than *S. cerevisiae*, probably due to its high resistance against the inhibitors and to the utilization of pentoses, and also resulted in the production of other value-added proteins and lipids. The same filamentous zygomycetes *M. hiemalis* was used by SSF in combination with sodium carbonate pretreatment [186]. The use of this chemical enabled to remove the high silica content from RS and consequently to enhance enzymatic hydrolysis and ethanol production by the fungus, that proved once more to perform better than *S. cerevisiae*. On BR, Gronchi et al. [100] found a great potential as ethanol producers by newly isolated yeast strains, performing better in a SSF than other well-known benchmark strains. This approach can be followed even with the objective to find superior outperforming phenotypes to be further selected at bioreactor scale for specific feedstocks and also in view of the construction of a recombinant strain for CBP.

Table 9. Bioethanol production from rice waste streams using SSF technology.

Feedstock	Chemical Pretreatment	Substrate Loading[a]	Enzymatic/Microbial Saccharification	Organism	Concentration[c]	Reference
					g/L	
RH	Alkali	5 % (w/w)	Cellulase	*Mucor hiemalis* CCUG 16148	9	[185]
			β-glucosidase	*Saccharomyces cerevisiae* Thermosacc®	6	
RS	Acid	15 % (w/v)	Cellulase	*Rhizopus oryzae*	12	[182]
				Saccharomyces cerevisiae	10	
				Mucor indicus	16	
RH	Acid	5 % (w/w)	Cellulase	*Saccharomyces cerevisiae* NCYC2826	4	[183]
RS	Acid	5 % (w/w)	Cellulase	*Saccharomyces cerevisiae* NCYC2826	7	[183]
RS	dAT	10 % (w/v)	Cellulase	*Kluyveromyces marxianus* NRRL Y-6860	20	[72]
RS	Alkali	5 % (w/v)	Cellulase	*Mucor hiemalis*	13	[186]
			β-glucosidase			
RS	NMMO	5 % (w/w)	Cellulase	*Saccharomyces cerevisiae* CCUG 53310	14	[77]
			β-glucosidase			
RS	Alkali	10 % (w/v)	Cellulase	*Pichia kudriavzevii* HOP-1[b]	24	[184]
			β-glucosidase			
			Pectinase			
RS	Alkali	60 % (w/v)	Cellulase	*Saccharomyces cerevisiae* YC-097	18	[74]
BR	-	20 % (w/v)	α-amylase	*Saccharomyces cerevisiae* L20	107	[100]
			glucoamylase			
RWC	-	77 % (w/w)	*Rhizopus sp.*	*Saccharomyces cerevisiae* KV25	133	[181]

Nr- Not reported; dAT- deacetylationAcid pretreatment; NMMO- N-methyl morpholine N-oxide; [a]- for pretreatment, [b]- GMO, RWC- Rice waste cake, [c]- Highest values of bioethanol reported (or calculated from available data)

4.6.3. CBP for bioethanol

The CBP of biomass into bioethanol is gaining increasing recognition as a potential breakthrough for low-cost biomass processing [187–189] where a single microbe is able to process one-step pre-treated feedstocks (Figure 3). A four-fold reduction in the cost of biological processing and a two-fold reduction in the overall production cost is projected when a mature CBP yeast will be available [172,188,190]. A CBP approach was proposed also from cellulosic- and starch-rich rice streams (Table 10), using engineered *S. cerevisiae* strain specifically developed for co-expression of efficient cellulases or amylases (Figure 4). Specific efforts were focused on RS, once pretreated with hot water (80°C, 16 h), which was converted into ethanol by the *S. cerevisiae* strain MNII/cocδBEC3 co-producing β-glucosidase, endoglucanase and cellobiohydrolase tethered to the cell surface [191]. Although the enzymatic activities of the CPB strain were promising, the ethanol levels obtained from 100 g/L HWP RS were low (with 33 % of the theoretical yield), pointing out that both substrate loading optimization and harsher pre-treatment conditions were the most important drivers towards higher ethanol yields. The same group indeed applied heavier pre-treatment on RS (Liquid Hot Water method, 130-300°C under the pressure of less than 10 Mpa). The resulting hydrolysate was converted into ethanol by the CBP *S. cerevisiae* strain MN8140/XBXX able to hydrolyzed hemicellulose by co-displaying the endoxylanase from *T. reesei*, the β-xylosidase from *R. oryzae* and the β-glucosidase from *Aspergillus aculeatus* and to assimilate the released xylose through the expression of *P. stipites* xylose reductase and *S. cerevisiae* xylitol dehydrogenase. The ethanol concentration reached was 8.2 g/L after 72 h fermentation, with an ethanol yield close to 82 % of the theoretical [192].

CBP applications were found to be very efficient in the case of starchy rice by-products such as RB, BR, UR and DR (Table 9). Two yeast strains, M2n[TLG1-SFA1] and MEL2 [TLG1-SFA1] co-expressing the glucoamylase TLG1 from *Thermomyces lanuginosus* and the α-amylase SFA1 from *Saccharomycopsis fibuligera,* previously reported for their promise as raw starch converting microbes [55] were effectively adopted to achieve high ethanol levels (Table 10). The higher the starch content (RB>UR>BR and DR), the higher ethanol concentrations were produced. Noteworthy, even higher ethanol levels were recently obtained by applying efficient amylolytic CBP strains on broken rice (20 % w/v). Two strains *S. cerevisiae* ER T12 and *S. cerevisiae* M2n T1, simultaneously secreting an α-amylase and glucoamylase originating from *Talaromyces emersonii*, were adopted in a CBP setting [53]. No substrate pre-treatment was needed, and the final alcohol titers (100 g/L) indicated that this process can be industrially viable.

Table 10. Bioethanol production from rice waste streams using CBP technology.

Feedstock	Physical pretreatment	Substrate loading % (w/v)	*Saccharomyces. cerevisiae* strain	Fermentation time (h)	Concentration (g/L)	Reference
RS	Milling,Thermal	100	MNII/cocδBEC3	72	8	[191]
RS	Autoclaving	80	MN8140/XBXX	72	8	[192]
BR	Milling	20	ER T12	168	101	[53]
			M2n T1		100	
BR	Milling	20	M2n[TLG1-SFA1	144	75	[61]
			MEL2[TLG1-SFA1]		68	
DR	Milling	20	M2n[TLG1-SFA1	144	79	[61]
			MEL2[TLG1-SFA1]		42	
RB	Milling	20	M2n[TLG1-SFA1	144	39	[61]
			MEL2[TLG1-SFA1]		68	
UR	Milling	20	M2n[TLG1-SFA1	144	66	[61]
			MEL2[TLG1-SFA1]		61	

4.7 Microbial Fuel Cell

Electricity is one of the most important energy forms that support most of the human activities. Recently, a new, future-promising segment has been added, i.e. electrical vehicles. Many personal cars and public transports are shifting to electricity run vehicles as they are more economical and less polluting. However, the current electricity supply is mostly based on thermal power, generated by coal burning, which unfortunately contributes to environmental pollution. To cope with this excessive demand, it is essential to find a renewable and non-polluting electricity source. Current studies indicate microbial fuel cell (MFC), as a possible future contribution. It is a strategy exploiting bacterial metabolism to generate electricity from a range of bio-wastes. The interest in this technology raised when the possible future use of the high producing bacterial strain *Geobacter sulfurreducens* KN400 was reported in 2009 by Time Magazine as one of the top 50 most important inventions [193].

MFC could be considered as a bioreactor with two chambers, an anode and a cathode separated by a PEM. Electrons, generated at the anode, move to the cathode through an external circuit and protons travel to cathode through PEM, where they combine with oxygen and electrons to form water molecules [194]. Few experiences on MFC exploiting rice by-products are available in the literature (Table 11). The PEMs used in MFC are generally polymeric membranes like Nafion, expensive and susceptible to fouling after repeated usage. Mashkour et al. compared Nafion to polymeric membranes modified with hydrophilic and antibacterial nanoparticles which lead to higher proton conductivity although more expensive [195]. Studies showed that blending of 10 % RH ash with soil to fabricate ceramic PEM gave higher volumetric power density as compared to that of control when rice mill wastewater was used as substrate and anaerobic sludge collected from the sediment of a pond was used as inoculum [80].

RH charcoal was also used as anode and cathode electrodes for MFC, showing the potential of RH to be used not only as a carbon source for microbes but also in the construction of MFC [196]. Jiao *et al.* [197] indicated that the power density is influenced by the surface area of the carbon electrode, i.e. porosity, used in MFC. Rezaei *et al.* [198] demonstrated for the first time that it was possible to generate electricity using MFC with cellulose as a carbon source and a single strain of *Enterobacter cloacae*. On the other hand, single strain, i.e pure culture of *S. cerevisiae*, did not give promising results if maximum power density is compared with mixed cultures and consortium [199]. When non-pretreated RS was used as a substrate and the mixed culture of cellulose-degrading bacteria as inoculum, MFC could generate power density up to 145 mW/m^2. When the same MFCs were connected in series, the power density increased more than three times. After an initial lag period of 110 h, the stable power density was

maintained for 10 days. The refuelling of the cell was done three times with a medium containing 1 g/L of RS and no lag period was observed, indicating that such MFCs can utilize RS for the production of energy [200].

RB was also used as a carbon source in single-chambered MFC inoculated with paddy field soil. The power density increased drastically when a mineral solution was used as liquid phase instead of pure water along with RB. The amplicon-sequencing showed the presence of *Geobacter* spp. at anode biofilm. The same MFC was continuously adopted for 130 days supplementing the system with RB after 10-20 days [201]. Phylogenetic analysis reveals the presence of a mutualistic behaviour between *Bacteroides, Clostridium* spp. and *Geobacter* spp. in the anode biofilm [202]. On the other hand, when pond bottom sludge was exposed to air, it gave higher volumetric power density as the methanogenesis was affected due to aeration. Schievano *et al.* [203] highlighted that rice waste streams can be usefully exploited in MFC applications. This is of great importance considering that the electricity can be obtained from MFC adopting the biorefinery approach after production of gaseous biofuels, such as biohydrogen and biomethane, from organic waste.

In the case of rice biowaste utilization using microbial fuel cells, pretreatment of the substrate does not improve the production of electric process integration (Table 11). Schievano et al. used rice waste streams for the production of gaseous fuels like biohydrogen as well as biomethane and then utilized the residues for MFC using a microbial consortium. This approach appears as the most efficient way to utilize the rice waste streams, as it depicts the biorefinery approach, also providing 477 mW/m^2 of power density. Moreover, the expensive PEM can be changed by ceramic membrane reinforced with RH to further reduce the cost.

Table 11. Production of electricity using microbial fuel cell from rice waste streams.

Feedstock	Pretreatment		Inoculum	Resistance applied	Power Density	Reference
	Physical	Chemical		Ω		
RH	-	Acid, Alkali	AS	1000	318 mW/m^2	[197]
RS	-	-	Consortium	1000	145 mW/m^2	[200]
RS	Milling	-	CDSM	1000	190 mW/m^2	[204]
RB	-	-	PFS	10000	520 mW/m^2	[201]
RB		HC	PB Mud	510	17 mW/m^2	[202]
RB	-	-	SM	500	477 mW/m^2	[203]
Rice washing water	-	-	*Saccharomyces cerevisiae*	320	1 mW/m^2	[199]
Rice mill wastewater	-	-	PB sludge	100	656 mW/m^3 ^{V}	[205]

AS- Anaerobic sludge, PFS- Paddy field soil, PB- Pond bottom , SM- Swine Manure, HC- Hydrodynamic cavitation, CDSM- Cellulose degrading soil microflora, ^{V}- Volumetric power density.

5. Biorefining of rice waste streams into added-value products

To ensure the cost-effective exploitation of rice waste streams, it is essential to recover all the potential co-products together with lower-value products such as bioethanol. As such, the overall process economics will be greatly improved. Once the cellulosic or starchy rice residues are hydrolyzed to monomers (ie, sugars, amino acids, fatty acids, etc.), the latter can serve as a feedstock for biological fermentation or chemical processing to various chemical building blocks. Besides biofuels, potential fermentation products from rice waste could be enzymes [206,207], biopolymers [208], organic acids [209–211] and vitamins [212]. Nevertheless, it is a hallmark to integrate processes for a mixture of products in a biorefinery setting to ensure the economic viability of a specific by-product [213,214]. For example, techno-economic modelling for the integrated waste streams-to-biofuels routes developed by IEA (International Energy Agency) demonstrated a positive outcome when 80 % of the hexose sugars were processed to bioethanol and 20 % to lactic acid [215]. Furthermore, the efficient integration of biorefineries into existing industrial plants can considerably contribute towards a sustainable bioeconomy [216]. This is particularly true in the case of rice milling residues which could be valorized into biofuels and higher values products nearby the paddy rice processing, thus reducing cost and greenhouse gas emissions related to their transport [54,188].

Few research initiatives, mostly on RS [40], already explored this perspective paving the way for additional and more in-depth research and development efforts. For instance, Zahed et al. [217] developed a continuous co-production of ethanol and xylitol from RS using a membrane reactor. Lignin can be recovered from rice residues and utilized for the production of phenolic compounds which are categories of fragrances. Lignin recovery was indeed successfully pursued from the solid waste of RS after producing relevant quantities of bioethanol in a pilot biorefinery plant [218]. Zheng et al. [219] produced vanillin from ferulic acid present in waste residue of rice bran oil using fungi. The few experiences of biorefining approaches from RS and rice bran indicated the promise of such substrates in a circular economy landscape relying on microbes as outstanding cell factories. Nevertheless, further research efforts are needed before large scale biorefinery plants can be installed from rice waste. Processes integration and the implementation of new hybrid technologies (i.e. thermo, chemical and biotechnological routes) and life cycle analysis will be useful. Furthermore, towards the future selection of the most efficient biorefining approaches, the holistic sustainability of the different valorization platforms should be assessed using advanced tools which combine life cycle, exergy and techno-economic analysis as recently and elegantly proposed [220] and reviewed [221–223] from a cluster of

lignocellulosic materials. These novel and comprehensive research activities will provide reliable insights on process costs, yields, efficiency, and overall sustainability of biofuels and bioproducts production systems from rice waste streams. In this framework, future governmental policies and regulatory legislations can take advantage of these evaluations before implementing carbon credits and tax incentives to support the industrial developments of rice by-products biorefining plants.

6. Conclusions and Prospects

The characterization of the residual starchy and lignocellulosic material globally available indicated that rice waste streams have great potential to be converted into energy, thus producing many gigalitres of biofuels, giving an important contribution to meet the world's energy demands and mitigate climate change. Towards their full exploitation, rice by-products can also be co-converted into a cluster of valuable compounds (i.e., organic acids, enzymes, pharmaceutical molecules, biopolymers). Among the biotechnological approaches adopted to convert rice waste into biofuels, ethanol production is one of the most successful applications. The whole process generally requires suitable physical, enzymatic and chemical pre-treatments, the key to extract maximum fermentable sugars. These findings are beneficial for even other liquid biofuels, but also for the most promising gaseous fuels such as biogas and biohydrogen.

As repeatedly emerging from the present review, the main actors of this story are microorganisms, able to metabolize the pretreated raw material into valuable products at the industrial level, thus considered as powerful cell factories. Moreover, microbes can be improved by genetic and/or evolutionary engineering to maximize product(s) yields and can be utilized as microbial consortia or as single bacterial or yeast strain. Biotechnological tools nowadays available and the production strategies extracted from the copious literature and here presented, indicate rice waste biofuels as economically viable. However, despite all these great promises, further research is still required on up-scale and industrial commercialization of the technologies so far developed. Moreover, future process integrations are needed toward biorefinery schemes where rice waste streams can be spun into biofuels and several other added-value products.

7. Acknowledgements

This work was supported by University of Padova [grants GRIC120EG8, DOR1931153/19, DOR1928058/19, DOR2087054/20, DOR2084579/20, DOR2027838/20].

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Biorefinery approach: Integration of process for multiple biofuels production

Research Article prepared for submission in 'Biomass and Bioenergy' Journal

Integrated production of bioethanol and biomethane from rice waste using superior amylolytic recombinant yeast

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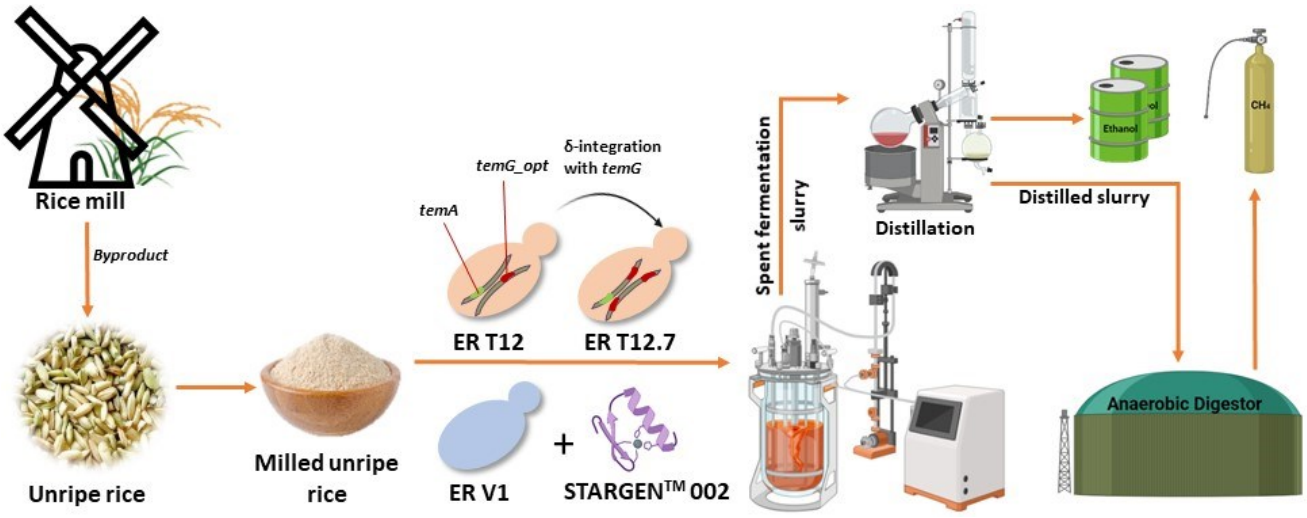
Graphical abstract



Abstract

Biorefinery approach was applied to convert a cheap rice milling industry by-product, unripe rice into high-value biofuels. Newly constructed, recombinant, amylolytic strain of *Saccharomyces cerevisiae* ER T12.7 was compared with its parental amylolytic strain ER T12 and industrial benchmark, non-amylolytic strain Ethanol Red® in combination with the commercial amylolytic cocktail, STARGEN™ 002. During hydrolysis trials with 20% (dry w/v) unripe rice, the recombinant enzymes secreted by the two engineered strains ER T12 and ER T12.7 showed hydrolysis equivalent to 50% and 100% STARGEN™ 002, respectively. The fermenting abilities of the two strains were then tested at a bioreactor scale. The novel strain ER T12.7 was able to produce the same amount of ethanol obtained in the case of the benchmark Simultaneous Saccharification and Fermentation (SSF) performed with the parental strain Ethanol Red®, thus proving that the novel engineered yeast can be used as proficient Consolidated Bioprocessing yeast with remarkable costs savings. Integrated biomethane production was explored using spent fermentation broth from two engineered amylolytic strains. Small-scale anaerobic digestion was carried out by substrate loading 2 g VS/L. Spent fermentation broth from engineered amylolytic strains ER 12 and ER T12.7 gave 370.20 and 372.61 mL CH_4/g VS. Overall, the obtained results indicated that the co-production of two biofuels from rice waste can be a feasible technology to fully exploit the feedstock in a biorefinery approach.

1. Introduction

In the last few decades of the 20th century, there was an extensive fascination towards the production and usage of liquid biofuels. Among biofuels, bioethanol stands out as a promising substitute for fossil fuels. Biofuels produced from plant-based feedstocks epitomize renewable energy resources. The use of this biomass would decrease fossil fuel demand saving the environment from deterioration (Bharadwaj, 2017; Khanal, 2008; Yu et al., 2004). Biorefinery is an integrative and multifunctional concept that exploits biological feedstocks for the sustainable production of a variety of intermediates

and final products as well as the maximum possible use of all feedstock components (Bušić et al., 2018). The concept includes a selective transformation of the different molecules available in the biomass into biofuels, and other value-added chemicals (Grilc et al., 2017; Kamm et al., 2005). Integration of different processes for the production of a variety of biofuels is the hallmark of biorefinery. This approach ensures the economic viability of the specific process as biofuels belong to the class of low-cost and high-volume commodities. Considering this fact, it is essential to convert the maximum possible carbon to biofuels (Gupte et al., 2022). The success of this approach depends upon making complex carbon bioavailable to microbial cell factories. Hence, when the complex substrate is used for the production of biofuel, more attention is given to the pre-treatment and saccharification of the substrate (Gupte et al., 2022).

Many scientific efforts were made to obtain two biofuels from all the available carbon in the feedstock wherein, leftover from anaerobic digestion (AD) was employed to get bioethanol (MacLellan et al., 2013; Wang et al., 2016; Yue et al., 2011; Zhu et al., 2017). The net energy acquired from the co-production of methane and ethanol is much higher than that of ethanol alone (Rabelo et al., 2011; Teater et al., 2011). Although this process appears to be effective, the drawback of this approach is C/N ratio which is an important factor in AD (Ge et al., 2016; Wu et al., 2010). This ratio should range between 20 and 30 (Chen et al., 2008; Yen et al., 2007). Generally, starchy as well as lignocellulosic feedstocks are rich in carbohydrates but possess less nitrogen, i.e., they have high C/N values (Giuliano et al., 2013; Ye et al., 2013). While the reverse approach i.e. production of ethanol before AD can be more fruitful as lower C/N can be achieved.

For efficient carbon utilization, it is necessary to select biomass which needs the least processing and better bioavailability of carbon. This paper specifically focused on unripe rice (UR), selected as an example of other rice waste streams abundantly available worldwide. Out of 1000 teragrams (Tg) of rice globally produced per year, 4.8 % go to waste (Wong et al., 2016). About 30.2 Tg of UR are available with a noteworthy ethanol potential (Gupte et al., 2022). Regardless of the amount of waste available

very less attention is given to UR. UR has around 69% of starch which can be converted into bioethanol and biomethane (Favaro, Cagnin, Basaglia, Pizzocchero, van Zyl, et al., 2017).

Combined production of bioethanol and biogas was previously reported from starchy feedstocks although in very few cases and after significant pretreatment and enzymatic hydrolysis. For instance, Moshi et al. (Moshi et al., 2015) pretreated cassava peels with alkali and enzymes. Sequential bioethanol and biogas production provided ethanol productivity of 1.3 g/L/h. Combined fuel energy was always higher than individual biofuels. The same strategy was applied to starch rich hydrophyte *Spirodela polyrhiza*, wherein, glucose was obtained from dilute acid pretreatment and converted to ethanol with the help of optimized *Saccharomyces cerevisiae* strain with 99.8% of the theoretical yield. Moreover, biogas yield of 0.88 NL/g VS was achieved., although only biogas production gave higher fuel energy than sequential production (Rana et al., 2021). In conclusion, this difference may be due to difference in biomasses and change in C/N ratio. Recent recombinant strain *S. cerevisiae* ER T12.7 was selected as proficient Consolidated BioProcessing (CBP) amylolytic recombinant strain with starch-hydrolysing activity even higher than ER T12 (R. A. Cripwell et al., 2019), from which it derived. The recombinant showed superior fermenting capabilities in the CBP at high substrate loadings (20% dw/v) of raw corn starch as well as rice bran and potato by-products such as potato peels and potato waste (R. A. Cripwell et al., 2019; Favaro et al., 2013; Favaro, Cagnin, Basaglia, Pizzocchero, Heber, et al., 2017).

In this study, the strain was further investigated for its hydrolytic properties on UR, selected as one of the most interesting industrial feedstocks. Moreover, the strain has been tested at 1 L bioreactor scale and confirmed its great promise as CBP yeast. In a biorefinery approach, to fully exploit UR, the spent fermentation feedstock was then efficiently converted into biogas. This is the first report describing the sequential production of ethanol using CBP strain and biogas from a starchy rice waste stream.

2. Methods and materials

2.1 Strains, media, and cultivation

Three *S. cerevisiae* strains used in this study are reported in Table 1. All the yeast strains were grown on YPD agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 15 g/L agar) at 30°C. Preinoculum was obtained by growing at 30°C the strains overnight in YPD broth. Unless stated otherwise, all media components and reagents were procured from Sigma-Aldrich (Steinheim, Germany).

2.2 Chemical analysis of unripe rice rice

UR was obtained from La Pila (Isola della Scala, Verona, Italy), dried in a forced-air oven at 55 °C for 48 h and milled to pass throughout a 1.25 mm screen. The raw material was stored at room temperature under vacuum until further use. The starch, cellulose, hemicellulose, protein, ash, and lignin content was determined according to international standard methods (AOAC, 2000).

2.3 Hydrolysis trials on raw unripe rice

The ability of the crude glucoamylase and α-amylase secreted by ERT12 and ERT12.7 to hydrolyze UR to glucose was evaluated by hydrolysis trials at 30 °C. Cell-free supernatants obtained from the recombinant strains ER T12 and ER T12.7, grown in YPD broth for 72 h at 30°C, were collected. 10 mL tubes (5 mL working volume) containing 20% dw/v UR and 0.02% w/v sodium-azide (to prevent microbial growth) were incubated with respective supernatants. Samples were removed at regular intervals and analyzed with an adapted DNS protocol and high-pressure liquid chromatography (HPLC) as described in section 2.5 Analytical methods and calculations.

Cell-free supernatant of *S. cerevisiae* ERT12 and ERT12.7 was compared to that of commercial enzyme cocktail STARGEN™ 002. STARGEN™ 002 was obtained from DuPont Industrial Biosciences (Palo Alto, California, USA). The dosage of the cocktail was used as a percentage of the manufacturer's recommendation (DuPont, 2012) i.e. 28.33 µl per 100 mL (1 g/kg of substrate) (Myburgh et al., 2019).

Supernatant was added into 125 mL serum bottles containing 20% dry w/v UR substrate (100 mL working volume) and, 0.02% w/v sodium-azide, to prevent microbial growth. The supernatant of the parental strain of *S. cerevisiae* ER V1 was loaded with 50, 100, and 200% of the recommended enzyme dosage of STARGEN^{TM} 002. Samples were withdrawn at regular intervals analyzed with an adapted DNS protocol and high-pressure liquid chromatography (HPLC) as described in section 2.5 Analytical methods and calculations. All experiments were performed in triplicate.

2.4 Fermentation of UR

All the fermentations were carried out in a 1 L (working volume) fermenter (Applikon Biotechnology, Schiedam, The Netherlands) using the BioXpert software version 1.13 (Applikon Biotechnology). Two different fermentation approaches were set up, i.e. simultaneous saccharification and fermentation (SSF) using commercial GSHE for starch hydrolysis, and conventional CBP. Concentrated YPD (5 g/L glucose), 20% dry w/v UR and a 10% v/v inoculum from 72 h aerobic pre-cultures (corresponding to 2 x 10^7 cells/mL) were used making a final volume of 900 mL. The inoculum size was distinctively chosen to compare the recombinants' fermenting abilities to those of other CBP amylolytic yeast strains (Alibardi et al., 2017; Favaro et al., 2015; Myburgh et al., 2019). Ampicillin (100 µg/mL) and streptomycin (50 µg/mL) were added to limit bacterial growth. Fermentation was performed at 30°C and 300 rpm. Daily samples were collected up to 99 h for HPLC quantification of ethanol, glucose, glycerol, acetic acid, and maltose concentrations. Spent ethanol fermentation slurries from CBP fermentations were analysed for starch, cellulose, hemicellulose and protein and then distilled until all ethanol was recovered as previously described (Cavka et al., 2014).

2.5 BMP batch setup

Biochemical methane potential (BMP) of a spent slurry of ethanol fermentation was studied. The slurry from recombinant strains ER T12.7 and ER T12 were labelled as F1 and F2, respectively.

An inoculum was obtained from an agricultural waste treating biogas plant named Pro.Energia (Ravanna, Italy). This mesophilic (about 42 ± 2 °C) biodigester, was fed with chicken manure, potatoes, onions, and maize silage at sampling time and operated in stable conditions with a hydraulic retention time of 85 days. The sampled inoculum was stored at the same temperature as original plant until starvation conditions were achieved, as suggested by Angelidaki et al. (Angelidaki et al., 2009).

BMP batches were set up in 120 mL glass serum bottles with 24 mL of inoculum and water in complementary quantity to reach the final working volume of 30 mL. Based on the outcome of previous experiments F/M ratio was not followed. An organic loading of 2 g VS/L was selected and glucose exploited as reference substrate. The two different substrates from ethanol fermentation have been distilled before use and their VS quantification assessed. All the experiments were run in triplicates. Bottles were flushed with nitrogen for three minutes to ensure an anaerobic atmosphere and sealed with a rubber cap to be finally crimped. All reactors were incubated at 37 °C. The endpoint of the experiment was defined as the reduction in daily gas production of less than 1% of the total gas production for at least three consecutive days (Angelidaki et al., 2009). Benchmark BMP experiments, containing only inoculum and distilled water, were also performed.

2.6 Analytical methods and calculations

Samples obtained at different time points were centrifuged at 3000 rpm for 5 min. The supernatant thus obtained was filtered through a 0.22 µm nylon syringe filter. HPLC was performed using the Shimadzu Nexera HPLC system equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). Phenomenex Rezex ROA-Organic Acid H^+ (8%) column (300 mm×7.8 mm) column was used for chromatographic separations. The analysis was performed using an isocratic system of 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min at 50°C. Keeping all the HPLC parameters constant, the temperature of the column was optimized to 60 °C to analyze volatile fatty acids (VFA). Maltose, glucose, acetic acid, ethanol, and glycerol were identified by correlating retention times and their

concentrations were calculated using standard calibration curves from external standards. Similarly, 10 mM analytical standard VFA mixture (Sigma-Aldrich, USA) was used as a standard for the calculation of VFA. Theoretical CO_2 yields were calculated based on ethanol production, assuming that ethanol and CO_2 are produced in equimolar fractions. The percentage of available carbon converted into the various fermentation products (referred to as estimated carbon conversion) was determined on a mole carbon basis (R. A. Cripwell et al., 2019). Ethanol yield ($Y_{E/S}$) is reported as a percentage of the theoretical maximum (0.51 g/L per glucose equivalent) based on the total available glucose equivalents.

The degree of saccharification (DS) of the unripe rice signifies the amount of soluble sugars released after hydrolysis and was calculated as given in the equation below. A conversion factor of 0.9 and 0.95 was included to reflect the addition of a water molecule during hydrolysis (R. Cripwell et al., 2015).

$$DS = \frac{[glucose\ g/L \times 0{,}9]\ + [maltose\ g/L \times 0{,}95]}{[available\ starch\ g/L]}$$

In the case of BMP experiments, the total solids (TS), volatile solids (TS), pH, and alkalinity were measured according to standard methods for the Examination of Water and Wastewater (Gandaseca et al., 2016). a gas chromatograph.

Biogas production was determined through the water displacement method (Alibardi et al., 2012). Biogas composition in terms of hydrogen, carbon dioxide, and methane were measured by a gas chromatograph (490 Micro GC, Agilent Technologies, CA, USA) equipped with a thermal conductivity detector (TCD) and two different capillary columns, one using argon as carrier gas and the other using helium, operating at 145°C, 30 psi, and 100°C, 28 psi, respectively. Data were analysed by SOPRANE 2 software (S.R.A. Instruments, France). CH_4 values were expressed in mL CH_4/g VS.

3. Results and Discussion

3.1 Substrate composition

Compositional analysis of UR indicated that starch was the main component with a content of 68.6% of TS. Whereas, low amounts of cellulose, hemicellulose ash and protein were detected without traces of lignin i.e. 1.8, 3.7, 1.5, 9.9 % TS, respectively. TS was found to be 87.9%. The quantities of cellulose, hemicellulose and ash were higher than broken rice (BR) and discoloured rice which are other byproducts of the rice milling industry (Favaro, Cagnin, Basaglia, Pizzocchero, Heber, et al., 2017). Although compositional analysis is necessary to understand the starch content of the substrate, other components also play an important part in the overall process of fermentation. Especially cellulose content has a key role on the reduction of amylolytic activity of α-amylase by probable adsorption of α-amylase (Dhital et al., 2015). Ji et al. (2018), for the first time, investigated the effect of cellulose nanocrystals (CNC) on the activity of amylase i.e. glucoamylase and α-amylase and observed the interaction between CNC and amylase (Ji et al., 2018). Moreover, an increasing quantity of CNC decreased the activity of these enzymes by changing the secondary structure of enzymes. This finding is in line with observations of Myburgh et al. (Myburgh et al., 2019) which showed higher enzyme activity using ER T12 when BR was used as a substrate which had nine times lesser cellulose and seven times lesser hemicellulose than those of UR, here adopted.

The presence of significant amount of protein (9.9% in UR) in the substrate may have positive or negative effects on starch saccharification. Commercial rice showed the presence of inhibitory proteins which affect the activity of α-amylase up to 65% (Ramli et al., 2018). Whereas, excess of protein may prevent non-selective adsorption of α-amylase on cellulose and improve saccharification. However, this amount is lower when compared to rice bran i.e. 14 % and other complex substrates, such as wheat bran i.e. up to 18% (R. Cripwell et al., 2015; Favaro et al., 2012; Favaro, Cagnin, Basaglia, Pizzocchero, Heber, et al., 2017). On contrary, presence of starch granular proteins in high amylose wheat hinders

amylolytic activity of amylase enzymes (Li et al., 2020). Overall, a suitable substrate should have higher starch content, lower cellulose content and moderate protein content to obtain maximum amylolytic and ethanologenic activity.

3.2 Assessment of hydrolysis performances on unripe rice of the recombinant enzymes versus a commercial STARGEN™ 002 cocktail

To assess the ability of the new recombinant enzymes to hydrolyse rice-starch at increased substrate loadings, the crude enzymes secreted by ER T12 and ERT12.7 were compared to commercial STARGEN™ 002, which is considered to be the most efficient commercial enzyme cocktail (Gronchi et al., 2019). The stability of recombinant enzymes was also checked for extended amounts of time. Hydrolysis trials were studied using a substrate loading of 20% dw/v simulating fermentation substrate loading.

When the three tested STARGEN™ 002 loadings (50, 100 and 200% of the recommended) were combined with supernatant from the ER V1 strain, a consistent increase in glucose concentration was observed over time (Fig. 1A). Importantly, a similar increase was observed for samples incubated in presence of crude enzymes from the recombinant ER T12 and T12.7 strain. ER T12.7 compared well to the parental strain supplemented with a 100% STARGEN™ 002 at 168 h producing 84.6±6.89 g/L glucose. Instead, ER T12 showed glucose production of 77.1±9.12 g/L which was comparable to 50% STARGEN™ 002.

On the same lines, steady increase in maltose concentration (Fig. 1B) along with glucose indicates the activity of enzyme. Interestingly, when glucose and maltose were compared for ER T12.7, a sharp increase in glucose concentration was observed after 96 hrs while simoultaneously the concentration of maltose clearly dropped. Combined together, these observations indicate the improved glucoamylase activity in ER T12.7 due to the further engineering of ER T12 for the expression of additional copies of

the optimized glucoamylase gene (*temG_Opt)* from *Talaromyces emersonii*. Noteworthy, maltose trends of 50, 100, and 200% STARGEN™ 002 showed interesting patterns. As concentration of STARGEN™ 002 increased, maltose concentration decreased (Fig. 1B). This suggests the presence in the crude ER T12.7 supernatant of glucoamylase activity as effective as that of 200% STARGEN™ 002.

Overall, the starch hydrolyzing ability of the novel ERT12.7 supernatant was remarkable, at the end of the hydrolysis, approached the performances of STARGEN™ 002 (100%). The improvement in glucoamylase activity in ERT12.7 was evident if compared with the glucose and maltose values released by the ER T12, which was able to produce yields similar to those of the 50% STARGEN™ 002, further confirming the data obtained from broken rice (Myburgh et al., 2019).

These considerations are furtherly confirmed considering the DS achieved by both recombinant supernatants (Fig. 1C). The longer incubation, the higher DS was detected by the crude ER T12.7 which DS values at 168 h statistically similar (p>0.01) to those of the 100% STARGEN™ 002 while that of ER T12 was comparable with 50% STARGEN™ 002. This is in agreement with previous studies reporting amylases with high enzyme stability over time at different temperatures (Görgens et al., 2015; Sakwa et al., 2018).

To the authors' knowledge, this is the first report presenting such high saccharification by crude enzymes produced by an amylolytic recombinant *S. cerevisiae* strain on a complex starchy substrate like UR, which is comparable to that of STARGEN™ 002, a specifically developed and industrially recognized commercial product.

3.3 1-L bench fermentation of unripe rice into bioethanol

Industrial feasibility is an important part of the development of technology in the biofuel industry. Apart from substrate composition as discussed in section 3.1, CBP strains are always preferred over the addition of exogenous enzymes. The ethanol productivity of the two recombinant CBP strains was

evaluated in 1-L bench reactor using 20 % w/v of unripe rice (Figure 2, Table 2). As a benchmark, fermentation with SSF (parental strain ERV1 with 100% STARGEN^{TM} 002 supplementation) was included.

Noteworthy, both recombinant strains displayed fermenting kinetics like those of the benchmark SSF by the parental strain, with final ethanol production after 72 h of about 45 g/L. Ethanol levels were similar to those achieved by the ER V1 up to 48 h of fermentation (Figure 2). Then *S. cerevisiae* ER T12.7 outperformed the parental strain and the ER T12 produced lower ethanol levels. This agrees with the promising hydrolysing capability of ER T12.7 enzymes detected after prolonged incubation in the presence of unripe rice (Figure 1).

Recombinant strain ER T12 was tested for its fermentation abilities at 20% (dry w/v) of unripe rice and showed 51% ethanol yield after 96 h of fermentation with a productivity of 0.42 g/L/h. Although the ethanol level increased consistently from 72 h (32.97 g/L) to 96 h (40.64 g/L), the productivity of the process decreased slightly. When SSF set-up of parental strain ER V1 was compared with CBP strain ER T12, SSF strain showed higher productivity of 0.45 g/L/h after 96 h with a slightly higher ethanol yield of 53%. Overall, carbon conversion was comparable at the end of fermentation for both parental strain ER V1 and recombinant strain ER T12 (Table 2). Interestingly, ER T12.7 showed comparatively superior results with an increased ethanol yield of 0.47 g/L/h and a corresponding ethanol yield of 59% in 96 h of fermentation. Eventually, ER T12.7 showed the highest carbon conversion of 64.58%. Thus the productivity of recently developed recombinant CBP strain ER T12.7 was comparable to the parental strain supplemented with STARGEN^{TM} 002 (100%) and distant from recombinant CBP strain ER T12 indicating higher amylolytic efficiency of ER T12.7. For the conversion of potato starch in conventional bioreactor, Jeon et al. (2008) co-cultivated *Aspergillus niger* and *S. cerevisiae,* to produce amylolytic enzymes from *A. niger* and ferment released sugars using *S. cerevisiae*. The system produced about 5 g/L of ethanol, while application of pulsed electric field of 4 volts could improve ethanol levels up to 19

g/L (Jeon et al., 2008). On other hand, liquefied and enzyme saccharified cassava starch, fermented for 72 h using *S. cerevisiae* at 2 L bioreactor scale produced 45.2 g/L ethanol with a yield of 0.23 and 52.1% fermentation efficiency (Wangpor et al., 2017). The results obtained by Wangpor et al. (2017) are comparable to current studies although 10% der w/v cassava starch was pretreated with amylolytic enzymes and the hydrolysate was used for fermentation trials.

Overall, as reported in Table 2. the ethanol yields of all the three bioreactor fermentations were low (on average 50%). Residual starch was indeed present in the spent fermentation broths after 96 h (on average 48 g/L, which accounts for only 65% utilization of the substrate). This can be most probably due to suboptimal environmental conditions during the reactor which affected both recombinant enzymes as well as the commercial STARGEN^{TM} 002. UR used in these studies was unpolished and coarsely milled. It is already reported that cellulose in raw flour restricts the enzymes from the degradation of starch (Englyst et al., 2005). When different rice waste streams were compared for the production of ethanol, the least enzyme activity was observed for unripe rice amongst all other starchy substrates of rice waste when fermentation was carried out by recombinant yeast strain (Favaro, Cagnin, Basaglia, Pizzocchero, Heber, et al., 2017). Production of recombinant protein by engineered yeast strains also significantly depends upon the nature and concentration of nitrogen (Favaro et al., 2012; Hahn-Hägerdal et al., 2005; Silva et al., 2010).

Scale-up studies in fermentation are also dependent upon many process parameters such as type and distance between impellers (Afedzi et al., 2022), and efficiency of agitation (Shupe et al., 2012). As particulate unripe rice is heavy, proper mixing of UR is another issue that rarely occurs in serum bottles as the magnetic bar rotates at the bottom of fermentation bottles. Foaming observed during fermentation also reduced active yeast biomass from liquid fermentation media.

3.4 Specific biogas and methane yields of batch anaerobic digestion experiment

After the bioethanol fermentation, the remaining substrates processed by ER T12 and ER T12.7 have been exploited for biomethane production. To ensure nutrient stability and remove the ethanol, deemed potentially harmful to the microbial population, the substrates from the first process were distilled and the analyses of their chemical composition repeated (Table 3). The substrate parameters like total solids (TS) and volatile solids (VS) were obtained. TS and VS values were similar for both spent unripe rice (Table 3).

As previously discussed, starch was mostly transformed into ethanol during the fermentation, reducing its relative quantity from 68.6 to an average of 21.3 (%TS) The direct consequence of this event is the change in the percentages of the other components. Cellulose and hemicellulose concentrations notably increased, the values of both components were 3 to 5 times higher than in unripe rice, indicating these carbohydrates have not been exploited by ER T12 and ER T12.7. Even protein concentration increased, quadrupling its value, in accordance with what reported for cellulose and hemicellulose.

The innovative idea of combining ethanologenic fermentation with AD processes seems to be an optimal strategy for exploiting a single substrate and bio-transforming the whole spectrum of carbon sources in green products like ethanol and biogas (Mahmoodi et al., 2018; Moshi et al., 2015). The daily and cumulative methane yields from the two fermented substrates were determined through a batch process. Both substrates were efficiently converted into methane with similar yields (Figure 3). This finding was in agreement with the composition of both spent fermented feedstocks characterized by quite similar amounts of polysaccharides and other polymers (Table 3). The spent unripe rice was rapidly utilized by the microbial population producing promising levels of methane (up to 372 mL CH_4/g VS). The production slowed down in both cases after the twelfth day and reached a steady state condition later on with a production peak after 38 days, when 372.61 ± 40.07 and 370.20 ± 51.42 mL CH_4/g VS were obtained from the ER T12.7 and ER T12 substrates, respectively. These values are in agreement with

those recently reported for another rice by-product, broken rice, mostly after acidogenesis of the substrate (Brojanigo et al., 2022). Interestingly, different reactors were employed to obtain biogas from potato juice, a potato starch processing industry byproduct, in a continuous process but batch assay could yield a maximum of 470 mL CH_4/g VS added (Fang et al., 2011). The results showed higher methane yield than the current study although 10% higher VS were added for AD. While lower yields were obtained when a two-phase system, acidogenic and methanogenic, was exploited for AD of cassava-based polymer yielding 249.1 mL CH_4/g VS (Cremonez et al., 2020).

Overall, the methane levels obtained are remarkable also considering the low C/N ratio available for methanogenesis. Optimal ratio was indeed reported to be in the range of 16-45, which is at least 2.5-fold higher than those of the spent unripe rice. The C/N ratio of 4-7 was considered very low and found the main culprit of releasing a large amount of ammonia which has a toxic effect on the methanogenic bacterial population of AD process (Kwietniewska et al., 2014). The high methane potential obtained in this study can be ascribed also to the presence of yeast biomass which can positively trigger methanogenesis because of the presence of additional growth cofactors and vitamins as was already described in the case of yeast extract and AD processes (Gonzalez-Gil et al., 2003)

4. Conclusion

The exploitation of alternative starchy byproducts such as UR in CBP configuration using superior amylolytic yeast strains can enhance economical ethanol production on an industrial scale when optimized scale-up process parameters are set up. In this study, crude enzymes from recombinant amylolytic yeast strains showed saccharification yields comparable to a commercial enzyme cocktail using untreated UR. During scale-up fermentation experiments the recently developed amylolytic recombinant strain ER T12.7 showed higher ethanol production than parental ER V1 in SSF set-up using commercial STARGEN^{TM} 002. Nevertheless, the fine-tuning of environmental parameters at the bench

scale seems to be crucial to optimize ethanol yields. In a biorefinery approach, this paper further exploits the spent fermentation from UR to produce methane, resulting in promising levels. Techno-economical evaluations are in progress to assess the overall feasibility of the process, in view of supporting the definition of biorefinery context converting organic waste into clusters of valuable biofuels.

5. Acknowledgments

This work was funded by Università degli Studi di Padova through BIRD210708/21, DOR1928058/19, DOR2084579/20, DOR208705/20, DOR2107797/21, DOR2114239/21. This work was also supported by "Centro per l'Agricoltura, la Sostenibilità e gli Alimenti" (CASA), CUP C26C18000190001, MIUR, Italy. The Authors are very grateful to BTS S.r.l. for providing the inoculum for AD and Valentino Pizzocchero (MSc, Università degli Studi di Padova) for valuable support in HPLC and GC analyses.

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Table 1. Yeast strains used during this study and their genotypes.

***S. cerevisiae* strains**	**Genotype**	**Reference/Source**
Ethanol Red^{TM} Version 1 (ER V1)	*MAT*a/α prototroph	Fermentis, Lesaffre, France
ER T12	δ-integration of *ENO1$_P$-temG_Opt-ENO1$_T$*; *ENO1$_P$-temA_-ENO1$_T$*	(R. A. Cripwell et al., 2019)
ER T12.7	δ-integration of *ENO1$_P$-temG_Opt-ENO1$_T$*; *ENO1$_P$-temA-ENO1$_T$*; *TEF1$_P$-kanMX-TEF1$_T$*	Stellenbosch University

Table 2: Summary of fermentation results after 72 and 96 hrs at 30 °C using 20% dw/v unripe rice in YPD broth.

Product (g/L)	ER V1		ER T12		ER T12.7	
	72 h	**96 h**	**72 h**	**96 h**	**72 h**	**96 h**
Glucose	nd	nd	0.51	0.51	0.27	0.27
Maltose	2.50	3.09	2.50	2.96	4.12	5.12
Glycerol	3.14	3.23	3.13	3.50	3.20	3.75
Ethanol	36.85	42.85	32.97	40.64	36.16	45.17
***Q* (g/L/h)**	0.51	0.45	0.46	0.42	0.50	0.47
$Y_{E/S}$(%)	46	53	41	51	48	59
Carbon conversion[c] (%)	49.40	57.40	44.92	54.98	52.76	64.58

nd- not detected *Q*- Ethanol productivity, *Y*- % yield of theoretical maximum, [c]- Carbon conversion was calculated on a mol C basis considering all products detected through HPLC, $Y_{E/S}$- percentage of the maximum theoretical ethanol yield as calculated from total available glucose equivalents.

Table 3: Characteristics of the distilled spent unripe rice

Parameters	After ERT12.7 fermentation	After ERT12 fermentation
TS (%)	96.64±5.40	97.71±5.21
VS (%)	90.95±6.21	93.39±4.9
TKN (%TS)	7.46±0.36	6.60±0.27
C (%TS)	44.75±2.71	43.40±2.10
N (%TS)	6.97±0.49	6.12±0.32
C/N	6.42±0.38	7.09±0.38
Protein (%TS)	46.63±3.15	41.28±2.73
Ash (% TS)	0.57±0.03	0.69±0.02
Hemicellulose (% TS)	14.31±0.82	13.26±0.91
Cellulose (% TS)	6.89±0.32	7.59±0.39
Lignin (% TS)	3.61±0.17	4.92±0.23

Figure legends

Figure 1: (A) Glucose and (B) Maltose concentration detected during hydrolysis of 20% dw/v unripe rice at 30 °C. Values for *S. cerevisiae* ER T12.7 (■) and ER T12 (●) supernatant as well as parental ER V1 supernatant supplemented with 50% (▲), 100% (♦) and 200% (○) STARGEN™ 002 dosages are reported. (C) Degree of saccharification (DS) of 20% dw/v unripe rice by *S. cerevisiae* ER T12.7 and ER T12 supernatant (with no addition of enzymes) and ER V1 supernatant supplemented with 50, 100 and 200 % recommended STARGEN™ 002 dose, respectively. Error bars represent standard deviation from the mean of three replicates.

Figure 2: Ethanol production in scale up batch of 0.9 L configured for SSF and CBP fermentation of UR. SSF was set up for ER V1(○) using commercial amylolytic cocktail STARGEN™ 002 while, CBP was set up ER T12(■) and ER T12.7(▲). All fermentations were performed in duplicate using 20% dw/v of UR. Error bars represent standard deviation from the mean of two replicates.

Figure 3: Trend of methane production using distilled spent fermentation slurry. Dashed line with (x) indicates distilled, spent fermentation slurry from ER T12.7 (F1), Continuous line with (○) indicates distilled, spent fermentation slurry from ER T12, VS- volatile solids

Figure 1

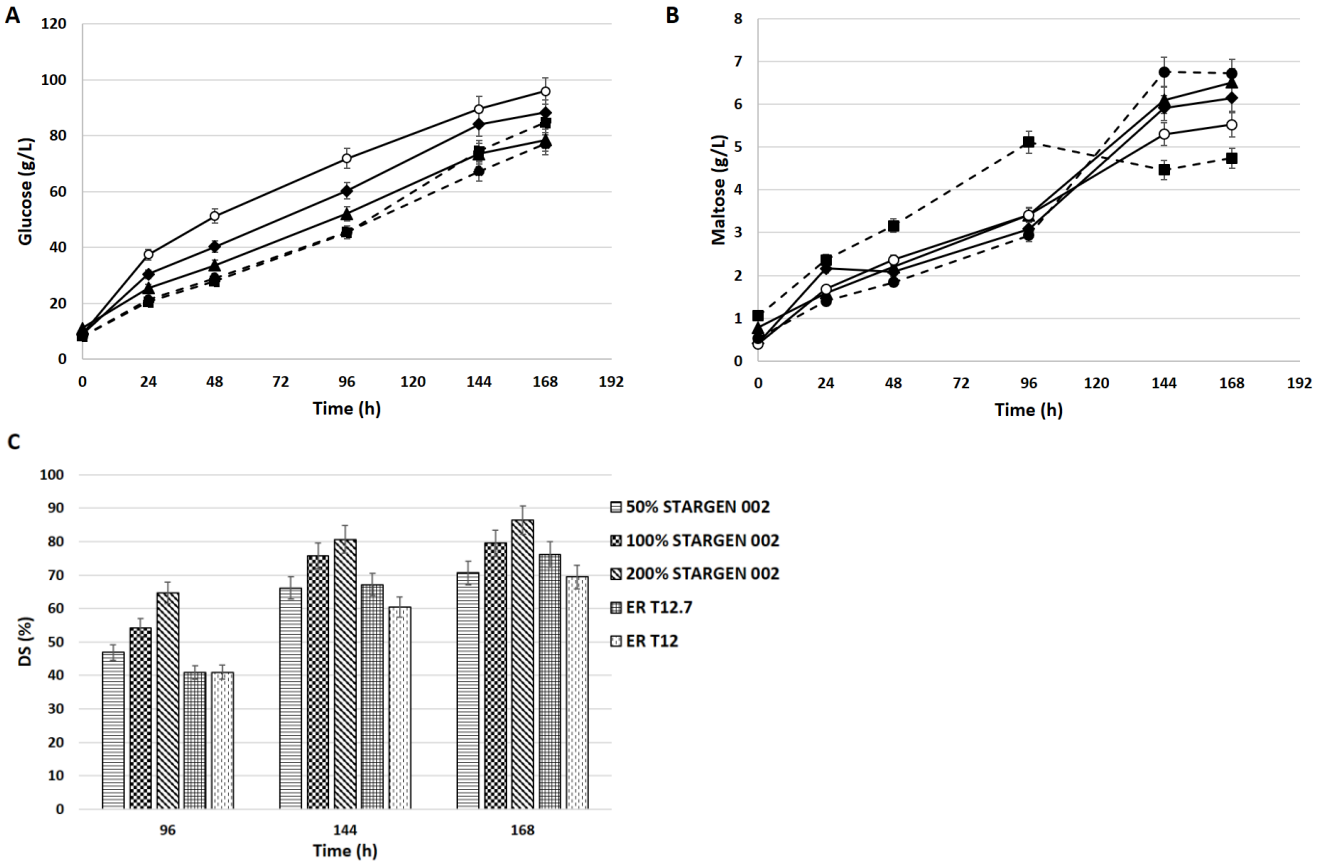

A
Glucose (g/L)
Time (h)
B
Maltose (g/L)
Time (h)
C
DS (%)
Time (h)
50% STARGEN 002
100% STARGEN 002
200% STARGEN 002
ER T12.7
ER T12

Figure 2

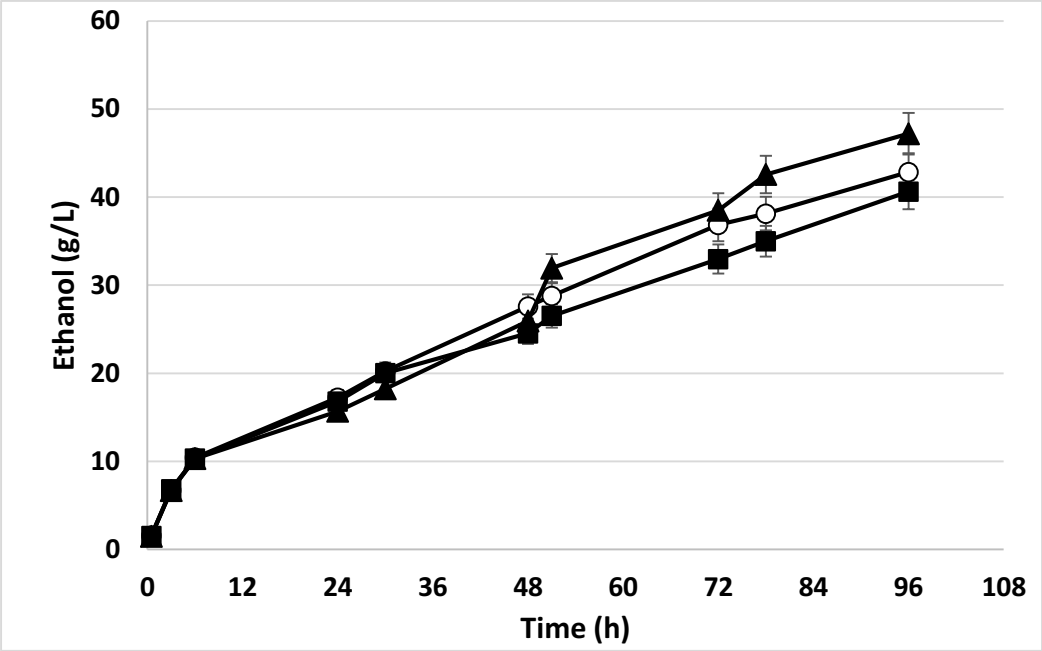
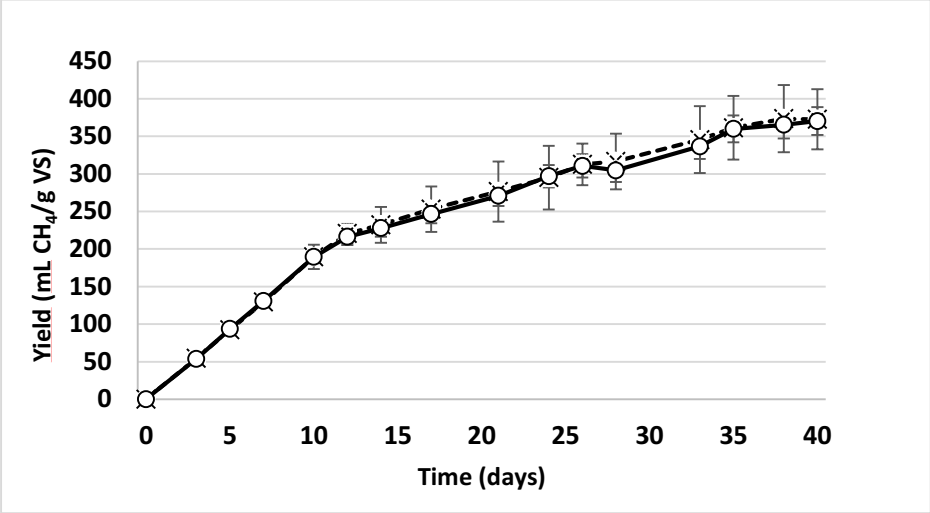


Figure 3


450
400
350
300
250
200
150
100
50
0
Yield (mL CH4/g VS)
0
5
10
15
20
25
30
35
40
Time (days)

Domestic waste:
a cheap, recurring and largely available feedstock for bioethanol production

Research Article prepared for submission in 'Waste Management' Journal

Consolidated Bioprocessing of the organic fractions of the municipal solid waste into bioethanol

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Abstract

Organic fraction of municipal solid waste (OFMSW) has a huge potential to sustain biofuels production. OFMSW is mainly converted into biogas by anaerobic digestion and limited research is available on the production of bioethanol. In this paper specifically employed, for the first time, a recombinant yeast strain co-expressing glucoamylase and alpha-amylase enzymes to process starch-rich OFMSW, into bioethanol. As such, OFMSW can be treated without the addition of costly enzymes with a Consolidated Bioprocessing concept. The OFMSW, sampled at an industrial anaerobic digestion (AD) plant, was processed to bioethanol with an outstanding yield, approaching 100% of the theoretical. Moreover, since it is located in proximity of the AD plant, a rice-milling industry is available with huge quantities of waste. The co-conversion of OFSMW and discolored rice was performed in view of testing the feasibility of processing different by-products at once. The ethanol levels reached 60 g/L displaying that both the developed process and yeast strain has important features towards the production of ethanol from organic waste streams.

1. Introduction

Recycle, reuse and reduce have emerged as high-priority plans due to strict waste disposal regulations, limitation of resources, effects of global warming, and environmental concerns. This strategy can be a great solution to the current municipal solid waste management practices which use landfilling as a predominant method; irrespective of the country's financial state. Overall, a big share (from 42 to 75%) of the municipal solid waste is composed of organic fractions (Seruga et al., 2020). This organic fraction of municipal solid waste (OFMSW) has the potential to be converted into biofuels. The green paper issued by the European Commission on the management of bio-waste defines it as biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers, and retail premises, and comparable waste from food processing plants (Communities, 2008). A major fraction of bio-waste is made up of food waste (FW), which contains raw or cooked food items and includes food materials

scraped at any step between ‘‘farm and fork’’. Generally, FW related to households is generated before, during, or after food preparation, including vegetable peelings, meat trimmings, and rotten or excess components or prepared food (Alamanou et al., 2015). Energy production from OFMSW could stand as a technical and economically viable alternative since it is highly available and free of cost.

European Union (EU) generates around 140 teragrams (Tg) of FW (López-Gómez et al., 2019), 42% of which is contributed by the domestic section. At present, OFMSW is managed by feeding animals, composting, anaerobic digestion, incineration, and landfills (Lin et al., 2013). Out of these methods, majority of FW goes to landfills and incineration while a small portion is utilized for composting and anaerobic digestion. Importantly, the outcome of the landfill or incineration process is groundwater contamination or emission of toxic gases and dioxins (Hong et al., 2011). European legislation aims to minimize landfilling practices in member states (Alibardi et al., 2015).

Generally, OFMSW is used largely for the anaerobic digestion. Wherein, the product obtained is biomethane. Although largely applied as biofuel, methane is rarely used in heavy commercial vehicles. Some studies also showed production of biohydrogen by dark fermentation but there is the need of a strict control on the chemical composition of OFMSW (Alibardi et al., 2016; Favaro et al., 2013), which is quite difficult at very large scale. On the contrary, bioethanol could be a better alternative as biofuel from OFMSW which can be used for blends (E10 or E20) in existing gasoline (Abel et al., 2021) or flexi-fuel (E100) vehicles (Saini et al., 2010). Previous studies mostly focused on the bioethanol potential of single and main components of OFMSW such as kitchen organic waste (KOW), green organic waste (GOW), and paper and cardboards (PCW). The theoretical yield estimated from these fractions were 363 mL/dkg (kilogram dry weight), 420 mL/dkg and 505 mL/dkg, respectively, but only after the adoption of additional steps of chemical and physical pretreatment as well as hydrolysis (A. Li et al., 2009). OFMSW treated with sulfuric acid at higher temperature were then saccharified with cellulolytic enzymes to get ethanol concentrations of 245.88 mL/kg of dry OFMSW (Mahmoodi et al., 2018a). While

another attempt with hydrothermal pretreatment and amylolytic as well as cellulolytic enzymes could yield up to 191.10 g of ethanol per kg of dry OFMSW (Mahmoodi et al., 2018b). Verhe et al. (Verhe et al., 2022) used paper and cardboard, a cellulose-rich fraction of OFMSW after acidic pretreatment and costly enzymatic saccharification with final concentration of ethanol of 66.4 g/L at substrate loading of 40% (w/w).

It is quite evident that the important part of OFMSW is starch and cellulose. Mahmoodi et al. (Mahmoodi et al., 2018b) used amylolytic and cellulolytic enzymes to achieve high glucose yield of 520 g/Kg of dry OFMSW followed by sequential bioethanol and methane production. While cellulose is considered major source of sugars for production of ethanol by pretreating the OFMSW using cellulolytic enzymes to produce 66.4 g/L ethanol in 57 h at 2 m^3 fermenter (Verhe et al., 2022).

This paper specifically focused on the one step conversion of OFMSW into ethanol by using the Consolidated Bioprocessing (CBP) amylolytic yeast Ethanol Red^{TM} T12.7 (ER T12.7). Specific efforts were spent on testing bread and pasta fraction of OFSMW, which can stand also as specific waste stream mostly in Europe (Ibenegbu et al., 2022; Narisetty et al., 2022) as well as a different composition of OFMSW to mimic the huge seasonal variability already reported in literature [7].

Along with OFMSW, agro-industrial waste streams obtained from rice mills can also be used to enrich the carbohydrate content of OFMSW, especially when OFMSW management plant, as in the case of this research, is in the proximity of industrial rice mill plant with high volumes of starchy by-products. Discolored rice (DR), a starchy waste from rice milling industry, was indeed selected as promising substrate to be co-processed together with OFSMW into ethanol. DR is definitely quite rich in starch and largely available worldwide with a bioethanol potential of about 2.9 Tg (Gupte et al., 2022).

2. Methods and Materials

2.1 Yeast strains and growth conditions

Two *Saccharomyces cerevisiae* strains, the engineered amylolytic CBP strain ER T12.7 and the parental Ethanol Red® (ER V1), were specifically selected for this study. The engineered ER T12.7 was obtained by further engineering of ER T12 which simultaneously expresses native α-amylase (*temA*) and codon optimized glucoamylase gene (*temG_Opt*) obtained from *Talaromyces emersonii* [22]. *S. cerevisiae* ER T12 was indeed further engineered for the expression of additional copies of the codon optimised glucoamylase gene (*temG_Opt*) resulting in the strain *S. cerevisiae* ER T12.7 (Stellenbosch University).

Strains were maintained in 20% glycerol stocks at -80 °C and usually plated on YPD agar (g/L: yeast extract 10, Peptone 20, Glucose 20, Agar 15) and incubated at 30 °C for 48 hrs. Pre-inoculum was made by inoculating single colony in YPD broth for 72 hrs. All the media were sterilized by autoclaving at 121 °C for 20 min before plating.

2.2 Characterization of OFMSW and discolored rice

The sample of OFMSW was collected in June 2021 from the waste reception area of an anaerobic digestion plant of organic waste located in Este, Italy. The OFMSW delivered at the plant was source segregated at household level and the collection area involves a population of about 150,000 inhabitants. About 100 kg of OFMSW was manually sorted and divided in the following fractions: fruit and vegetable (FV); meat, fish and cheese (MFC); bread and pasta (BP); shells and bones (SB), paper, rejected materials; undersieve 20 mm. Plastics, shoppers, metals and glass were classified as rejected materials. Results of manual sorting procedure are reported in Table 1.

Using the sorted fractions, a composition of organic waste was prepared by maintaining the same proportion of the single fractions as given in Table 1, without the rejected materials. After mixing and

grinding, a slurry was obtained from this sample and referred as OFMSW. Additionally, Bread and Pasta (BP) fraction was collected separately to simulate seasonal variation in starch fraction, as studied by Alibardi et al. (Alibardi et al., 2015). OFMSW and BP were stored at -20°C until further use. Discolored rice (DR) was obtained from a rice milling industry in the proximity of Este, dried in forced-air oven at 60°C for 48 hours, milled in hammer mill and then sieved through a 1.25 mm screen. Chemical composition of OFMSW, BP and DR was determined according to international standards (AOAC, 2000) and reported in Table 2.

2.3 Fermentation experiments

S. cerevisiae strains were inoculated in 200 mL culture medium in 500 mL Erlenmeyer flasks and incubated on a rotary shaker (30 °C) at 150 rpm for 72 h. Small-scale fermentation experiments were conducted in 120-mL serum bottles containing 100 mL of fermentation medium. Different substrates, once autoclaved (121°C, 15 min) were used singly or in combination with different substrate loadings: 7.5% dw/v (dry w/v) was adopted for OFMSW, BP and the OFMSW enriched with BP to simulate the winter OFMSW composition where BP can account for 15% of the wet OFMSW [7], 10% dw/v was adopted for DR and 17.5% (dry weight/v) when combining 10% of DR and 7.5% of enriched OFMSW. 7.5% dw/v was found as optimum after several trials, to reduce the viscosity of substrate that hampers the rheology of fermentation.

All the experiments were carried out in triplicate and bottles were inoculated with 10% (v/v) pre-inoculum. A needle was inserted through the rubber stopper of fermentation bottles for CO_2 removal and fermentations were performed under oxygen-limited conditions. Samples (2 mL), taken daily throughout the fermentation, were kept at −20 °C for future high performance liquid chromatography (HPLC) quantification of glucose, maltose, ethanol, glycerol, and acetic acid.

2.4 Analytical methods, calculations and statistical analysis

Samples, taken throughout the fermentation experiments, maintained at -20 °C were analysed for glucose, maltose, ethanol, glycerol and acetic acid and the other VFA. All the samples were thawed and centrifuged at 10000 rpm for 10 min and filtered through 0.22 μm filter prior to HPLC analysis which was performed using a Shimadzu Nexera HPLC system, equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The chromatographic resolution was achieved using a Phenomenex Rezex ROA-Organic Acid H+ (8%) column (300 mm× 7.8 mm). The column temperature was set at 60 °C and the analysis was performed at a flow rate of 0.6 mL/min using isocratic elution, with 5 mM H_2SO_4 as a mobile phase. Concentration of compounds were calculated by plotting calibration curves of external standards.

The ethanol yield, $Y_{E/S}$, (g of ethanol/g of utilized glucose equivalent) was determined considering the quantity of glucose/cellulose/starch utilized during the fermentation and compared to the maximum theoretical yield of 0.51 g of ethanol/g of spent glucose equivalent. The volumetric productivity (Q) was computed as grams of produced ethanol per liter of fermentation medium per hour (g/L/h) and the maximum volumetric productivity (Q_{max}) was defined as the highest volumetric productivity exhibited by the *S. cerevisiae* strains. Statistical analyses were performed using the Graphpad Prism 5 package (Graphpad Software, Inc., San Diego, California). Descriptive statistics, mean values and standard deviations were computed. Data were analysed also by two ways factorial ANOVA (Analysis of Variance) with Duncan test.

3. Results and discussion

3.1 OFMSW sampling and composition

Segregation of waste at source of generation would surely be a winning strategy and separation of biodegradable from non-biodegradable materials improves the efficiency of waste to wealth process. The waste obtained for this study was the representative sample of around 100 Kg obtained from different

sites of Padova province, Italy. Results obtained after manual sorting of OFMSW are given in Table 1. A total of 96.3 Kg of OFMSW waste was collected in June and sorted manually. 89.3 Kg resulted as compostable, while 7 Kg was composed of plastics bags, metals, rubber etc. By considering the biodegradable fraction, fruits and vegetable amount to 55.5% (w/w), while starchy component such as bread and pasta to only 5.4% of the total. A similar trend was reported in a noteworthy study taking into account the variability of OFMSW fractions with time (Alibardi et al., 2015), where May and June showed 7.7 and 1.3% bread and pasta contribution, respectively. Data collected by Hanc et al. (Hanc et al., 2011) from urban settlements presented 58.2% of fruits and vegetables in OFMSW and similar numbers (around 43%) were obtained by Favaro et al. (Favaro et al., 2013). Hence, it was quite evident that fruits and vegetable fraction has a major contribution in OFMSW. Out of all the fermentable components, meat, fish and cheese fraction showed lowest percentages. Undersieved fraction was observed to be constant along the year, ranging between 18-22% (Alibardi et al., 2015). The numbers obtained in this study and those of Favaro et al. (Favaro et al., 2013) i.e. 13.7% and 13% ,respectively, are not far different than the mentioned range.

Chemical analysis of OFMSW confirmed that the occurrence of polymers and macromolecules was strictly linked to the shares of food fractions. Table 2 shows that the highest fermentable component per dry weight was found to be protein. Separately collected and analysed fraction of meat-fish-cheese and vegetable fractions showed 52 and 11% proteins, respectively, while fruit fraction only 3% (Alibardi et al., 2016). OFMSW showed the presence of 25.05% protein which is in accordance with Magdalena et al. (Magdalena et al., 2021), while very less (8.30 and 7.89 %) protein components were reported by Mahmoodi et al. (Mahmoodi et al., 2018b, 2018a) in two different reports. The range of proteins component in three different mixtures tested by Alibardi et al. (Alibardi et al., 2015) were 15-17%. Protein component of OFMSW from different countries showed diverse chemical composition depending upon feeding habits, way of segregation, and seasons. The range of different components are

as follows: Starch (11.7-56.5%), Cellulose (3.2-49.0%), Hemicellulose (1.8-16.0%), Lignin (1.8-29.1%), Protein (6.8-25.8%), Lipid (5.6-24.7%) (Barampouti et al., 2019). Lower concentrations of cellulose (10.19%) and hemicellulose (7.27%) were detected in OFMSW (Table 2) although vegetable and fruit fraction was the highest (Table 1). Starch was the less abundant polysaccharide present in the OFMSW tested in this study. Interestingly almost 37% of dry matter was made of non-fermentable fractions such as lignin and ash (Table 2). In the case of BP, the most abundant polysaccharide was starch, with much lower amount of hemicellulose and very limited quantity of cellulose. Protein composition contributing to 15.44% was in agreement with other BP fractions as reported by Alibardi et al. (Alibardi et al., 2016). As expected, DR was characterized by the highest share in starch and very limited amount of hemicellulose and cellulose, with values similar to those recently reported (Favaro et al., 2017; Gupte et al., 2022).

3.2 Fermentation of OFMSW

Ideally, substrates used for bioethanol production would be fermented via CBP without the need for any exogenous enzyme addition (Bala et al., 2022; Cripwell et al., 2020; den Haan et al., 2021; Gupte et al., 2022). The same should also apply in the case of OFMSW. Nevertheless, despite many efforts spent on separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) settings with (Mahmoodi et al., 2018b, 2018a) or without (Magdalena et al., 2021) pre-treatments showed that OFSMW can be indeed converted into bioethanol, no CBP approaches have been so far adopted. This paper specifically focused on OFMSW to be processed via CBP relying on the efficient amylolytic strain ER T12.7 recently reported for its high promise as starch-to-ethanol converter. OFMSW can be indeed quite rich in starch, whose composition can vary from 2 to 56.5% of dry matter (Barampouti et al., 2019). Moreover, significant fluctuations along the seasons were detected, with winter having the highest values and summer the lowest.

Before testing the recombinant yeast strains and its parental ER as benchmark, the most suitable substrate loading of the obtained OFMSW was firstly tested at small scale fermentation settings. OFMSW is indeed quite viscous and has a lot of particulate matter which also include partially milled solids like leaves, seeds, rinds of fruits etc. This particulate matter makes the slurry non-homogenized and difficult to mix. Different substrate loadings were then tested (5, 7.5, 10, 12.5, 15 % dry w/v) and the substrate concentration of 7.5% dw/v was found to be efficient for mixing by using magnetic stirrer (data not shown). This loading is well in line with the values usually adopted for full scale AD applications (Amiri et al., 2014; Linde et al., 2007; Sassner et al., 2006). The engineered strain ER T12.7 was firstly adopted for the one step production of ethanol from 7.5% dry matter of OFMSW as well as BP fraction (Figure 1 and Table 3).

From OFMSW, the recombinant yeast produced up to 6.4 g/L of ethanol with a complete depletion of starch as well as simple sugars available (data not shown). The fermenting performances, although limited, were significantly higher than those of the parental yeast (3.9 g/L) obtained from the monosaccharides in the OFMSW, whereas starch content remained constant along with the fermentation. As reported in Table 3, ethanol productivity of the CBP yeast was almost 1.6-fold that of the parental with Q_{max} values even higher (1.9-fold), further supporting the significant improvement in terms of ethanol production ensured by adopting the recombinant strain for the processing of OFSMW.

In the case of BP (7.5% dw/v), the engineered yeast ER T12.7 readily processed starch into ethanol with levels of about 25 g/L after 48 h of fermentation (Figure 1) with a complete consumption of starch. On the contrary, the benchmark strain ER V1 produced up to 6.7 g/L of alcohol, consuming all the monosaccharides available in the feedstock. Volumetric productivities of the engineered strain were again outperforming those of the parental yeast: both final (0.32 g/h/L) and maximum volumetric (0.81 g/h/L) parameters were indeed 3.5-fold than those of the benchmark yeast with a sharp increase compared to the performances obtained from OFMSW (Table 3).

Considering the data of Figure 1 and Tables 2 and 3, starch content was the limiting factor for ER T12.7 ethanol performances. BP indeed greatly supported ethanol production by the recombinant yeast because of the much higher starch content (Table 2). For the sake of assessing the promise of T12.7 also in OFMSW samples typical of winter seasons, where BP shares can account for up to 8-15 % of wet organic waste (Aggarwal et al., 2022; Alibardi et al., 2015), OFMWS sampled at the waste management plant was specifically enriched with up to 15% of BP fraction. As such, the resulting feedstock, named hereafter enriched OFMSW, turned to be fortified in starch content with lower quantities of mostly cellulose, protein and ash (Table 2).

As reported in Figure 1, the engineered strain took advantage of the increased starch availability with a final ethanol production, after 72 h, of about 13.9 g/L, which was 2.5-fold of the parental *S. cerevisiae* ER V1. The final productivity of the recombinant yeast was 0.19 g/L/h and Q_{max} approached 0.47 g/L/h after 24 h of fermentation. Both values were again much higher than those detected for the parental yeast pointing out that the engineered strain has great promise for the conversion of different composition of OFMSW into ethanol.

The recombinant amylolytic strain, ER T12.7, produced indeed levels of ethanol during the Consolidated BioProessing (CBP) of tested substrates always higher than those of the parental yeast (Figure 1). The low residual amounts of glucose and maltose in the fermentation broth indicate a rapid sugar uptake by the recombinant strain (Table 3). Moreover, limited glycerol concentrations were detected suggesting that the carbon metabolism was mainly directed to ethanol production (Table 3).

This performance should be pondered furtherly promising once considered the high content of VFA available in the systems (acetic propionic and heptanoic acid were the most abundant with 4.16, 2.37 and 10.06 g/L, respectively) which were reported to inhibit growth and fermenting activities in many *S. cerevisiae* strains from OFMSW (Du et al., 2022; B. Li et al., 2020) as well as other pretreated lignocellulosic materials (Cagnin et al., 2021; Favaro et al., 2019; Gupte et al., 2022). Moreover, the

ethanol levels achieved in this proof of concept from OFMSW repeated fermentations, higher substrate loadings and upscaling experiment are likely to further enhance the ethanol levels of this proof of concept.

The ethanol yields and productivities so far obtained are indeed of great importance when compared to the literature. The limited amount of ethanol increased in the presence of OFMSW, with very restricted starch content. After pretreating OFMSW and following hydrolysis the ethanol levels were indeed limited to 8.32 g/L with a final volumetric productivity of 0.17 g/L/h (Hafid et al., 2017). Slightly higher ethanol levels and performance were obtained adopting the parental yeast ER V1 on OFMSW but with hydrothermal pretreatment and massive amounts of costly cellulases and amylases (Miezah et al., 2017). Ethanol levels similar to those obtained in this work (up to 23.3 g/L) were obtained from the hydrolysate of kitchen waste only after acidic pretreatment (sulfuric acid, 60°C, 3h) and/or enzymatic hydrolysis with both commercial amylolytic and cellulolytic enzymes (Cekmecelioglu et al., 2013).

Generally, the volumetric productivity of ethanol in both *S. cerevisiae* ER T12.7 and the parental *S. cerevisiae* ER V1, reaches its maximum at around 24 hours of fermentation (Table 3). After 72 h of fermentation, the OFMSW substrate reaches an ethanol concentration of 6.39 g/L using ER T12.7, which was found to be the lowest level compared to the other adopted feedstocks (Figure 1). This is due to the low starting starch concentration (Table 2) but also to the presence of organic acids (data not shown). Consequently, Q_{max}, was also low (Table 3). Taking into consideration BP, the fraction of OFMSW, almost 40% of the dry matter contains starch and with a yield equal to the theoretical one it reaches an ethanol concentration much higher than OFMSW (about 23 g/L) and with a Q_{max} of 0.81 (Table 3). As far as OFMSW enriched by BP is concerned, the analysis of this raw material was important in order to be able to evaluate the different yields obtained on the basis of the seasonal changes, in fact, values have been obtained which are higher than those of OFMSW and lower than BP (Table 2). The recombinant

strain also achieved high performance when BP was used as substrate, about 29.7 g/L of the available starch content was fermented to 16.6 g/L (subtracted ethanol produced by ER V1) of ethanol corresponding to 100% of the yield theoretical (Table 3).

3.3 CBP co-processing of discolored rice and OFMSW

OFMSW was efficiently converted into ethanol by the *S. cerevisiae* T12.7. Nevertheless, the final ethanol titers are not yet suitable for any industrial development of the technology. To further improve the overall process viability, besides the above-mentioned improvement and optimization of fermentation process parameters (substrate loading, continuous or repeated batch fermentations, upscaling), OFMSW composition clearly suggested the need for a CBP yeast capable of hydrolyzing also cellulose and, possibly, hemicellulose which should be a significant OFMSW fraction worldwide (Barampouti et al., 2019). All this calls for a next wave of CBP yeast strains co-expressing at least amylases and cellulases at once to almost fully exploit OFMSW towards bioethanol. Alternatively, ethanol levels can be readily enhanced by adopting the efficient CBP amylolytic yeast in the co-processing of OFMSW and a starch-rich byproduct.

Since in the close proximity of the MSW management plant there is a rice milling industry, DR was selected as suitable feedstock because of very high starch content (Table 2), with an outstanding global bioethanol potential of 2.9 teragrams (Tg) of alcohol from the 7.5 Tg of DR yearly produced. In this study, DR was then adopted to enhance the starch content of OFMSW with the aim that conversion of mixture of two substrates into ethanol could make bioethanol production economically viable.

The recombinant yeast strain was tested for the first time on a rice by-product and displayed great promise from 10% (dw/v) of DR (Figure 2, Table 3). The highest ethanol levels were detected after 72 h (53.6 g/L) pointing at a complete starch consumption and an outstanding starch-to-ethanol yield of 99% of the theoretical. The CBP strain exhibited ethanol productivity of great interest (Cripwell et al., 2020)

with Q_{max} of 1.22 g/L/h (Table 3) which is at industrial level. On the contrary, the parental yeast *S. cerevisiae* ER V1 produced limited amount of ethanol from the simple sugars available in the broth and the resulting ethanol performances were very scarce. Q_{max} and final productivity were found to be at least 2-fold less than those of the engineered yeast and the difference may reach up to 5-folds depending on substrates. Overall, the fermenting ability of the T12.7 strain is hallmark once compared with the literature. DR was already processed into ethanol at higher substrate loading with an ethanol yield of 88 and 91% by two strains MEL2[*TLG1-SFA1*] and M2n[*TLG1-SFA1*], respectively, co-expressing glucoamylase (*TLG1*) and alpha-amylase (*SFA1*) (Favaro et al., 2017). While conversion of DR to ethanol, productivity of 0.77 and 0.65 g/L/h was achieved using M2n[*TLG1-SFA1*] and MEL2[*TLG1-SFA1*], respectively after 72 h of fermentation.

Although substrate composition and loading is different, when ER T12, the closest strain to ER T12.7, and M2n T1 were used for broken rice (BR) fermentation, productivities were 0.97 and 0.82 g/L/h after 96 h while productivity of ER T12.7 on DR as substrate could reach up to 0.69 g/L/h. Higher productivity of 1.07 and 1.04 g/L/h could only be achieved by supplementation of 10% commercial amylolytic cocktail (Myburgh et al., 2019).

Once tested the new recombinant yeast on DR, the co-processing of both feedstocks into ethanol was finally assessed. The substrate loading of DR was specifically adopted to ensure good mixing conditions once combined with 7.5% (dw/v) enriched OFMSW. From 17.5% (dw/v) substrate loading, the ethanol concentrations achieved 66.2 g/L after 96 h of fermentation. Most of the starch available was consumed within 48 h (data not shown) resulting in a noteworthy Q_{max} of 1.51 g/L/h (Table 3), which was even higher than that detected in the sole DR fermentation. This value is very promising as 1 g/L/h is the industrial requirement for ethanol strains (Cripwell et al., 2020; Favaro et al., 2019). Once again, the parental strain confirmed its poor fermenting abilities, with up to 12 g/L ethanol, mostly obtained

from the simple sugars available. As such, the recombinant engineered strain demonstrated nearly 3- and 5-times higher ethanol levels and productivity values (Table 3)

Overall, the combining of both substrates was pivotal to boost ethanol performances and final titers, which was above the industrial threshold of 60 g/L (Favaro et al., 2019). Moreover, this is the first report in the co-processing of two waste streams originating from different plants into bioethanol. Although further efforts in term of process optimization and integration are ongoing towards the industrial application of ER T12.7 on such feedstocks, this approach will pave the way for future exploitation of different byproducts with various compositions and origins into bioethanol.

4. Conclusion

This paper clearly demonstrates that OFMSW can be beneficially converted into bioethanol by the means of highly efficient amylolytic CBP yeast strain, mostly exploiting the OFMSW starch content. This is the first proof of concept also indicating that the OFSMW composition can greatly be influenced by many parameters like geographical location, feeding habits and season which alters the overall ethanol yield. Further investigations are needed to increase substrate loading and to make the process continuous, while improving the rheology of the system to increase the final ethanol titers towards the industrial development. The co-conversion of OFSMW and DR revealed a promising perspective in order to achieve higher ethanol levels while reducing cost of fermentation. Increased ethanol yield will make the suitable for industrial application and open novel research routes towards the co-processing of different industrial by-products into valuable compounds such as ethanol, paving the way towards biorefinery concept to obtain biofuels and other value added chemicals.

5. Acknowledgements

This work was funded by Universit`a degli Studi di Padova through BIRD187814/18, BIRD210708/21, DOR1928058/19, DOR2084579/20, DOR208705/20, DOR2107797/21, DOR2114239/21. Authors are grateful to S.E.S.A. spa for providing resources.

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Figures legends

Figure 1: Ethanol production during fermentation of OFMSW, Enriched OFMSW, and BP (Bread and Pasta) by *S. cerevisiae* ER T12.7 and ERV1. OFMSW (●), Enriched OFMSW (■), BP (♦). Continuous lines and dashed lines describe ethanol production by parental (ER V1) and recombinant (ER T12.7), respectively. The bars represent standard deviation

Figure 2: Ethanol production during fermentation of DR (Discolored rice), Enriched OFMSW+DR by *S. cerevisiae* ER T12.7 and ERV1. DR (●), Enriched OFMSW+DR (■). Continuous lines and dashed lines describe ethanol production by parental (ER V1) and recombinant (ER T12.7) respectively. The bars represent standard deviation

Figure 1

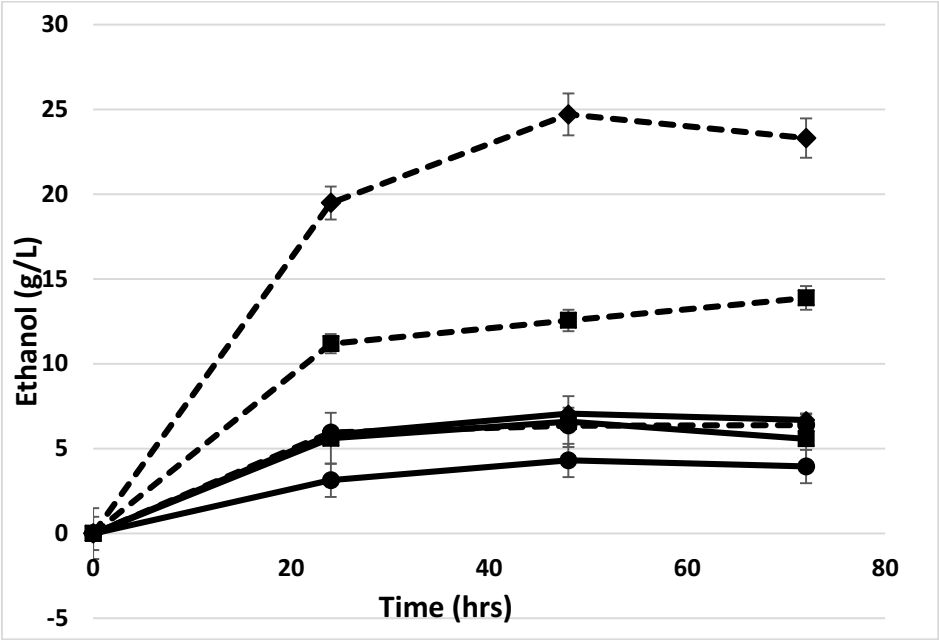

Figure 2

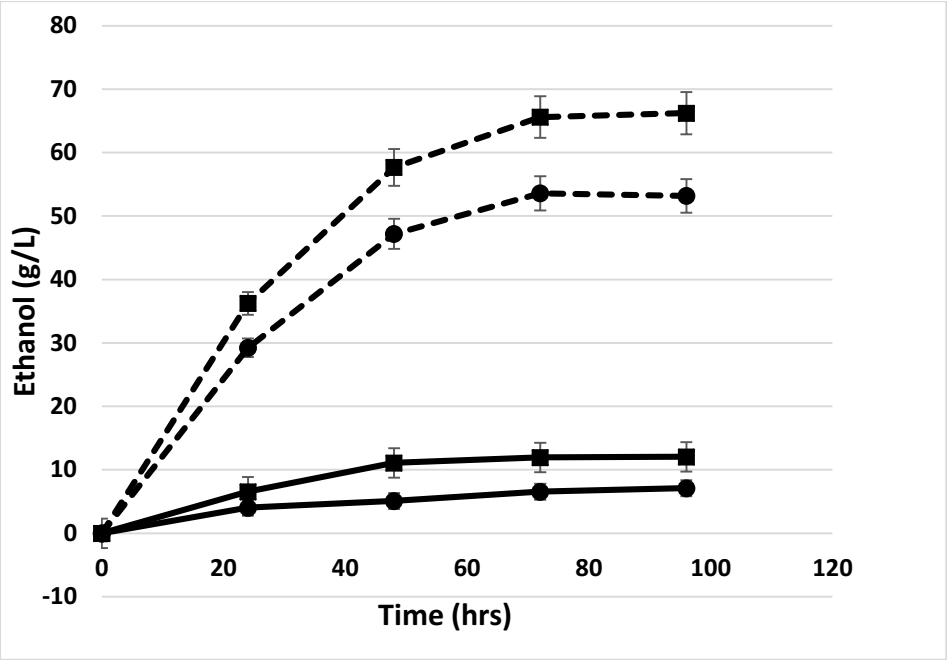
80
70
60
50
40
30
20
10
0
-10
Ethanol (g/L)
0
20
40
60
80
100
120
Time (hrs)

Table 1: Fractions of OFMSW: manual sorting and segregation

Fraction	Weight (kg)	Percentage
Fruit and Vegetables	53.4	55.5
Meat, fish, Cheese	3.8	3.9
Bread and pasta	5.2	5.4
Undersieve (20 mm)	13.2	13.7
Paper	10.4	10.8
Shells and bones	1.2	1.2
Rejected	7	7.3
Total	96.3	100

Table 2: Chemical composition of OFSMW, bread and pasta (BP) and discolored rice (DR).

Waste stream	Starch	Cellulose	Hemicellulose	Lignin	Protein	Lipid	Ash
	(% dry matter)						
OFMSW	3.53±6.2	10.19±5.9	7.27±8.2	5.91±7.4	25.05±7.2	2.85±8.1	31.44±4.8
Enriched OFMSW	10.65±5.9	8.55±7.2	6.47±3.9	5.00±6.3	22.89±8.1	3.19±3.5	28.23±6.9
BP	39.62±8.6	0.43±7.2	9.74±4.8	0.91±7.5	15.44±7.1	14.20±8.2	2.69±4.6
DR	84.60±8.4	0.10±8.6	0.90±9.1	-	8.0±6.8	-	0.50±3.8

Table 3: Conversion of OFMSW, BP and DR to ethanol and other byproducts, separately or in combination, using *S. cerevisiae* parental strain ER V1 and its recombinant ER T12.7, after 72 and 96h

Product (g/L)	OFMSW [b]		BP [b]		Enriched OFMSW [b]		DR[c]		Enriched OFMSW +DR[c]	
	ER V1	ER T12.7	ER V1	ER T12.7	ER V1	ER T12.7	ER V1	ER T12.7	ER V1	ER T12.7
Glucose	0.07 ± 0.02	0.38 ± 0.09	0	0.12 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.30 ± 0.02	0.27 ± 0.02	0.18 ± 0.01	2.39 ± 0.16
Maltose	0.07 ± 0.01	0.24 ± 0.05	0.11 ± 0.02	0.65 ± 0.01	0.12 ± 0.01	0.78 ± 0.04	0.08 ± 0.005	0.87 ± 0.05	0.22 ± 0.01	0.64 ± 0.04
Glycerol	0.38 ± 0.03	0.49 ± 0.02	0.20 ± 0.18	2.62 ± 0.04	0.92 ± 0.06	1.07 ± 0.07	1.21 ± 0.07	4.02 ± 0.23	0.97 ± 0.07	4.85 ± 0.31
Ethanol	3.95 ± 0.15	6.39 ± 0.45	6.68 ± 0.44	23.30 ± 0.55	5.58 ± 0.31	13.88 ± 0.76	7.10 ± 0.47	53.18 ± 2.56	12.05 ± 0.47	66.22 ± 3.43
***Q* (g/L/h)**	0.05	0.09	0.09	0.32	0.08	0.19	0.07	0.55	0.13	0.69
***Q_{max}* (g/L/h)[a]**	0.13	0.25	0.24	0.81	0.23	0.47	0.17	1.22	0.27	1.51

BP- bread and pasta, DR- discolored rice, Q- ethanol productivity, Q_{max}- maximum productivity, [a]- Q_{max} was detected after 48 h, [b]- fermentation conducted till 72 hrs, [c]- fermentation conducted till 96 hrs

Yeast classical genetics:
a tool to produce variation in S. cerevisiae

Article published in Journal of Fungi

Renewing lost genetic variability with a classical yeast genetics approach

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Abstract: Due their long domestication time course, many industrial *Saccharomyces cerevisiae* strains are adopted in numerous processes mostly for historical reasons instead of scientific and technological needs. As such, there is still significant room for improvement of industrial yeast strains relying on yeast biodiversity. This paper strives to regenerate biodiversity with the innovative application of classic genetic methods in already available yeast strains. Extensive sporulation was indeed applied on three different yeast strains specifically selected for their different origin as well as backgrounds with the aim to clarify how new variability was generated. A novel and easy method to obtain monosporal colonies was specifically developed and, to reveal the extent of the generated variability, no selection after sporulation was introduced. The obtained progenies were then tested for their growth in defined medium with high stressors levels. Considerable and strain-specific increase in both phenotypic and metabolomic variability was assessed and few monosporal colonies were found to be of great interest towards their future exploitation in selected industrial processes.

Keywords: yeast classical genetics; metabolomic fingerprint; sporulation; recombination; stress, glucose; formic acid; copper; FTIR

Citation: Gupte, A.P.; Pierantoni, D.C.; Conti, A.; Donati, L.; Basaglia, M.; Casella, S.; Favaro, L.; Corte, L.; Cardinali, G. Renewing Lost Genetic Variability with a Classical Yeast Genetics Approach. *J. Fungi* **2023**, *9*, x. https://doi.org/10.3390/jof9020264.

Academic Editor: Chang-Hui Shen and Milena Georgieva

Received: 28 December 2022
Revised: 6 February 2023
Accepted: 13 February 2023
Published: 16 February 2023

1. Introduction

Apart from being a powerful model system to answer hallmark biological questions, the species *Saccharomyces cerevisiae* plays a key role in many industrial applications [1,2]. The domestication of *S. cerevisiae* strains independently occurred in many processes even before the first microbes were observed [3–5]. After centuries of continuous growth under favorable conditions, with nutrients readily and abundantly available, many domesticated *S. cerevisiae* strains have partly or entirely lost the ability to reproduce sexually [6], gotten more and more tolerant to specific stressors frequently faced in industrial plants, and metabolized a few sugars more rapidly than natural strains [7]. This is reminiscent of the so-called "domestication syndrome," already described in 1868 by Darwin, where organisms under domestication tend to drop undesirable and/or unselected traits and acquire attributes that make them successful in human-shaped environments [8,9].

In the past centuries, brewers, bakers, and, to some extent, even winemakers were used to perform subsequent fermentations using yeast strains from an old batch [5,10]. The back-sloping procedure was indeed essential to maintain the

stability of the final product, thus ensuring the economic sustainability of the process of interest. Both refrigeration and the advent of pure cultures to start the fermentation further enhanced the stability of the final industrial and/or artisanal products [5,11]. Paradoxically, as soon as bakers and brewers recognized the pivotal role of *S. cerevisiae* strains in the fermentation and began to isolate pure cultures, the yeast genetic diversity severely decreased as pure cultures were more and more adopted and clonal batches were maintained by refrigeration [12]. As such, many yeast strains industrially used today, primarily those adopted in bioethanol, wine, and beer fermentations, are often utilized mostly for historical reasons rather than scientific ones [3,13–15]. Furthermore, since the demand of customers as well as industries has turned and continued to turn, there is still significant scope for improvement of industrial strains despite their long domestication time course. The non-genetically modified organisms (non-GMO) approaches, particularly for food and beverage yeast applications, should be considered the most since they do not suffer from any issues with consumer acceptance and/or specific legislation [16,17].

There are multiple non-GMO strategies to provide suitable yeast strains for specific industrial goals (Figure 1), as elegantly reviewed [18]. A very powerful approach is to look for natural biodiversity by selecting a yeast able to operate best in a specific industrial process [13,18]. Indeed, recent metagenomic surveys underpin the fact that the natural yeast biodiversity is immense and largely unexplored, with the existing industrial strains corresponding to only a small share of the natural biodiversity [19–21]. An alternative route is to regenerate biodiversity with the innovative application of classic genetic methods to already available yeast strains [18]. Both the search for natural biodiversity and the regeneration aim at selecting the best phenotypes.

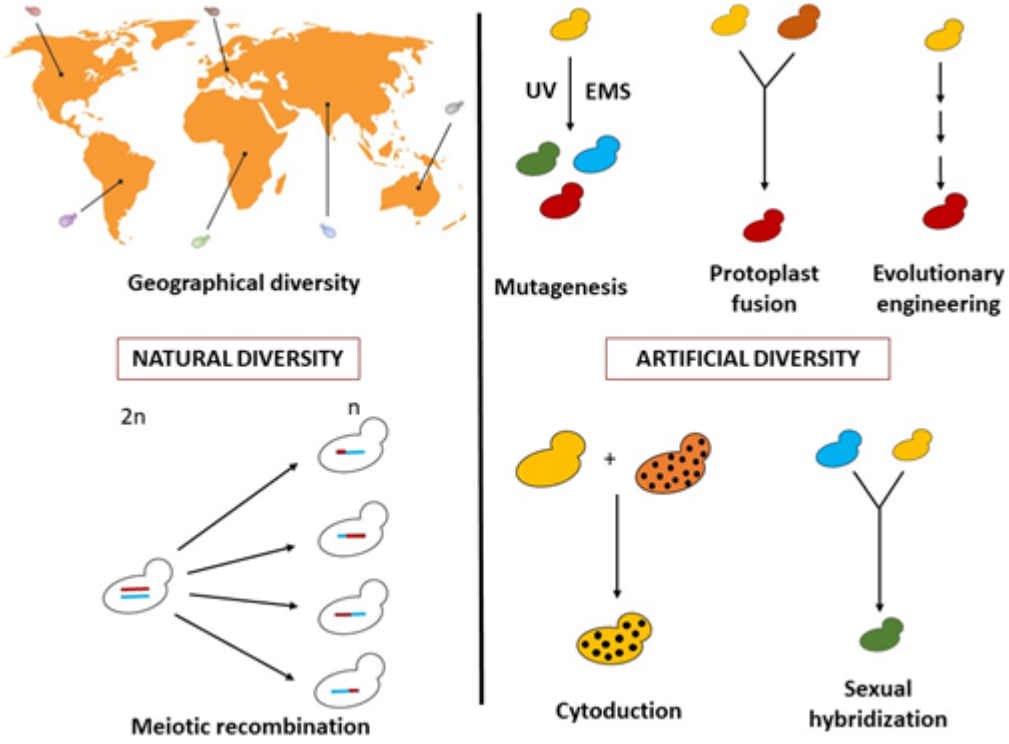

Figure 1. Methods for obtaining genetic variability in yeast adapted from Steensels et al. [18] (UV ultraviolet, EMS, ethyl methane sulfonate)

This work specifically focused on the latter strategy, choosing *S. cerevisiae* as a yeast candidate with a long biotechnological history as well as being a model organism with a homothallic nature. In contrast to higher eukaryotes, yeast gametes enter a haploid life cycle that is substantially similar to the diploid mitosis-based cycle. By taking of this feature, it is possible to produce recombination of important traits by extensive sporulation, obtaining as many combinations as the spores. As an example, *Drosophila* was suggested as a possible niche for sporulation and mating as, different strains of *Schizosaccharomyces japonicus* isolated from Drosophila showed variation for pheromone-related genes [22]. Various sporal cultures can be directly tested or induced to undergo homothallic switching and subsequent diploidization. The output of this route is a collection of diploid cultures that are homozygous at all loci since they are derived from the conjugation of genetically identical cells. These cultures are theoretically very stable, since mutations would rarely affect the phenotype due to the very low, if any, heterozygosity and would provide the most extreme effects of quantitative trait loci. Whether the genome renewal [23] could reintroduce heterozygosity and to what extent is a matter of obvious importance at both the theoretical and practical levels [24].

This work applied extensive sporulation to three different yeast strains, precisely selected for their different origins as well as backgrounds, with the aim of clarifying how new variability was generated from sporulation. Three different hypotheses were investigated: (i) the isolation of diploid homozygous mono-spore colonies (MSCs) allows to verify the amount of variability produced in the sporulation of each parental genotype, (ii) the quantitative traits analyzed show significant differences from the parental strains; and (iii) the obtained variability strictly depends on the starting parental genotype under sporulation. A new method for obtaining MSC without the use of a micromanipulator has been specifically developed, avoiding the introduction of any form of selection to exclusively focus on the extent of variability generated by recombination under sporification. For this purpose, mono-spore colonies (MSCs) were indeed randomly chosen and sequentially analyzed with increasingly informative tests also considering the presence of specific stressors (i.e., high levels of glucose or formic acid, or copper sulphate).

2. Materials and Methods

2.1. Yeast strains and growth conditions

Three *S. cerevisiae* strains with different backgrounds and geographical origins were specifically selected for this study (Table 1). Strains were maintained in 20% glycerol stocks at -80 °C and usually plated on YPD agar (Yeast extract- 10 g L^{-1}, Peptone- 20 g L^{-1}, Glucose- 20 g L^{-1}, Agar- 15 g L^{-1}) and incubated at 30 °C for 48 hrs. Screening for sporulation was performed at 30 °C for 7-21 days on SM1 (potassium acetate- 10 g L^{-1}), McClary's Acetate medium (sodium acetate- 8.2 g L^{-1}, glucose- 1 g L^{-1}, yeast extract- 2.5 g L^{-1}, potassium chloride- 1.8 g L^{-1}, Agar- 15 g L^{-1}) and modified sporulation medium (MSM) (potassium acetate- 10 g L^{-1}, yeast extract- 0.5 g L^{-1}, glucose- 1 g L^{-1}, Agar- 15 g L^{-1}) [25]. All the media were sterilized by autoclaving at 121 °C for 20 min before plating.

S. cerevisiae TC1517 has been isolated from grape marcs [26] and has shown great promise in terms of fermenting abilities. *S. cerevisiae* YI30 was chosen as a strong candidate for lignocellulosic ethanol because of its high inhibitor and temperature tolerance [27]. The Canadian strain *S. cerevisiae* YVGC13A was chosen to evaluate the variability of a strain directly isolated from vine bark, which is

currently considered the main natural reservoir of *S. cerevisiae* strains that could participate in alcoholic fermentation [28].

In addition, stressing experiments and metabolomic studies using Fourier-Transform Infrared Spectroscopy (FTIR), were carried out by inoculating yeast cultures at OD_{600} = 0.1 in 100 mL of filter sterilized (0.22 μm) synthetic defined (SD) medium containing 6.7 g L^{-1} of Yeast Nitrogen Base medium (YNB, Difco Laboratories, USA) and 20 g L^{-1} of glucose and grown them for 16-18 hrs at 30 °C under shaking at 120 rpm.

Table 1. Strains used in this study: background, origin and tested stressing agents.

Strain	Background	Geographical Location	Genotype	Reference	Stressing Agent	Low stress	High stress
TC1517	Grape marcs	Italy	2n, homotallic	[26]	Glucose (g L^{-1})	250	300
YI30	Industrial distillery	South Africa	2n, homotallic	[27]	Formic acid (g L^{-1})	0.3	0.6
YVGC13A	Vineyard, isolated from vine bark	Canada	2n, homotallic	University of Perugia	Copper sulfate (Cu-ppm)	5	7.5

2.2. Spore Production and Sporulation Efficiency

A fresh single colony of each strain was inoculated into 5 mL YPD broth, and microaerophilic conditions were maintained while shaking at 30 °C for 16 hrs. The suspension was centrifuged at 3000 rpm for 5 min and the obtained pellet was washed twice with a sterile 9 g L^{-1} NaCl solution. Washed yeast cells were resuspended in 0.5 mL saline solution, and aliquots of 100 μL were plated on MSM. Plates were then incubated at 30 °C for 7-21 days. To avoid moisture loss, plates were sealed with Parafilm® (Bemis Company, Inc., USA). Microscopic observation was performed every week to observe spore development. The spores were counted after the addition of methylene blue (MB) to the spore suspension to allow the distinction of the living cells from the dead ones that were excluded. The number of dyads, triads, and tetrads was counted using a counting chamber (Thoma, Germany). Sporulation efficiency, a measurement of the amount of cells that undergo sporulation, was calculated by microscopic observation of the sum of triads and tetrads divided by the total asci. Sporulation efficiency (SE) [29] was then calculated as follows:

$$\%\ \text{Sporulation efficiency} = \frac{\text{Number of triads} + \text{Number of tetrads}}{\text{Number of total spores}}\ \text{X}\ 100$$

2.3. Screening of temperature tolerance of PS and spores

In order to develop a quick method to produce MSCs, the minimum temperature required to kill vegetative cells of each parental strain (PS) within a population of spores was screened. Each strain was grown in YPD broth for 16 hrs at 30 °C and centrifuged at 3000 rpm for 5 min. Cells were suspended in sterile saline to a final density of 1×10^7 cells mL^{-1}, and 0.5 mL of cell suspension was transferred to a sterile 1.5 mL tube and exposed for 10 min at different temperatures from 55 to 67 °C at 2 °C intervals.

Each treated suspension was observed microscopically using the MB viability assay [30], and a proper dilution was plated on YPD agar plates in triplicate. The

asci of yeast were broken using zymolyase treatment, as explained in Section 2.4 [31], and the related spores' sensitivity was tested at 63, 65, and 67 °C. The quantification protocol was the same as for vegetative cells.

2.4. Production of Mono-spore colonies

The ascospore isolation method described by Bahalul et al. [31] was modified to avoid the use of diethyl ether. Briefly, colonies grown on MSM agar were scraped and resuspended in sterile, demineralized water. This high-density suspension of asci was heat-treated at 65 °C for 10 min to kill vegetative cells and then processed with zymolyase treatment (Zymolyase®-100T, ICN; 100 U mL−1 in 1M sorbitol) by extending the incubation time to 1 hr. Sterile glass beads (400-600 μm) were used to apply shear force on ascus walls. The resultant spore suspension was observed microscopically using MB to check for the presence of asci or viable vegetative cells. Each suspension was then properly diluted and plated on YPD agar plates supplemented with 5% (w/v) glucose. Thus, obtained *colonies were referred to as* MSCs. Up to 100 MSCs of each PS were stored in 20% glycerol stock at -80 °C. All MSCs were then grown on YPD 5%, transferred to MSM, and incubated at 30 °C for 7-21 days to test their sporulation ability. Microscopic observation was used to check the occurrence of asci and confirm the homothallic phenotype of the parental strains.

2.5. Phenotypic Variation in MSCs

Thirty MSCs were randomly selected from each PS and grown in SD broth at 30 °C for 16 hrs. These pre-cultures were inoculated in the same broth to obtain a final OD_{600} of 0.1 in a final volume of 200 μL. The experiment was run in 96-well plates in triplicate (TECAN Spark® 10M, Austria) at 30 °C (flat-bottom cell culture plate with instrument lid; interval time- 5min; shaking- 60 sec; shaking mode-orbital; amplitude- 2.5 mm). Growth curves were plotted using the *Pyphe-growthcurves* tool. Growth parameters such as maximum growth rate (max_slope), time at max_slope (t_max), and lag phase (Lag) were obtained with the same tool [32]. The definition of the growth parameters given by *Pyphe-growthcurves* are as follows: max_slope- maximum slope of growth curve, t_max- time at which maximum growth slope of curve is reached, lag- lag phase.

Principal component analysis (PCA) was performed [33] considering these growth parameters, and the principal component scores and loading vectors were combined in a biplot used for the selection of specific MSCs for further studies. Additionally, a Student's *t*-test was performed to determine if the observed differences were statistically significant. Moreover, at least one MSC with growth parameters similar to those of the parents was also included.

The resulting selected 12 MSCs and their parental strain were then grown under specific stressing conditions to observe growth parameters and, as reported in paragraph 2.7, metabolomic changes at different stressing levels. The MSCs and PS were pre-inoculated in SD medium and grown at 30 °C for 16 hrs. Each PS and respective MCS were inoculated (OD_{600} = 0.1) in SD medium with no stressing agent and in the presence of low and high concentrations of the stressing agent. Each test was performed in triplicate at 30 °C in a 96-well microtiter plate (TECAN Spark® 10M, Austria) with the same protocol described above.

2.6. Metabolomic Fingerprint at the End of Growth

Cell suspensions, prepared as detailed in Section 2.1, were centrifuged (4500 x g, 5 min), washed twice with distilled sterile water, and re-suspended in 5 mL HPLC (High-Performance Liquid Chromatography) grade water to the final concentration of OD_{600} = 12. From each culture, 105 µL volume were sampled for three independent FTIR readings (35 µL each, according to the technique suggested by Essendoubi and colleagues [34].

2.7. Metabolomic Fingerprint under Stress

The FTIR analysis was also applied to investigate the metabolomic response under the stress of the selected MSCs cultures compared to their respective parental strains. MSCs and parental strain cultures were grown under different concentrations of stressing agents, as detailed in Table 1. However, yeast cultures were pre-inoculated at OD_{600} = 0.1 in 15 mL tubes with 7 mL of SD medium and grown at 30 °C under shaking at 120 rpm. Cell growth was stopped after 15 hrs. Each cell suspension was adjusted to an OD_{600} = 0.2 in a 2X fresh SD medium. A total of 100 µL of each standardized cell suspension was seeded in each selected well of a flat-bottom 96-well microtiter plate and brought to the final volume of 200 µL by adding 100 µL of a 2X solution of the respective stressing agent. Control (0% stressor concentration) was obtained by re-suspending cells in sterile, distilled water. All tests were carried out in triplicate. The growth was monitored in the TECAN as described above. The samples were collected at the end of the log phase of growth and processed for FTIR analysis [34].

2.8. FTIR data analysis

FTIR spectra were recovered from the OPUS software version 6.5 (Bruker Optics GmbH, Ettlingen, Germany) and transferred to MS Excel. Principal Component Analysis (PCA) and Significant Wavelengths Analysis (SWA) were performed in an R environment. SWA was employed to select the FTIR spectral regions with statistically significant differences in the comparison between the spectra of parental and MSCs cultures from the different experimental conditions tested [35]. In addition, pairs of spectra, each with three replicates, were compared using the Student's t-test for each wavelength separately. For each wave number, the calculated *p*-value was recorded. Significant wavelengths were selected based on $p < 0.01$. Hierarchical cluster analysis was performed with MetaboAnalyst 5.0 [36]. Data were filtered based on interquartile range, normalized to the sample median, and scaled by Pareto scaling. Hierarchical cluster analysis (HCA) was employed to highlight the metabolic differences under stress between MSCs and PS cultures, using the Euclidean correlation method and the Ward.D clustering algorithm. Significant wavelengths were selected based on these criteria: t-test (*p* adjusted < 0.05) and one-way ANOVA (*p*-value < 0.05).

3. Results and Discussion

3.1. Efficiency of Sporulation (SE) and Development of an Easy and Effective Protocol for MSCs Production

The three strains of *S. cerevisiae* were specifically selected for their different geographical origins and phenotypic backgrounds (Table 1). To develop a simple yet efficient protocol for obtaining high numbers of MSCs, the parental strains were

first tested for their sporulation efficiency (SE) once plated on different media [25,37,38]. The highest SE was obtained on MSM plates, confirming literature data on the role of nutrient deficiency and non-fermentable carbon sources, such as acetate, in inducing sporulation [39] and on the involvement of the salt acetate cation in promoting the SE of yeast strains [38]. Sporulation media was indeed modified by Petersen et al. [40] to increase the SE. The addition of yeast extract to MSM also improved the SE, as reported by Tremaine et al. [41]. Notably, YI30 showed the highest SE (85.5%), and the other two yeast strains displayed slightly lower values (*S. cerevisiae* TC1517 and YVGC13A, at 68.4, and 64.1% SE, respectively).

Dawes and Hardie proved that vapors of diethyl ether in an agar plate or ether in liquid media kill the vegetative yeast cells, keeping spores alive [42]. This was previously applied once a protocol combining glusulase treatment, sonication, and separation of hydrophobic spores using diethyl ether was developed [43]. The diethyl ether protocol was also adopted after clubbing it with zymolyase and microbead treatment [31].

In the present study, temperature rather than diethyl ether was employed to kill vegetative cells [31], thus avoiding the use of an extremely flammable and volatile chemical solvent. Once exposed to high temperatures, the ascospores displayed greater tolerance than the respective vegetative cells. Separate experiments showed that vegetative cells tolerated temperatures up to 63 ± 0.5 °C. When the temperature was increased to 65 ± 0.5 °C, the parental strain was unable to grow, but the ascospores were able to produce colonies on YPD agar medium. These results are in line with those of Rachon et al. [44], who already observed a significant difference in temperature tolerance for vegetative cells and ascospores at 65 °C.

In order to optimize the ascospore separation protocol step, sporulated parental strains were scraped from MSM agar plates and suspended in sterile demineralized water. The zymolyase treatment, developed by Bahalul et al. [31], was found to be efficient with the extension of the zymolyase treatment to one hour. The combination of zymolyase and glass beads treatment was crucial to separate ascospores from broken asci, followed by heat treatment at 65 °C for 10 min.

Microscopic observation showed that around 60% of the asci were disrupted, releasing circular and refractive spores in suspension. These suspensions gave rise to individual colonies, referred to as MSCs, once plated on YPD agar medium.

Around 100 MSCs from each parental strain were thus obtained, and their homothallic nature was investigated as detailed in the 2.4 section. All parental strains were confirmed to be phenotypically homothallic, since all MSCs tested were able to produce spores.

3.2. Growth of MSCs from Each Parental Strain in SD Broth

Thirty randomly selected MSCs from each parental strain were first screened for their growth at 30 °C in SD medium with 2% glucose. OD_{600} was monitored for 24 hrs at 30 °C using a 96 well plate reader (TECAN Spark® 10M, Austria). The generated growth curves were processed using the *pyphe-growthcurves* tool to assess specific parameters such as max_slope, t_max, and lag used for the PCA analysis of Figure 2.

The first two principal components explained 97% of the variance between all the MSCs cultures (PC1: 62% and PC2: 35% of the variance). The spatial distribution of the MSCs cultures indicated a clear signature of the respective parental strains, suggesting that both ecological origin and geographical background are of great importance for the phenotypic variation triggered by sporulation. Camarasa et al.

[45] observed similar results when metabolic traits were considered as differentiating parameters to understand the origin of *S. cerevisiae* strains. Interestingly, the growth performances of each parental strain remained outside the confidence ellipse, indicating higher variation between the growth of the parental strain and that of the corresponding MSCs. The highest variability was found within the monosporal progeny of the environmental yeast YVGC13A. Conversely, most of the MSCs from the YI30 and TC1517 strains formed a compact group, except for a few MSCs positioned outside the confidence ellipse.

According to PC1, the Lag Phase parameter was the most differentiating between groups. The other two parameters mainly contributed to the separation of the YVGC13A cluster from those of YI30 and TC1517 along the PC2.

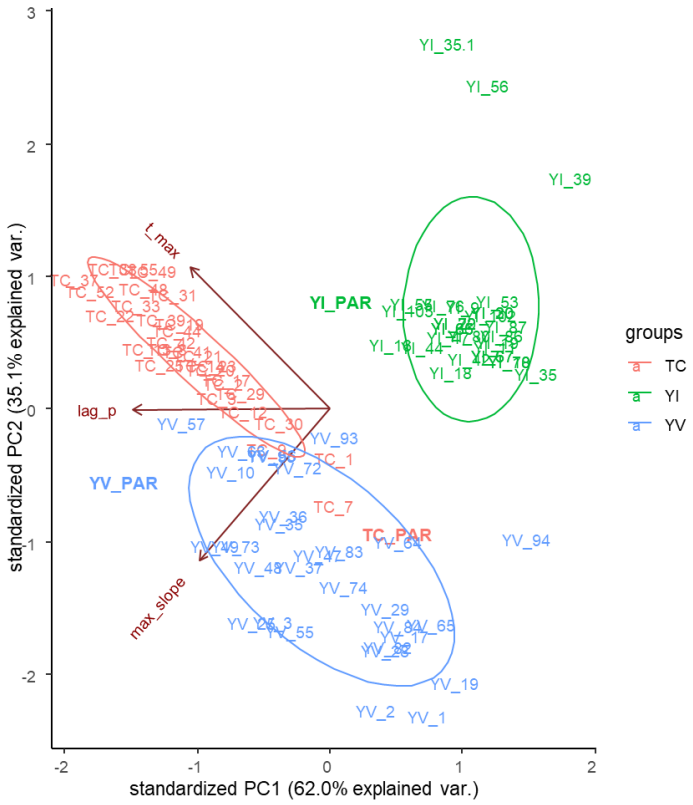

Figure 2. PCA biplot obtained from the growth parameters of all the 90 MSCs selected for the study. Input variables: Lag, t_max and max_slope growth parameters obtained using the *pyphe-growthcurves* tool from the growth curves of YVGC13A (YV, blue), TC1517 (TC, red), and YI30 (YI, green) cultures in SD with 2% glucose

The same analysis was then carried out separately for each tested progeny (Figure 3). In all cases, most of the variance is distributed along the PC1, specifically 62.3, 77.3, and 55.9% for the YVGC13A, TC1517, and YI30 strains, respectively.

Interestingly, as already underlined in Figure 2, the parental strain was not part of the distribution of the variance of the relative MSCs cultures. Moreover, the three parameters differentially shaped the variance within each population, with lag and t_max as the main drivers for PC1 in the YVGC13A and TC1517 populations, while separately contributing along both PC1 and PC2 for the YI30 MSCs cultures.

Overall, these data already suggest that sporulation triggered phenotypic differences during aerobic growth in the presence of glucose. To further assess this evidence, twelve out of the 30 MSCs tested in each group were selected to undergo FTIR fingerprinting. MSCs were selected according to their statistically different growth parameters (p-value < 0.01) with respect to their parental strain (Tables S1–S3). Moreover, at least one MSCs with growth parameters such as those of the parental strain was included in the shortlist.

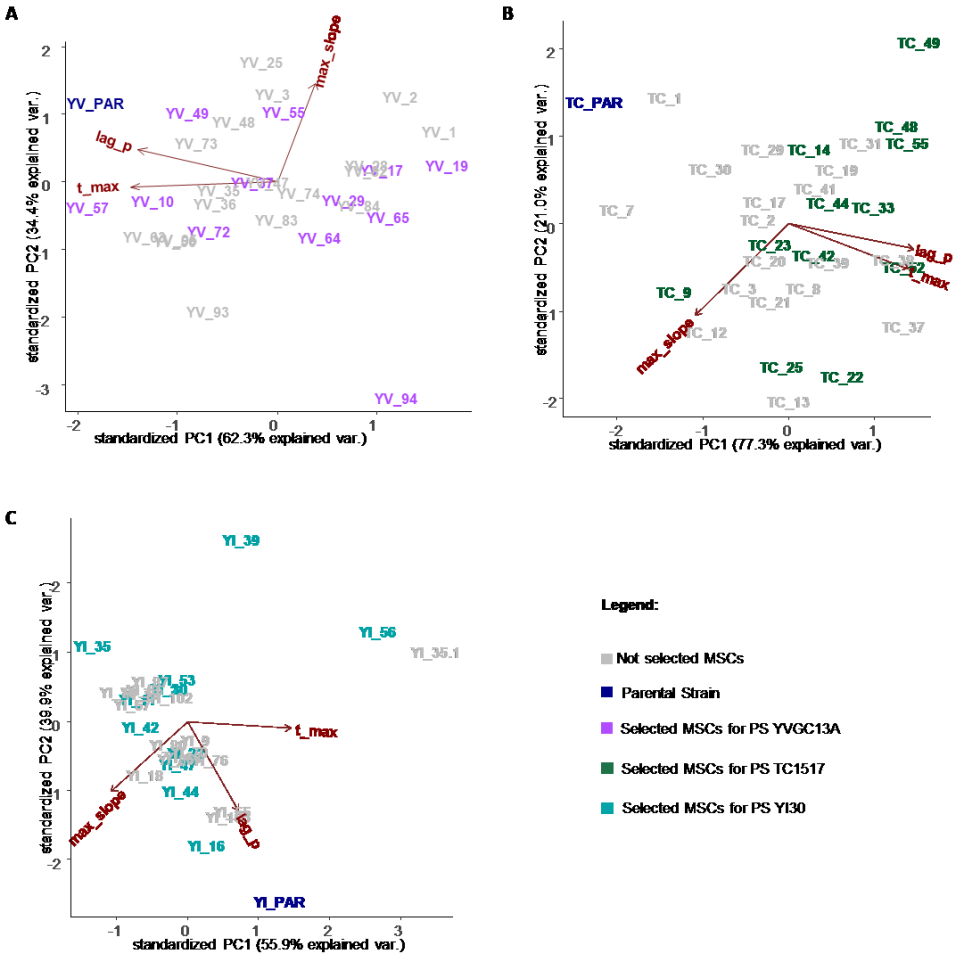

Figure 3. PCA biplot of growth parameters obtained from the 30 MSCs selected from the sporulation of each *S. cerevisiae* parental strain. Input variables: Lag, t_max and max_slope growth parameters obtained using the *pyphe-growthcurves* tool from for 30 MSCs of the parental *S. cerevisiae* strain YVGC13A (**A**), TC1517 (**B**) and YI30 (**C**). Parental strains are reported in blue, not selected MSCs cultures in grey and the twelve MSCs cultures selected for the next step of the analysis in violet (A), green (**B**) and light blue (**C**)

3.3. Metabolomic Fingerprinting of Selected MSCs

The selected MSCs were grown in SD broth supplemented with 2% glucose, and the cells were harvested at the end of the exponential phase to analyze the metabolomic fingerprint of their primary metabolism. The "R" script for Significant Wavelengths Analysis (SWA) was then adopted to compare all the statistically relevant differences between the spectra of PS and each related MSC [35]. Significant

wavelengths were selected based on the Student's t-test ($p < 0.01$, and their number was computed within each spectral region (Figure 4).

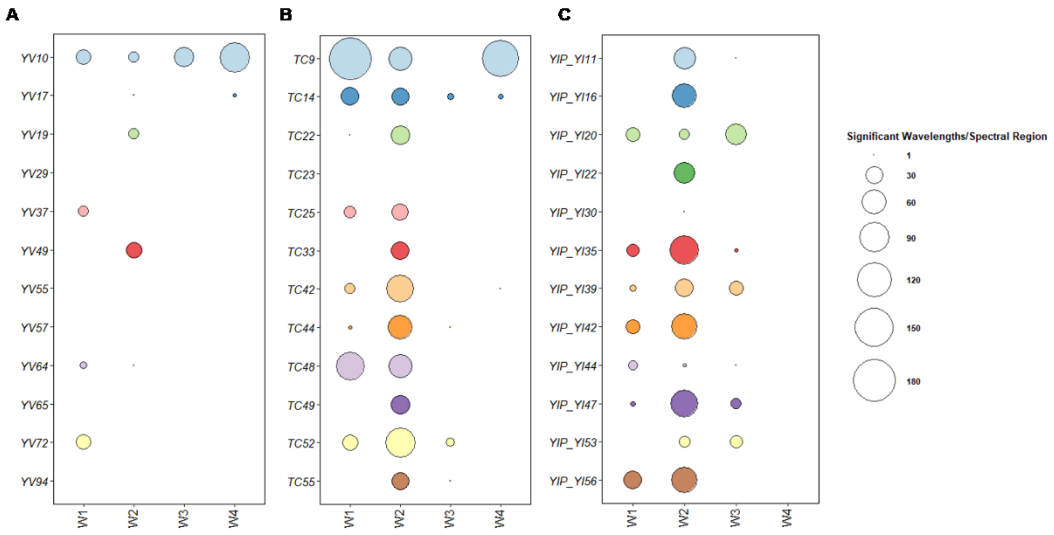

Figure 4. Number of significant different wavelengths detected in the comparison between each *S. cerevisiae* parental strain and the selected 12 MSCs. Spectra were compared using the Student's t-test for each wavelength separately. The number of wavelengths with statistically significant difference ($p < 0.01$) was calculated for each specific spectral area separately, namely: fatty acids (W1), amides (W2), mixed region (W3) and carbohydrates (W4) regions. (**A**–**C**): MSCs of YVGC13A, TC1517 and YI30, respectively

The FTIR fingerprints of monosporal cultures from the parental strain YVGC13A (Table S4, Figure S1) showed little to no variability, except for the MSC YV_10, which displayed significant differences in all the spectral regions tested. Notably, the highest variation was observed in the carbohydrate region of the FTIR spectrum (Figure 4A).

Although the five MSCs YV_29, YV_55, YV_57, YV_65, and YV_94 showed significantly different growth kinetics from their parental cells (Table S1), these differences did not induce significant changes in their metabolome.

On the contrary, higher variation of the metabolomic profiles was observed in most MSCs cultures from *S. cerevisiae* TC1517 (Table S5, Figure S2) and YI30 (Table S6, Figure S3). As reported in Figure 4B, within the TC1517 progeny, the greatest variability was focused on the amide (W2) and fatty acid (W1) regions.

Huge variations were observed in TC_9 in the fatty acid, amide, and carbohydrate regions (Figure 4B). Statistical analysis of growth parameters showed significant differences for all the tested MSCs in comparison to the parental strain except TC_9, whose t_max was the only one significantly different ($p < 0.05$) from the parental yeast. TC_23, which displayed a metabolome similar to the parental, was characterized by a t_max statistically divergent from the parental ($p < 0.01$).

Considering YI30, MSCs also showed significant differences in W1 and W2 regions (Figure 4C), with seven out of the twelve selected MSCs carrying metabolomic changes also in the mixed region (W3). No metabolomic alteration was instead detected for the carbohydrate metabolism (W4). The MSC YI_30 shared the metabolome of its parental strain except for a few wavelengths in the W2 region. Of

the eleven MSCs exhibiting metabolomic differences in SWA, only four responded differently to the statistical analysis of growth parameters, while YI_16, YI_20, YI_44, and YI_53 showed no significant differences ($p < 0.01$) compared to the parental strain (Table S3).

Overall, FTIR fingerprinting of the tested MSCs clearly indicates a specific progeny signature. The lowest metabolomic changes were detected within the YVGC13A-derived MSC. Conversely, the sporulation of TC1517 and YI30 parental strains pushed the metabolomic variability of MSCs into the amides (W2) region, also triggering a response in the W1 and W3 regions for TC1517 and YI30 MSCs, respectively.

Based on both metabolomic and growth phenotypes, six MSCs for each parental strain were further selected to be representative of the variability produced by sporulation by choosing those with lower, higher, and PS-like growth phenotypes as well as similar or different metabolomic traits.

3.4. Growth and metabolomic phenotypes under stressing conditions

In order to further assess the phenotypic changes due to the genetic reshuffle mediated by sporulation, the second set of selected MSCs were tested for growth and metabolomic changes (Tables S7–S9; Figures S4–S6) once exposed to stressors specific to the parental strain background (Table 1).

The choice of copper as a stressor for the YVGC13A strain, isolated from vine bark in Canada, is based on the evidence that copper-based fungicides have been used in vineyards for more than 100 years and copper sulphate-based fungicides are the only chemicals allowed under organic standards [46]. High glucose concentrations can damage yeast cells and hamper their normal growth and metabolism [47]. The effect of high glucose levels on altering cell metabolism is therefore particularly interesting for a strain such as TC1517 isolated from grape marc [26]. Finally, since *S. cerevisiae* YI30 has been described as a promising candidate for second-generation bioethanol [13,27,48], formic acid was chosen as one of the most toxic weak acids [26,27] generated during the pre-treatment of lignocellulose wastes and their conversion to ethanol [13].

Overall, when grown under increasing concentrations of stressing agents, both parental strains and related MSCs displayed a dose-dependent response (Figures 5–7).

3.4.1. Phenotypes under Copper Sulphate Stress

S. cerevisiae YVGC13A-progeny were tested for the ability to withstand increasing concentrations of copper sulphate. Growth performances in the benchmark broth SD (Figure 5A) revealed significant differences in lag and max_slope values ($p < 0.05$) for all MSCs tested, except for YV_49 and YV_57. YV_10, YV_64, and, to a lesser extent, YV_94, showed the most interesting phenotypes for the simultaneous increase in the max_slope and decrease in the lag phase, significantly improving the growth kinetics of these MSCs.

Additionally, differences with respect to the parental strain were even more intense once considering the metabolomic reactions (Figure 5D, Table S7, Figure S4). Although with similar growth kinetics, the YV_57 MSC exhibited a metabolomic pattern more divergent from the PS, downregulating bands in mixed and carbohydrate regions (W3 and W4) and up-regulating those in W1. Conversely, YV_72 and YV_94 MSCs, which showed significantly different lag phases, displayed more similar metabolomic patterns, except for an increase in W4 and a decrease in

W2 band intensities. The other three MSCs, YV_10, YV_49, and YV_64, had a pattern slightly reversed from that of PS, reducing W4 and increasing W2.

Once exposed to 5 ppm of copper, most MSCs showed higher sensitivities than *S. cerevisiae* YVGC13A, characterized by a longer lag phase and a lower max_slope (Figure 5B). Only the YV_72 displayed a lag phase shorter than PS and can therefore be considered the most tolerant MSC at this copper concentration. The heatmap of the significantly altered FTIR peaks (Figure 5E) highlighted how the increased sensitivity of the MSCs corresponded to a general decrease in intensity of the whole spectra except for the W4_2 bands in the YV_10, YV_57, YV_64, and YV_72 MSCs. Among all MSCs, only the YV_49 clustered together with the PS.

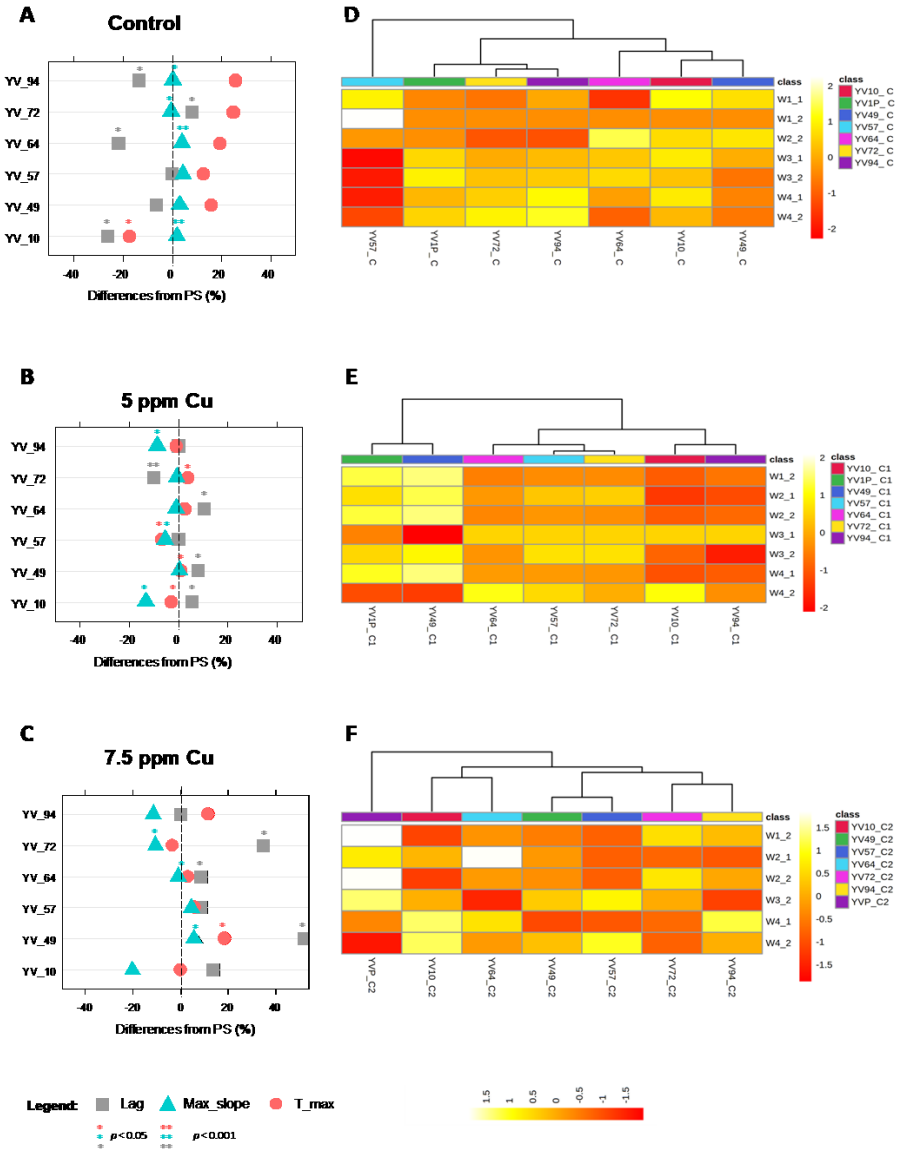

Figure 5. Growth and metabolomic phenotypes of YVGC13A *S. cerevisiae* parental strain and its derived MSCs during growth at increasing concentrations of copper (5–7.5 ppm). Panels (**A–C**): Lag time (grey square), max_slope (light blue triangle) and t_max (light red circle) parameters reported as percentage difference respect to the parental strain. Panels (**D–F**): Heatmap of the significantly altered FTIR peaks (distance measure using Euclidean, and

clustering algorithm using ward.D). The coloured boxes indicate the relative intensities of the mean of peaks in the corresponding spectral region. The colour scale is log2 transformed value and indicates relatively high (yellow) and low (red) peak intensities. Spectral regions have been divided into sub-regions, namely: Fatty acids (W1_1 from 3200 to 3100 cm^{-1}–W1_2 from 3098 to 2801 cm^{-1}); Amides (W2_1 from 1800 to1649 cm^{-1}–W2_2 from 1647 to 1501 cm^{-1}); Mixed region (W3_1 from 1499 to 1352 cm^{-1}–W3_2 from 1350 to 1202 cm^{-1}); Carbohydrates (W4_1 from 1200 to 1053 cm^{-1}–W4_2 from 1051 to 902 cm^{-1})

As the copper concentration increased (7.5 ppm), a quite different scenario was depicted (Figure 5C, Table S7, Figure S4). YV_10, YV_57, and YV_94 MSCs seem to increase their tolerance, by showing the same phenotype as *S. cerevisiae* YVGC13A. Conversely, YV_64 and YV_72 MSCs displayed increased sensitivity, attributable to the longer lag and the reduction of the max_slope values. Noteworthy, the YV_49 displayed higher values than the PS for all growth parameters considered. Despite the longer lag phase, this culture was then able to grow more rapidly during the log phase, giving a higher cell density than the parental strain YVGC13A.

In reaction to this copper concentration, we observed an increase in the variability of metabolomic profiles, resulting in MSC-dependent signatures (Figure 5F). Overall, the metabolomic patterns of MSCs mirrored those of PS, depicting a general slowing of metabolism to the exclusion, in a few cases, of carbohydrates in the W4_2 region.

With respect to its progeny, *S. cerevisiae* YVGC13A reacted to copper's supplementation by inducing genes responsible for carbohydrates metabolism and protein biosynthesis as a general mechanism of stress response in this species (Figures 5E,F) [45,49]. Conversely, the higher intensities in fatty acids may be the result of higher reactive oxygen species (ROS) accumulation, already described as a specific *S. cerevisiae* response to stress conditions triggered by copper [50,51].
Generally, the sporulation of YVGC13A has produced a significant amount of variability, resulting in some improved phenotypes, both at rest and under stressful conditions. However, the MSCs that performed better in control broth were not the same ones that displayed increased tolerance to copper supplementation. These data supported the hypothesis that the extensive sporulation applied in this study increases the amount of available variability compared to those procedures implying sporulation under selection conditions.

3.4.2. Phenotypes under Glucose Stress

In the case of TC1517-derived progeny, the control in SD broth was useful to confirm and further investigate the differential behaviors of the selected MSCs both in terms of growth parameters (Figure 6A) and alteration of cell metabolomes (Figure 6D).

Only the TC_44 displayed growth parameters such as those of the parents. The other MSCs were able to grow faster than the parental strain, with significantly higher t_max and max_slope values (Figure 6A). Remarkably, the increase in the growth rate of TC_22 and TC_23 was linked to specific metabolomic changes induced by the up-regulation of fatty acids and amides bands (W1 and W2_1) coupled with the down-regulation of mixed and carbohydrates ones (W3_2 and W4) (Table S8, Figure S5). The fingerprints of these MSCs were reversed with respect to those of *S. cerevisiae* TC1517 (Figure 6D). The other four MSCs displayed lower changes and were mainly located in the W1 and W2_1 regions.

At increasing glucose concentrations, significant alterations were evident in both the growth and metabolomic phenotypes of the selected MSCs. In the presence of 25% glucose, TC_52 was the only MSC that significantly increased the growth rate

(max_slope) compared to that of the parental, as already revealed in the control condition. No significant differences were detected for the other MSCs, except for TC_44 and TC_55, both affected by a significant increase in t-max values and a reduction in max slope values ($p < 0.05$), resulting in a reduced growth rate (Figure 6B).

Furthermore, once challenged with 25% glucose, the parental strain expressed metabolomic changes opposite those observed in the benchmark broth (Figure 6E). The increase in glucose concentration prompted higher intensities of fatty acid and protein bands (W1 and W2_1) together with a reduction of those in mixed and carbohydrate regions (W3_2 and W4). The same response was observed for TC_9 MSC, which, noteworthily, exhibited growth performances such as those of *S. cerevisiae* TC1517. On the contrary, TC_23, TC_52, and TC_55 MSCs had an antithetical regulation in these spectral regions. Additionally, the last two MSCs, sharing similar metabolomic footprints, showed opposite growth behaviors. Finally, the TC_44 exhibited band intensities around neutrality for all spectral regions.

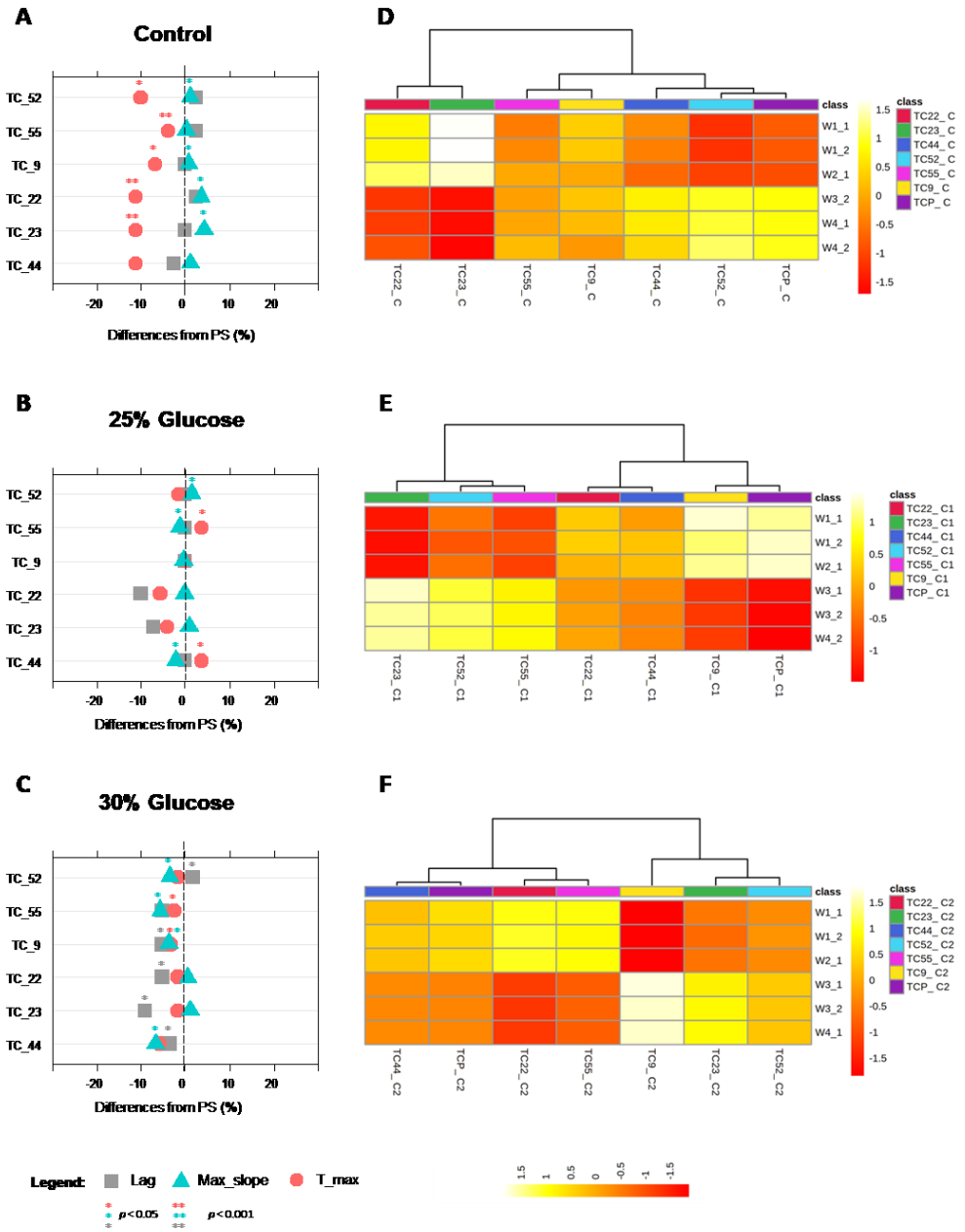

Figure 6. Growth and metabolomic phenotypes of *S. cerevisiae* TC1517 strain and its derived MSCs during growth at increasing concentrations of glucose (25-30%, w/v). Panels (**A–C**): Lag time (grey square), max_slope (light blue triangle) and t_max (light red circle) parameters reported as percentage difference respect to the parental strain. Panels (**D–F**): Heatmap of the significantly altered FTIR peaks (distance measure using Euclidean, and clustering algorithm using ward.D). The coloured boxes indicate the relative intensities of the mean of peaks in the corresponding spectral region. The colour scale is log2 transformed value and indicates relatively high (yellow) and low (red) peak intensities. Spectral regions have been divided into sub-regions, namely: Fatty acids (W1_1 from 3200 to 3100 cm^{-1}–W1_2 from 3098 to 2801 cm^{-1}); Amides (W2_1 from 1800 to1649 cm^{-1}–W2_2 from 1647 to 1501 cm^{-1}); Mixed region (W3_1 from 1499 to 1352 cm^{-1}–W3_2 from 1350 to 1202 cm^{-1}); Carbohydrates (W4_1 from 1200 to 1053 cm^{-1}–W4_2 from 1051 to 902 cm^{-1})

The glucose supplementation up to 30% significantly affected the growth parameters ($p < 0.05$) and led to a general reduction of all MSCs' growth (Figure 6C). In TC_9, growth reduction was accompanied by a substantial alteration of cell metabolism with the down-regulation of fatty acids (W1) and proteins (W2) and the up-regulation of mixed (W3) and carbohydrate (W4) regions (Figure 6F). A similar response, though of lesser intensity, was displayed by TC_23 and TC_52 MSCs. The TC_22 and TC_55 clustered separately because of the opposite response to that of these three MSCs. At glucose 30%, TC_44 was the only MSC that displayed the same metabolomic alteration as the parental TC1517.

Overall, the sporulation of TC1517 has triggered a renewed level of variability that has impacted both the growth and metabolomic phenotypes of the six MSCs selected. In the absence of glucose stress, the growth phenotypes of most spores improved compared to the parental strain. Conversely, high glucose levels induced a general worsening of growth performance by reducing the growth rate of the MSCs. The only exception was the TC_52 culture, which maintained a growth rate higher than that of the parental cells even under 25% glucose.

Interestingly, TC_22 and TC_23 MSCs, which demonstrated the best performance under control conditions, reacted to glucose addition by significantly shaping their metabolism in the direction of a reduction of the lag phase, a typical response of strains with increased tolerance to a specific stressor [52,53].

The metabolomic fingerprint of these strains, despite having a dose-dependent pattern, showed a peculiarity in the constant clustering of the responses of W1 and W2 in opposition to those of W3 and W4, both under control and in stressed conditions. This evidence could be attributed mainly to the opposite regulation of genes involved in protein and carbohydrate metabolism. The fact that some stressing conditions induce carbohydrate metabolism genes by down-regulating those involved in protein biosynthesis has already been observed in *S. cerevisiae* [54]. It is well documented that *S. cerevisiae* cells accumulate some carbohydrates in response to different types of stress [45,49,55]. In the presence of 15 g L^{-1} of glucose, the production of intracellular glycerol and trehalose was found to be significantly increased [47]. Furthermore, glucose concentration has been reported to have a proportional effect on intracellular ROS, which increases intensity in the W1_1 region [56].

3.4.3. Phenotypes under formic acid stress

When grown in control broth, most of the MSCs of *S. cerevisiae* YI30 were affected by a significant increase in the lag phase ($p < 0.05$) with respect to the parental strain (Figure 7A). The variability induced by sporulation also interested the max slope value in YI_11, which exhibited the worst phenotype together with YI_22.

Based on the metabolomic alterations (Table S9, Figure S6), MCSs were grouped into two main clusters (Figure 7D). The first included four MSCs, of which YI_20 and YI_35 are closest to the PS, whereas YI_22 and YI_39 are in a separate subcluster characterized by a general downregulation of bands in all spectral regions. The second group consisted of YI_11 and YI_53 MSC, which were separated from the PS mainly by reduced intensities in the amide bands and increased signals for carbohydrates.

The phenotypes described for growth in a resting condition significantly changed in response to formic acid, according to the increase in dose (Figures 7 B,C). The presence of 0.3 g L^{-1} formic acid modified growth phenotypes except for YI_11, which maintained the same pattern displayed in the control broth (Figure 7B). No

significant differences from the parental strain were detected for YI_22, YI_39, and YI_53, while YI_20 and YI_35 were faster thanks to the significant reduction in Lag and t_max ($p < 0.05$).

At the highest tested concentration (0.6 g L^{-1}), formic acid clearly prompted the growth of all MSCs by reducing the lag parameter with respect to the PS ($p < 0.05$), with the only exception of YI_39 MSC (Figure 7C).

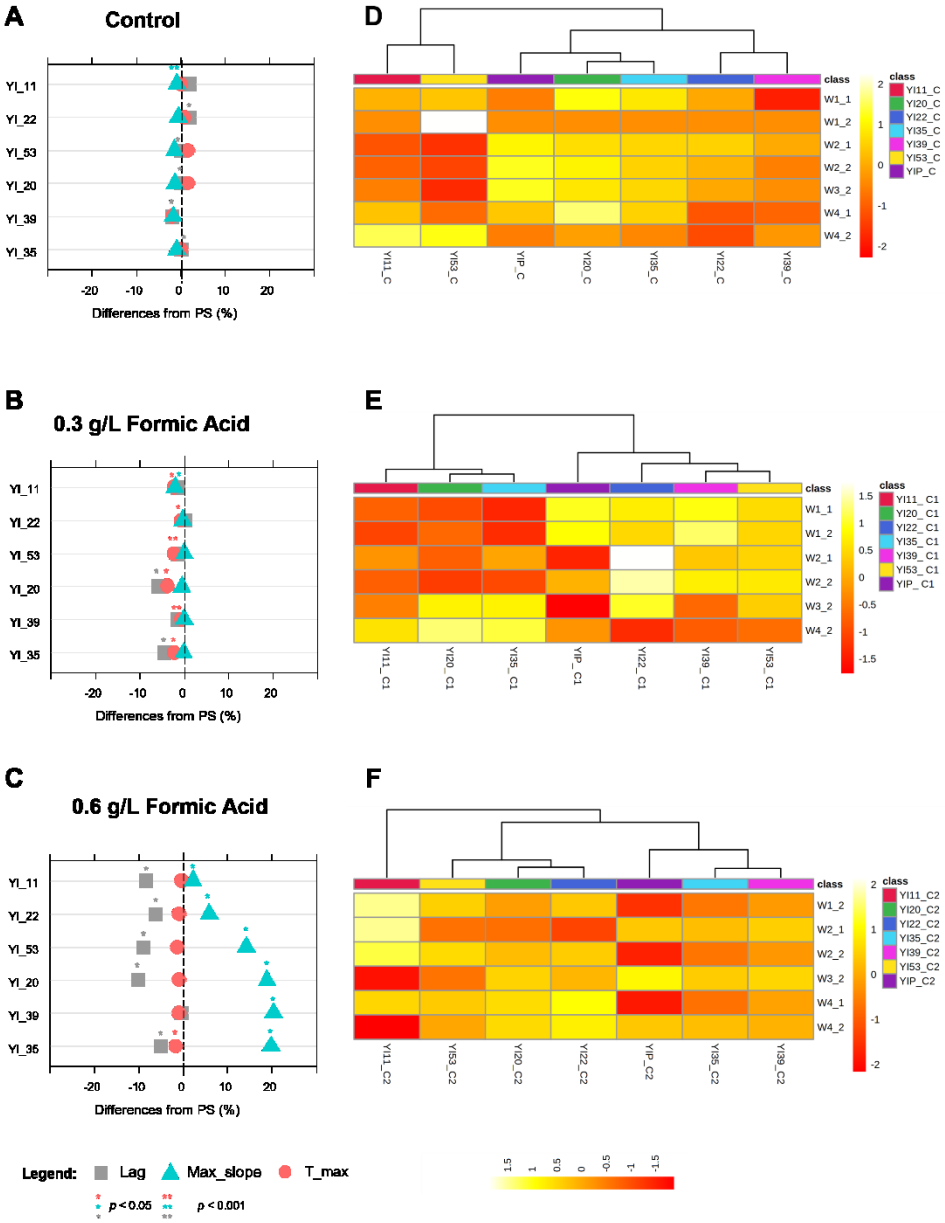

Figure 7. Growth and metabolomic phenotypes of YI30 *S. cerevisiae* parental strain and its derived MSCs during growth at increasing concentrations of formic acid (0.3 and 0.6 g L^{-1}). Panels (**A–C**): Lag time (grey square), max_slope (light blue triangle) and t_max (light red circle) parameters reported as percentage difference respect to the parental strain. Panels (**D–F**): Heatmap of the significantly altered FTIR peaks (distance measure using Euclidean, and clustering algorithm using ward.D). The coloured boxes indicate the relative intensities of the mean of peaks in the corresponding spectral region. The colour scale is log2 transformed value and indicates relatively high (yellow) and low (red) peak intensities. Spectral regions have been divided into sub-regions, namely: Fatty acids (W1_1 from 3200 to 3100 cm^{-1}–W1_2

from 3098 to 2801 cm^{-1}); Amides (W2_1 from 1800 to1649 cm^{-1}–W2_2 from 1647 to 1501 cm^{-1}); Mixed region (W3_1 from 1499 to 1352 cm^{-1}–W3_2 from 1350 to 1202 cm^{-1}); Carbohydrates (W4_1 from 1200 to 1053 cm^{-1}–W4_2 from 1051 to 902 cm^{-1})

The heatmaps of the significantly altered FTIR peaks (Figures 7E,F) showed that the differential ability to withstand formic acid was mediated by a fine tuning of carbohydrates, proteins, and fatty acid pathways. In addition, the improved performance of YI_20 and YI_35 in the presence of 0.3 gL^{-1} formic acid (Figure 7E), supported by the strong down-regulation in W1 and up-regulation in W3_2 and W4_2 regions, could be explained considering that *S. cerevisiae,* under some stressful conditions, induced genes involved in carbohydrate metabolism while down-regulating those involved in protein biosynthesis. In addition, the metabolomic pattern shown by YI_11 at higher concentrations (Figure 7F) suggests that other mechanisms may be involved in the response to formic acid stress. ROS, which are potentially responsible for providing tolerance to toxic formic acid, fall in the fatty acid region (W1_1). The higher intensity in W1_1 bands displayed by YI_11 can possibly be related to the higher accumulation of ROS [56]. This hypothesis is under investigation using a focused LC/MS approach.

Overall, the sporulation of YI30 resulted in MSCs with differential ability to withstand increasing concentrations of formic acid and was useful for the selection of a few candidates with promising phenotypes to be further studied to both shed light on the still poorly investigated mechanism of formic acid tolerance in *S. cerevisiae* and to develop superior yeast strains with increased resistance to this weak acid [57,58]. Few MSCs, indeed, showed a lower lag phase, thus reacting much faster than the parental strains thanks to strong and strain-specific intracellular metabolomic reactions.

4. Conclusions

The main hypothesis of this paper is that the proposed non-GMO approach was efficient in renewing genetic variability through the extensive sporulation of three *S. cerevisiae* strains with different origins and backgrounds. The procedure involved an initial randomized sampling of the MSCs produced by the extensive sporulation of each strain, without any preliminary selection. In addition, a series of sequential steps focused on the analysis of growth performances and metabolomic reactions allowed the analysis to be restricted to six MSCs for each strain, screened at rest and under specific stress conditions. Overall, data confirmed that *i.* the genome renewal reintroduced a quote of variability, selectable following the approach presented in this study, *ii.* the extensive sporulation generates variability in both growth and metabolomic phenotypes; and *iii.* this variability depends on the starting parental strain, proving that the geographical location and ecological origin of yeast have a major signature on its phenotypic pattern. Although the ongoing whole genome sequencing of selected MSCs will clarify the nature and stability of this variability, this novel procedure looks very promising for renewing yeast genetic variability as a tool to obtain improved organisms with specific phenotypes and industrial fitness. Further, selected MSCs are indeed of great metabolomic interest towards the identification of molecules with deep impact on the yeast resistome against specific stressors

Supplementary Materials: Table S1: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Probability associated with the Student's t-test (*p*-value) of all MSC compared to the parental strain (YVGC13A), **Table S2:** The probability associated with the Student's *t*-test (*p*-value) of all MSC compared to the parental strain

(TC1517); **Table S3:** Probability associated with the Student's *t*-test (*p*-value) of all MSC compared to the parental strain (YI30); **Table S4:** FTIR absorbance spectra of the YVGC13A strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose**, Table S5:** FTIR absorbance spectra of the TC1517 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose**, Table S6:** FTIR absorbance spectra of the YI30 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose**, Table S7:** FTIR absorbance spectra of theYVGC13A strain under copper sulphate supplementation**; Table S8:** FTIR absorbance spectra of the TC1517 strain under glucose supplementation**; Table S9:** FTIR absorbance spectra of YI30 strain under formic acid supplementation**, Figure S1:** FTIR absorbance spectra of YVGC13A strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose**, Figure S2:** FTIR absorbance spectra of TC1517 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose**, Figure S3:** FTIR absorbance spectra of YI30 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose**, Figure S4:** FTIR absorbance spectra of YVGC13A strain under copper sulphate supplementation**, Figure S5:** FTIR absorbance spectra of TC1517 strain under glucose supplementation**, Figure S6:** FTIR absorbance spectra of YI30 strain under formic acid supplementation**.**

Author Contributions: Conceptualization, L.F., L.C. and G.C.; methodology, L.F., L.C.; software, A.P.G., D.C.P. and L.C. ; formal analysis, A.P.G., D.C.P., L.C.; investigation, A.P.G., D.C.P., A.C, L.D.; resources, L.F., L.C., M.B., S.C. and G.C.; data curation, A.P.G., D.C.P.; writing—original draft preparation, A.P.G., L.F. and L.C.; writing—review and editing, L.F., L.C. and G.C.; visualization, D.C.P., L.C.; supervision, L.F., L.C.; funding acquisition, L.F., L.C., M.B., S.C. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially funded by Padova University with the following research projects: DOR2297411/22, DOR2251254/22, BIRD210708. A.P.G. is recipient of a University of Padova PhD fellowship.

Data Availability Statement: Not applicable here.

Acknowledgments: Prof. Willem van Zyl (Stellenbosch University) kindly provided *S. cerevisiae* YI30.

Conflicts of Interest: The authors declare no conflict of interest

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Final Remarks

The quest for the vestigial starchy and lignocellulosic material globally available suggests that rice waste streams have enormous potential to be transformed into energy, generating biofuels, and thus giving a vital contribution to meeting the world's energy demands and mitigating climate change. The biorefinery approach entails the complete exploitation of the maximum available carbon. In this perspective, rice byproducts could be co-converted into a constellation of value-added compounds (i.e., organic acids, enzymes, pharmaceutical molecules, biopolymers). Among all the biofuels, ethanol production from rice waste could be one of the most successful applications irrespective of any biotechnological methods adopted. During the process of biofuel production from rice waste, pretreatment is an inseparable step to obtain maximum fermentable sugars. Biogas is another promising biofuel that can be obtained from rice waste streams. The main actors of all processes are microorganisms that act as cell factories where the pretreated raw material is metabolized into valuable products. Moreover, the use of microorganisms in microbial consortia or as a single bacterial or yeast strain, narrow down the specificity of the product. However, despite all the available technologies, additional research is still required on up-scaling the technologies developed so far. Process integration is the key to economically sustainable biofuel production in the biorefinery approach where rice waste streams can transform into biofuels along with several other added-value products. Unripe rice, a rice milling industry byproduct, is rich in starch and has huge availability although remains highly underexploited. For these reasons, unripe rice was selected and employed in a CBP configuration at 1L scale, using the recently developed efficient amylolytic CBP strain *S. cerevisiae* ER T12.7 Its performance was compared with the previously best known amylolytic CBP strain *S. cerevisiae* ER T12 and the parental SSF strain *S. cerevisiae* ER V1. The highest ethanol yield of 56% was obtained for *S. cerevisiae* ER T12.7 in 96h. These results were comparable with those obtained using the parental strain ER V1 (53%) in SSF set up using commercial amylolytic cocktail STARGEN^{TM} 002, while the recombinant amylolytic CBP strain ER T12 showed the lowest yield (51%)

among the three strains. Higher yields could be obtained by fine-tuning the fermentation parameters like the distance between impellers, rpm, foaming control etc.

To adopt the integrated approach and obtain methane as secondary biofuel, spent fermentation slurry from CBP configuration of *S. cerevisiae* ER T12 and *S. cerevisiae* ER T12.7 was lyophilized and a substrate loading of 2 g VS/L was used for anaerobic digestion. Both substrates gave methane yields of around 370 ml CH_4/g VS. Although, the C/N ratio was far below the optimum range, higher methane yield was obtained from lyophilised spent fermentation slurry indicating that the yeast biomass debris present in the spent slurry sustained AD. Hence, the sequence of duel biofuel production in the biorefinery set-up was confirmed for better efficiency of a process wherein, ethanol has to be the primary biofuel and methane can be the alternative.

The organic fraction of municipal solid waste (OFMSW) was also considered as a substrate for bioethanol production. Starch was considered the main fermentable carbon source for the study and thus the amylolytic recombinant CBP strain *S. cerevisiae* ER T12.7 was adopted as inoculum and compared with parental *S. cerevisiae* ER V1. The seasonal variation observed in OFMSW composition was simulated in the study by increasing bread and pasta content (collected separately from the same waste) to understand the overall ethanol potential of OFMSW. Interestingly, fermentation trials of OFMSW and enriched OFMSW, in a simulation of the composition during the winter season, showed an increase in ethanol concentration with an increment in starch content. Hence, another rice milling industry byproduct, discolored rice, was considered to top up the starch. The prominent reason behind selecting rice waste was the existence of the rice milling industry close to a domestic organic waste disposal site. The study reveals that starch in OFMSW could constructively be converted into bioethanol by the means of a highly efficient amylolytic CBP yeast strain. It was clearly observed that OFSMW composition can greatly get influenced by many parameters like geographical location, feeding habits, and seasonal

variation that could alter the overall ethanol yield. Although substrate loading was kept low owing to the viscosity and presence of particulate matter, further investigations are necessary to raise substrate loading. make the process continuous and increase the final ethanol yields in an industrially established process. The co-conversion of OFSMW and DR revealed a promising outlook to achieve higher ethanol levels while reducing the cost of fermentation. Thus co-processing of different industrial byproducts into valuable compounds such as ethanol paves the way towards the biorefinery concept to obtain sustainable biofuels and other value-added chemicals.

When OFMSW was analyzed for the presence of VFA, a higher concentration of some acids raised a need for superior yeast strains which can tolerate even higher VFA concentrations. To obtain tolerant strains of *S. cerevisiae,* the non-GMO approach was effective in acquiring genetic variability through extensive sporulation of three *S. cerevisiae* strains with diverse origins and backgrounds. An initial randomized sampling of the monospore colonies (MSCs) produced by the extensive sporulation of each strain was performed, without any preliminary selective pressure. A sequence of consecutive steps focused on the analysis of growth performances and metabolomic reactions, allowed to narrow down the analysis to 6 MSCs for each strain, which were then screened with and without specific stressing conditions with a metabolomic approach. Overall, data confirmed that the extensive sporulation was able to create phenotypic variability confirmed by growth kinetics and FTIR metabolic spectrum analysis. The variability was dependent on the parental strain proving that the geographical location and ecological origin of yeast have a significant signature on its phenotypic configuration. The novel process appears promising as a tool to revive yeast genetic variability and obtain better microorganisms with specific phenotypes and superior industrial fitness. Moreover, selected superior MSCs are of great metabolomic interest for the identification of the molecules of the *S. cerevisiae* resistome against specific stressing agent.

Acknowledgements

First of all, I would like to thank my mother Vidula for supporting me to stay focused on my work. A special thanks to my friend Priyanka, who encouraged me to shift back from corporate research to pursue Ph.D. I would like to thank also my family, my father Pankaj, cousin Amogh, aunt Vaishali, and uncle Vinay for supporting me every now and then.

I would like to thank my supervisors Prof. Sergio Casella and Prof. Lorenzo Favaro and my co-supervisor Prof. Marina Basaglia for introducing me to their research group and guiding me during my PhD as well as in bureaucratic work. The care taken by entire team of University of Padova to provide a sanitised and secured work place under critical COVID-19 situation was commendable.

I would like to extend my gratitude to Dr. Nicoletta Gronchi for initial hand holding and Valentino Pizzocchero, who helped me for the analytical work and all the ex-colleagues of the Microbiology group.

I would like appreciate the help of Sara, Rebecca, Paolo, Gloria, Sofia and Wessel who were always ready to assist me. Master students, Serena and Noemi who produced their thesis under my supervision has a crucial role in taking this work to completion.

I would like to thank also the entire team of CRIBI, Department of Biology, University of Padova, to avail the necessary resources. My special acknowledgements to Prof. Laura Corte, Prof. Gianluigi Cardinali and Dr. Debora Casagrande Pierantoni, University of Perugia, for extending a hand of help for instrumentation, resources and statistical analyses. I warmly thank the Microbiology group of Stellenbosch University for their help in making experties and resources avilable for my research. Importantly, my sincere gratitude to all the technicians, secretariat who helped me during my entire Ph.D. period.

Finally, I would like to thank also all my peers and the other people I met during this path.

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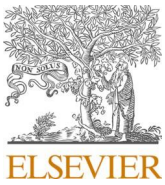

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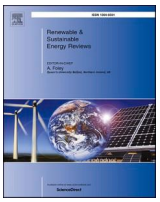

Rice waste streams as a promising source of biofuels: feedstocks, biotechnologies and future perspectives

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A R T I C L E I N F O

Keywords:
Rice biowaste
Bioethanol
Biogas
Microbial fuel cells
Biorefinery
Microbial cell factories

A B S T R A C T

Increased environmental concern over climate change due to higher oil usage has made human being to shift to cleaner and greener alternatives. The utilization of abundant agricultural waste streams as renewable feedstock for biofuels production can be a pivotal strategy. Among others, rice is one of the most largely grown crops, and more than 4.8% of the total production goes to waste. Although previous reviews are related to biofuels obtained from some rice waste, most of those are focused on lignocellulosic rice residues with much attention to thermochemical processes. The present paper, instead, reviews for the first time the biotechnological approaches to convert all rice wastes, like rice husk, rice straw, broken rice, discolored rice, unripe rice, into liquid (bioethanol, biobutanol, biodiesel), and gaseous (biogas, biohydrogen) biofuels through the use of pure or mixed microbial cultures. The global availability of each rice byproduct has been also investigated and the potential of rice waste as a 'fuel farm' has been estimated for bioethanol. The physical, chemical, enzymatic, or microbial pretreatments, which play a key role in making carbon available for hydrolysis and fermentation, are here discussed and evaluated. Despite the great promise of technologies so far developed, further research is still required for their up-scale and industrial commercialization. Moreover, future process integrations will open the landscape to biorefinery schemes where rice waste streams can be processed into biofuels and other added-value products, towards the full exploitation of the feedstocks and the economic and environmental sustainability of the overall process.

1. Introduction

Considering the highest economic growth, the energy consumption in 2050 is expected to increase by almost 70% [1], with an overall energy demand rising to almost 680 quadrillions BTU by 2030 [2]. Up to 85% of this demand will be fulfilled by fossil fuels, thus continuing to contribute to environmental pollution by the release of greenhouse gases (GHG) in the atmosphere (50% higher than in 2011) [3]. These are carbon monoxide (CO), nitrogen oxides (NO_X), particulate matter (PM), sulfur oxides (SO_X), methane, and volatile organic compounds (VOC) [4]. To overcome the continuous increase of energy demand, alternative solutions for cleaner and more environmentally friendly fuels than the available fossil ones, are needed [5].

Among various alternatives, biomass can unambiguously play a pivotal role as a source of renewable energy, with great potential in the production of biofuels for heat, electricity and transportation [6–9]. Moreover, biofuels show a significant reduction in NO_X, PM, and SO_X [4], thus helping to mitigate climate change. Within this scenario, the most promising biofuels are represented by bioethanol, biomethane, biobutanol, biohydrogen [10] from waste.

Lignocellulose is by far the main component of farm residues like bagasse, straw, husks, brans and it is the most abundantly available raw material on the Earth. It contains an aromatic polymer (lignin) and 80% of polymeric carbohydrates (cellulose, hemicellulose) [11], suitable for the production of biofuels. Moreover, since 140 x 10^3 Tg) of agricultural waste biomass is generated every year worldwide, the improper management of such organic material could lead to pollution [12]. For instance, the excess of biomass burned in the open [13] results in an important loss of resources potentially available for fuel production. In fact, the yearly generated lignocellulosic biomass is theoretically equivalent to 50 x 10^3 Tg of oil [14]. Some surveys have been developed and published on the evaluation and characterization of agro-food residues for biofuels production [15–20] and, among a number of different

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https://doi.org/10.1016/j.rser.2022.112673
Received 30 January 2022; Received in revised form 25 May 2022; Accepted 2 June 2022
Available online 17 June 2022

Abbreviations

ABE	Acetone Butanol Ethanol
ADSS	Anaerobically digested sewage sludge
AFEX	ammonia fiber expansion
AFP	Acid fungal protease
AS	Anaerobic sludge
ASS	Activated Sewage Sludge
ASSP	Anaerobic sludge from sediment of pond
ATCC	American type culture collection
BR	Broken rice
BTU	British thermal unit
C/N	Carbon to nitrogen ratio
CBP	Consolidated bioprocessing
CD	Cow dung
CDSM	Cellulose degrading soil microflora
CDTD	Combinative dispersion thermochemical disintegration
CDW	Cell dry weight
CO	Carbon monoxide
CRF	Cow rumen fluid
dAT	Deacetylation acid pretreatment
DDGS	Distillers' dried grains with solubles
DM	Dairy manure
DR	Discolored rice
DRB	De-oiled rice bran
DS	Digested sludge
FAME	Fatty acid methyl ester
FW	Food waste
GHG	Green house gases
GL	Gigalitres
GMO	Genetically modified organism
GSHE	Granular starch hydrolyzing enzyme
HC	Hydrodynamic cavitation
HRT	Hydraulic retension time
HTB	Hybrid thermochemical-biological
IEA	International Energy Agency
LCA	Life cycle Assessment
Mg	Megagram
MFC	Microbial fuel cell
MTCC	Microbial type culture collection
MWTPS	Municipal wastewater treatment plant sludge
NMMO	*N*-methyl morpholine *N*-oxide
NMR	Nuclear magnetic resonance
NO_X	Nitrogen oxide
nr	not reported
OLR	Organic loading rate
PB	Pond bottom
PEM	Proton echchange membrane
PFS	Paddy field soil
PM	Particulate matter
RB	Rice bran
RBDW	Rice Bran De-oiled wastewater
RH	Rice husk
RRC	Rice residues from canteen
RS	Rice straw
RWW	Rice washing water
S/I	Substrate to inoculum ratio
SHF	Simultaneous hydrolysis and fermentation
SHS	Slaughterhouse Sludge
SM	Swine manure
SO_X	Sulfur Oxides
SRSH	Synthetic rice straw
SS	Sewage sludge
SSF	Separate saccharification and fermentation
SWOT	Strength, Weakness, Opportunity, Threat
Tg	Teragram
TS	Total solids
UR	Unripe rice
VFA	Volatile fatty acids
VOC	Volatile organic compounds
VS	volatile solids
[BMIM][OAc]	1-butyl-3-methylimidazolium acetate

starchy and lignocellulosic residues, rice waste biomass has been indicated as one of the most abundant and promising feedstocks [2]. Globally, rice is the second most-produced food grain after wheat and generates around 972 Tg of waste per year. Noteworthy, the processing of rice crop results in a unique combination of lignocellulosic and starchy waste streams largely available at the proximity of the milling site. As such, the complex variety of composition is one of the challenges to be still faced at the industrial level towards the full exploitation of all rice byproducts into biofuels. Biofuels from rice waste can indeed be obtained by thermochemical as well as biotechnological pathways. Furthermore, the combination of the thermochemical and biotechnological processes can be an hybrid platform which catches the advantages while alleviating the weaknesses of the standalone thermochemical and biotechnological pathways [21]. Thermochemical processes can indeed overcome the recalcitrance of rice biomass and thus eliminates the complex pretreatment step and the need for costly saccharification processes. On the contrary, the high instability of the thermochemical outputs, mostly in the case of pyrolysis, and their usually high toxicity can be finely alleviated by specific biotechnological approaches to produce high value products [22]. Despite these promising perspectives, hybrid thermochemical-biological (HTB) processes from rice waste streams have not yet been reported and can be an outstanding future research area.

Many thermochemical efficient technologies to process rice waste, like pyrolysis [23,24], gasification [25], combustion [26] etc. are available, whereas the biotechnological routes received less attention and the present review is focused on the latest biotechnological

Table 1
Reviews on rice waste to biofuels: main findings and timeline.

Year	Main Topic	Reference
2010	Pretreatment of rice straw for the production of ethanol.	[27]
2010	Rice husk as a cellulosic feedstock to meet one-fifth of world energy demand.	[28]
2012	Physico-chemical characteristics and pretreatment techniques, thermochemical as well as biochemical technologies available to convert rice straw and husk into energy.	[2]
2012	Effect on socio-economics of Thailand due to the conversion of rice straw into ethanol and power	[29]
2016	Novel techniques to convert rice mill effluent into energy and value-added products with curbing pollution caused due to effluent.	[30]
2018	The efficiency of the solid-state digestion method over liquid anaerobic digestion for lignocellulosic rice husk.	[31]
2019	Insights on pretreatment, co-culture approaches, consolidated bioprocessing, and strain improvements for biobutanol tolerance using rice straw.	[32]
2020	Utilization of rice husk and straw for energy generation, environmental adsorbents, construction materials, and speciality products.	[33]
2020	Socio-economic effects, LCA and SWOT analysis of biofuel production from rice straw.	[34]
2022	Biotechnological routes to convert all rice waste streams into biofuels	This article

approaches devoted to biofuels production. Table 1 provides the timeline and important findings of previous reviews which are mostly related to biofuels obtained from the rice waste mostly using the biotechnological route.

Many papers described details of lignocellulosic rice waste with much attention to physico-chemical processes for the production of biofuels [2,30]. Other reviews dealt with LCA [34] and socio-economic effects [29,34] of producing biofuels from specific rice byproducts, with great emphasis mainly on rice straw. The present paper, instead, reviews for the first time the biotechnological approaches to convert all rice waste, as rice husk (RH), rice straw (RS), broken rice (BR), discolored rice (DR), unripe rice (UR) and rice bran (RB), into liquid and gaseous biofuels using pure or mixed microbial cultures. Moreover, the global availability of each rice waste has been investigated and their bioethanol potential has been calculated. As such, this manuscript is specifically devoted to researchers with expertise and interests in biotechnology and process engineering, as well as to agronomists and industries seeking potential and more promising valorization routes of the widely available rice by-products. Furthermore, this review can be of interest also for governmental agencies and institutions which can be supported in the implementation of tax incentives and commercial carbon credits related to bioeconomy approaches dealing with rice residues.

2. Rice waste biomass: global availability and composition

Oryza sativa and *O. glaberrima* are the two domesticated rice species that originated in Asia and Africa. Although both can grow in dry land and deep water, *O. sativa* has a higher yield and milling quality and thus is cropped in at least 112 countries [2] with approximately 90% of production in Asia [35]. Rice is a staple food for about half of the world's population and thus it is one of the most important crops with a worldwide production of almost 1000 Tg in 2018 [35]. Around 88% of cropped rice goes for human consumption and 2.6% for animal feed, thus, due to the global economic and population growth, rice demand is likely to remain robust in the next decades. Although eating preferences of Asian countries are shifting to a diet including food other than rice, the rice industry should remain significant for a long time and thus the availability of rice waste high.

Besides food, feed and seed, more than 4.8% of total rice grains go to waste [36]. For instance, in North America, 12% of produced rice is wasted and in Asia around 22 Tg of dry rice are discharged. Rice agricultural waste include crop and agro-industrial residues: crop residues are parts of the plant remaining on the field after harvesting and include RS, while agro-industrial residues are generated by cleaning and milling processes and comprise RH, removed from the seed during milling, RB and BR, DR and UR.

The disposal of high quantities of RS can cause multiple hazards to environment and ecosystem. Traditionally it was used as animal fodder [37], fertilizers [38], hatching, manures, burnt [13,39] or is incorporated into the soil or used as mulch potentially harbouring rice diseases [33,40]. Freshly generated RS burning in open field is commonly adopted by the farmers all over the world but especially among the Asian countries accounting for more than 70% of the world's rice production [41,42]. This approach causes the release of pollutants and greenhouse gasses into the atmosphere, thus contributing to increasing the global warming [43] and adversely affecting the environment and human health [44–46]. In addition, Carlson et al. [46] demonstrated that the highest concentrations of the GHG emissions zones are in the major rice-growing areas of Asia as a result of open field burning of the waste straw. Indeed, the management of rice wastes is a critical issue also taking into consideration the volume generated in the world [47]. In this perspective, many countries such as those of European Union, restricted field burning and adopted the "waste-to-resource" approach [42].

In recent years, along with traditional utilization ways RS was also exploited for the production of biofuels, biochar, compost, mushroom, fuel-briquette, fuel-pellets, and paper etc. [39,48]. In this perspective, RS and RH were proposed as construction materials or adsorbents of heavy metals or for the production of energy and fuels [2]. The exploitation of these agro-industrial residues could give an income to farmers but poses logistic problems: in fact, while RH and RB are easily available at rice mills, RS needs the activation of short local chains because must be collected from the fields [29]. RB, obtained from the milling industry, is already used by the food industry for functional food due to its ability to inhibit colonization of *Salmonella* in the gastrointestinal tract [49] or as a source of oil with beneficial properties. RH, which is the seed cover obtained during grain processing (40% wet weight), is cheap and can be easily collected and utilized for different purposes such as a component of polymeric composite resins or as an energy source [33]. RH is abundant, constitutes 20–22% of total rice biomass and it is presently disposed by rice mill industry as a waste. RH contains SiO_2 large amounts ranging from 8.7 to 12.1%, with a medium average 10.6% [50]. Silica-rich RH could be converted to biofuels (e.g., bio-oil, vapors) and biochars simultaneously via thermochemical processes such as pyrolysis or gasification. Although proposed for other low-value products [51], it is mainly underutilized, on-site burned or landfilled, leading again to serious environmental problems [52].

The most desirable eco-friendly alternative is the conversion of this material by biological methods, alone or coupled with the above mentioned ones, which are already available in a large part. Indeed, the chemical composition of biomasses from rice cropping and industry includes both lignocellulosic and starch-rich residues (Table 2). RS and RH mainly contain hemicellulose and cellulose but low amounts of starch, while BR, DR, and UR contain large quantities of starch. Both lignocellulosic and starchy rice residues could be used as feedstock for the biochemical conversion into biofuels after their hydrolyses into fermentable sugars. The potential of bioethanol, here chosen as representative of other biofuels, was theoretically assessed considering the bioethanol attainable from the fermentation of starch, cellulose, and hemicellulose after pretreatments and/or enzymatic saccharification as previously recommended [17–19] considering both conversion coefficients as well as experimentally obtained values. The polymers' hydrolysis yields for starch, cellulose, and hemicellulose were supposed to be 91, 81 and 96% respectively. The stoichiometric ethanol yields of monosaccharides were estimated 92.5% for glucose and 86% for xylose with an ethanol recovery yield after distillation of 99.5%.

Lignocellulosic waste streams are the most abundant with up to 836 Tg. RS, the crop residue available on the field when the product is harvested (approximately 22% wet weight), accounts worldwide for 685 Tg, with a potential ethanol of nearly 194 Tg. RH, which is the seed cover obtained as an agro-industrial waste during grain processing (40% wet weight) can be converted into up to 41 Tg ethanol (Table 2). Starch-rich residual biomasses from rice could be also utilized for fuels production (Table 2). Their starch levels vary from 29 to 80% of dry matter with a high content of proteins which were shown to support nitrogen requirements of microbial strains involved in their fermentation [53]. BR is a promising feedstock with an availability of up to 45 Tg an ethanol potential of 16 Tg. RB, UR and DS are also largely available, with significant ethanol applications [54–57].

Overall, rice cropping results in the generation of large amounts of biomasses that are underutilized. This led the scientific community to identify potential uses, such as the generation of energy and fuels, that will be implemented only if specific legislation and taxation will make them more attractive and economically convenient.

3. Pretreatment of rice biomass

Pretreatment of rice waste streams is one of the most important and cost determining steps for their conversion into biofuels. This is necessary for the separation of lignin and hemicellulose, to reduce the crystallinity of cellulose and to increase the accessibility of hydrolytic enzymes [68]. Pretreatments should meet the following criteria: 1.

Table 2
Average composition and availability of rice waste.

Waste	Average composition (% dry matter)						World biomass availability (Tg)	Bioethanol potential (Tg)	References
	Starch	Cellulose	Hemicellulose	Lignin	Protein	Ash			
Lignocellulosic material									
RH	6.9	40.1	20.6	22.3	3.4	18.2	151.1	41.4	[2,31,54,58–60]
RS	11.8	34.3	25.1	18.6	1.3	15.0	685.0	193.7	[2,27,31,58,61–66]
Starchy material									
BR	77.7	0.2	0.5	–	8.3	0.5	45.3	16.0	[54]
DR	84.6	0.1	0.9	–	8.0	0.5	7.5	2.9	[54]
RB	29.6	6.9	15.7	4.1	14.5	8.0	52.9	11.5	[61,67]
UR	68.6	1.8	3.7	–	9.9	1.5	30.2	9.9	[54]

Lignocellulosic rice byproducts (RH, RS) and starchy waste streams (BR, DR, RB, UR), Yearly ethanol potential (Tg) from each feedstock has been calculated as previously described [19] considering both the availability and average composition.

obtain high efficiency of sugars formation either by the chemical, physical or enzymatic way [69]; 2. reduce loss of carbohydrates; 3. reduce inhibitory byproducts formation; 4. be cost-effective [70]. In principle, the treatment of lignocellulosic feedstocks is more complex than the processing of starch-rich substrates. Many efficient pretreatments of lignocellulosic and starchy rice byproducts have been recently developed to optimize the production of various biofuels and added valued compounds. Table 3 reports a selection of the most used physical, enzymatic and chemical methods.

Considering RS as raw material, a number of attempts have been reported to improve the efficiency of the enzymatic hydrolysis. For instance, a novel lime-pretreatment process was proposed without solid-liquid-separation. In the same vessel, xylan, starch and sucrose are present together and inhibitory effects on saccharification and fermentation were found to be not significant [62]. When the same pretreatment was applied on RH, no generation of detectable furfural and hydroxymethyl furfural was also observed [71]. Castro et al. focused on deacetylation of RS using alkali which resulted in a reduced concentration of inhibitors in pretreated hydrolysate [72]. NaOH combined with urea helped to increase the availability of cellulose and hemicellulose by effectively disrupting the structure of RS and increased maximum hydrogen production by over 160% than control [73]. Zhu et al. combined microwaves along with NaOH to reduce reaction time and enzyme loading. This combination yielded around 5% more ethanol than only alkali pretreatment [74]. Two-step pretreatment process consisting of aqueous ammonia and sulfuric acid helped in selective removal of lignin and hemicellulose respectively [75]. Teghammar et al. used *N*-methyl morpholine *N*-oxide (NMMO) for pretreatment of RS which increased the methane production by seven times than that of untreated RS. Also, 98% of the solvent used during pretreatment was recovered, making this pretreatment method environmentally friendly and economically feasible [76]. When the same method was adopted for bioethanol production and compared with 1-buthyl-3-methyl imidazolium acetate, NMMO was found to be more efficient in producing bioethanol [77].

Glycerol, a byproduct of the biodiesel industry, was used in two forms (i.e., acidified aqueous glycerol and glycerol carbonate) for pretreatment of RH. Results showed that glycerol carbonate gave better bioethanol production than acidic counterpart [78]. Saha et al. [71] treated milled RH with 1.5% NaOH at 121 °C along with a cocktail of three commercial enzymes (i.e cellulase, β-glucosidase and hemicellulase), whereas Ebrahimi et al. [79] used ammonium carbonate to

Table 3
Selection of the most used and efficient physical, chemical and/or enzymatic pretreatment recently adopted for rice waste streams. For the sake of clarity, this table also consider enzymatic or microbial hydrolysis as a pretreatment.

Feedstock	Pretreatment		Enzymatic or microbial	Product	References
	Physical	Chemical			
Lignocellulosic material					
RH	Wet air oxidation	–	–	Bioethanol	[60]
RH	Milling, Autoclaving	2% H_2SO_4, 3% NaOH	–	Bioethanol	[84]
RH	Milling	$(NH_4)_2CO_3$	Cellulase	Bioethanol	[79]
RH	Thermal	–	–	Electricity	[80]
RH	Milling	Acidified aqueous glycerol	Cellulase	Bioethanol	[78]
RH	Milling	Glycerol carbonate	Cellulase	Bioethanol	[78]
RH	Milling, Autoclaving	1.5% NaOH	Cellulase, β-glucosidase, hemicellulase	Bioethanol	[71]
RS	–	3.5% H_2SO_4	–	Biolipids	[85]
RS	Steam explosion	10% NaOH	–	Glucose	[86]
RS	Thermal	2% $Ca(OH)_2$	–	Biogas	[87]
RS	Extrusion	–	–	Biogas	[88]
RS	Extrusion	3% H_2SO_4	–	Bioethanol	[89]
RS	Autoclaving	–	–	Biogas	[90]
RS	Ozone	aqueous ammonia	–	Biogas	[91]
RS	Gamma irradiation	1% NaOH	–	Biogas	[92]
RS	Milling, Autoclaving	0.4% NaOH	*Trametes hirsuta*	Bioethanol	[93]
RS	Milling, Autoclaving	–	*Pleurotus ostreatus*	Biogas	[94]
RS	Autoclaving	–	*Pleurotus ostreatus Trichoderma reesei*	Biogas	[95]
RS	Milling, Autoclaving	2.5–3% HCl	Cellulase	Biohydrogen, Bioethanol	[96]
RS	CDTD	–	–	Biohydrogen	[97]
Starchy material					
BR	–	–	α-amylase, amyloglucosidase	Bioethanol	[98]
BR	–	–	AFP, GSHE	Bioethanol	[99–101]
BR	–	–	Hyper active α-amylase	Bioethanol	[57]
BR, DS, RB, UR	–	–	GSHE	Bioethanol	[53,54]

AFP- Acid fungal protease, GSHE- Granular starch hydrolyzing enzyme, CDTD- Combinative dispersion thermochemical disintegration.

improve the ethanol yield from 10 to 47% in the 72 h fermentation. This indicates that usage of alkali for pretreatment of RH is helpful to boost bioethanol production. Treating RH at 900 °C produced ash that provided the economic and efficient source of proton exchange membrane (PEM) for the production of electricity [80].

When above mentioned and recent alkali, acid, or ammonia based pretreatment methods on lignocellulosic waste are compared, it was prominent that alkali is widely used for pretreatment of lignocellulosic rice waste and can be one of the most important future research topics. Alkali breaks down easter bonds between lignin, cellulose and hemicellulose with release of less furfural, 5-hydroxymethyl furfural and vanillin than acidic pretreatment. Some processes combine alkali and/or acids with thermal pretreatments to improve product yield. These practices are well established in the industry [81], due to low energy demand, low temperature, short reaction time, and easy to scale up. The major disadvantages of these combined pretreatments are the corrosion of the reactors due to the extreme pH, the formation of fermentation inhibitors (phenolic), and thus the increase in operational cost [82].

Special attention must be given to the development of pretreatment exploiting solvents fully biodegradable and, hopefully, recyclable at industrial settings, as it was demonstrated for NMMO pretreatment on RS [77]. NMMO has been used to reduce crystallinity of cellulose by breaking the intramolecular hydrogen bonds and van der Waals forces [76]. Moreover, NMMO does not act as enzymes inhibitor [83], thus enhancing the saccharification of polysaccharides into monosaccharides. Although high theoretical yields (>80%) were obtained for production of biomethane and bioethanol, utilization of recycled solvent and scale up studies must be improved.

Considering now starchy-rich rice waste pretreament, it is clear that such materials are usually more prone to pretreatment than the lignocellulosic one (Table 3). As such, efficient enzymatic pretreatment is enough to release glucose and a cluster of mostly commercial amylolytic blends were tested resulting in high saccharification yields. Overall, towards the efficient processing of rice by-products into biofuels, with the large varieties of pretreatment technologies available, an in-depth assessment should consider the economic trade-off associated with pretreatment handling and transportation costs.

4. Biofuels production from rice waste streams

The world energy demand along with the pollution due to overuse of fossil fuels and potential of rice biomass as alternative raw material were well described in section 1 and 2 respectively. To overcome the continuous increase of energy demand, cleaner and more environmentally friendly fuels can be obtained by thermochemical as well as biotechnological ways. Many technologies like pyrolysis [23], gasification [25], combustion [26] etc. are available which can also be applied to rice biowaste streams; but, this review is focused on the biotechnological strategies and their updates for the production of biofuels from rice biowaste streams. Hence, only those technologies are discussed in detail in following sub-sections.

4.1. The key role of microorganisms as cell factories

In general, the microbial conversion of a waste into a product is an approach that is becoming increasingly popular as microorganisms can be considered powerful cell factories, capable of metabolizing raw materials and producing useful substances at the industrial level [102–104]. Moreover, microorganisms can be further improved by genetic as well as evolutionary engineering approaches to maximize the desired product(s) yields and productivities. In this perspective, microorganisms can play an essential role in the transition from fossil fuels to biofuels from rice waste streams. Essentially, after the optimization of the pretreatments, two approaches have been developed to converting pretreated rice products into biofuels, namely the utilization of microbial consortia or the use of single bacterial or yeast strains (Fig. 1).

Mixed cultures are typically adopted for biohydrogen and biogas applications. The production of these biofuels provides that the process conditions select specific groups of microorganisms, naturally present in the inoculum or the feedstocks, acting sequentially to convert complex substrates into hydrogen or methane. Thus, the research is mostly focused on pretreatments optimization of the feedstocks as well as on the fine-tuning of process conditions aimed to select and facilitate the most efficient microbial populations. Pure cultures are mainly used to obtain bioethanol, biobutanol biodiesel and electricity. This approach considers the utilization of single strains and specific efforts were spent

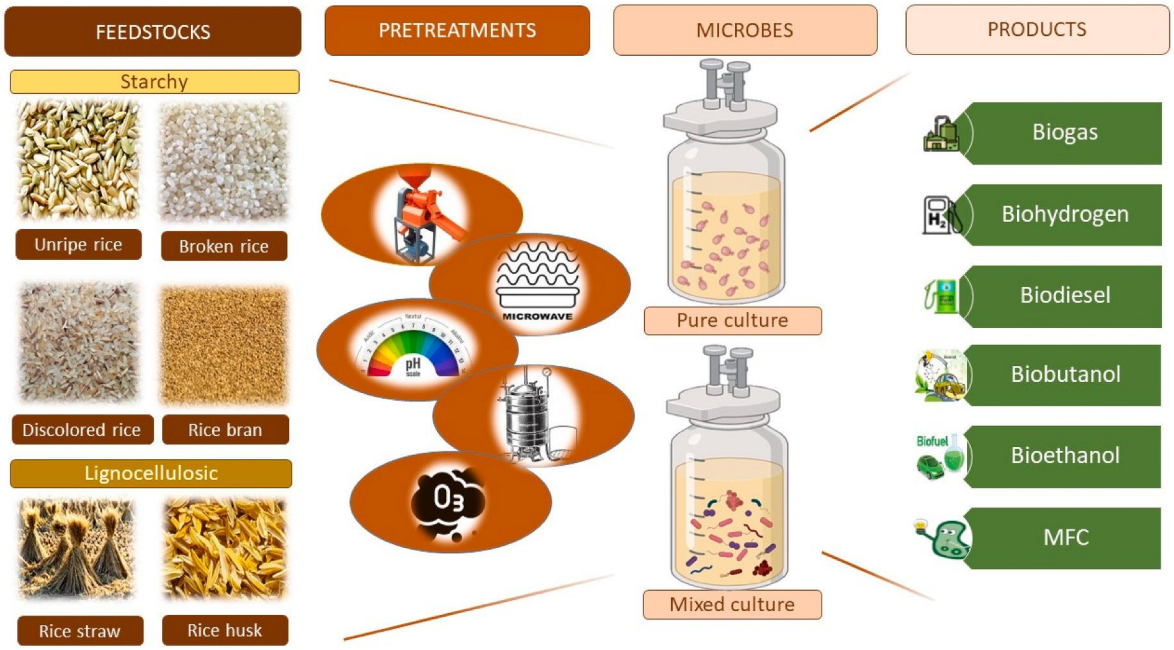

Fig. 1. Biofuels production from different rice waste streams. Once subjected to a single or a combination of pretreatment(s), rice byproducts can be processed into different gaseous or liquid biofuels and electricity by using a pure or a mixed culture approach. MFC-Microbial fuel cell.

towards efficient biotechnological routes by exploiting properly selected and/or genetically modified bacterial and yeast strains.

In the next sections, biofuels applications (biogas and biohydrogen) dealing with mixed microbes will be firstly described. Pure cultures strategies to produce biodiesel, biobutanol and bioethanol will be then discussed.

4.2. Biogas

Anaerobic digestion is one of the proven technologies for converting organic waste into biogas. The generation of biogas, mainly a mixture of methane and carbon dioxide, is considered eco-friendly and contributes to the reduction of soil and water pollution [105], thus encouraging the circular economy [106]. The entire process of anaerobic digestion, from the recent biological innovations to downstream strategies to improve biogas production has been deeply reported by Tabatabaei et al. [107,108] who detailed and discussed the biological innovations and optimizations including upstream, mainstream, and downstream in biogas production from different feedstocks.

Methanogenesis is a complex process (Fig. 2) that needs multiple reactions conducted by bacterial and archeal consortia under anaerobic conditions [106]. Insoluble organic compounds, mainly carbohydrates, proteins, and fats, are hydrolysed into soluble molecules, monosaccharides, amino acids, and fatty acids by extracellular enzymes synthesized by specific hydrolytic bacteria. Then, lactate, ethanol, propionate, butyrate, and higher volatile fatty acids (VFA) can accumulate and are converted to hydrogen by a specific microflora (Fig. 2). In the following acetogenesis process, the acetate bacteria convert the acid phase products into acetic acid and hydrogen, used by methanogenic bacteria to produce methane [109,110] (Fig. 2). Thus the syntrophic degradation of complex organic compounds to methane and carbon dioxide is a difficult process and requires the cooperation of diverse groups of microorganisms occurring in the natural environments and usually introduced in the industrial plants through specific inocula. Proportions of CO_2 and CH_4 in biogas are related to the degree of oxidation of carbon in the organic substrates [111]. Once biogas is generated, methane must be separated from carbon dioxide. As it is cost imposing process, methane yield in biogas is equally important.

The use of rice wastes to feed biogas plants has been proven feasible and sustainable, although anaerobic bacteria can hardly degrade lignocellulosic materials such as those contained in RS and RH (Table 2),

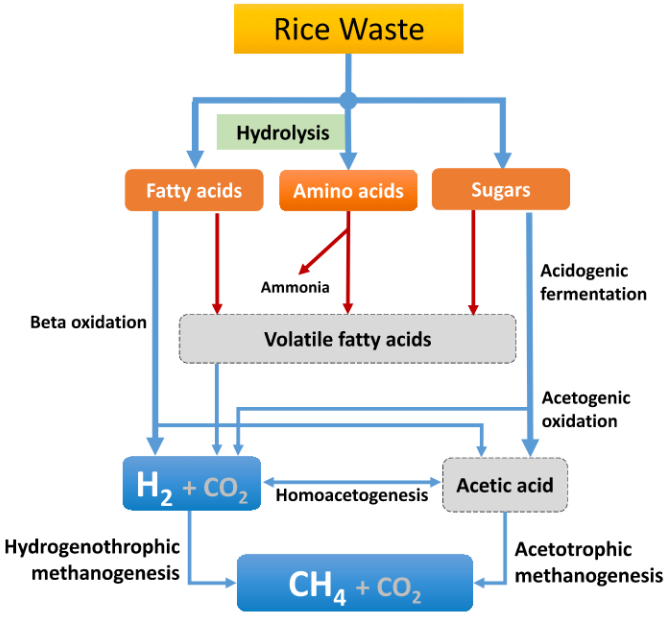

Fig. 2. Steps of anaerobic digestion of rice waste.

due to the high C/N ratio, cellulose crystallinity, and great lignin content. The most important parameters controlling the efficiency and stability of anaerobic digestion are, among others, the chemical and physical characteristics of the substrate, the inocula and the feedstock to inoculum ratios, trace elements, C/N ratio, temperature, pH etc [111]. Operation temperature significantly influences reaction velocity. In general, the growth rate of microorganisms is best at mesophilic and thermophilic temperature ranges [112,113], killing most of the microbial pathogens [114] speeding up the digestion procedure, although the thermophilic system needs additional energy to sustain the higher temperature of the reactor [112]. Furthermore, at mesophilic temperatures, a wider range of microorganisms are involved [115] and the entire process is more stable [116]. In addition, methanogenic bacteria are sensitive to thermophilic temperature and require time to adapt to higher temperature [117].

C/N ratio is another key factor in anaerobic digestion [118] because an imbalance in C/N ratio could cause accumulation of VFAs or ammonia [119,120]. The recommended C/N ratio for hydrolysis is 16–45 and 20–30 for methanogenesis [121]. The C/N of RS reported in the scientific literature varies between 25 and 75 based on substrate origin and can be balanced by co-digesting with nitrogen-rich substrates such as pig urine, cow manure, and food waste [111]. Co-digestion of farm waste is the most applied method for increasing methane yields [122]. It is a treatment strategy in which several feedstocks are mixed [119] and thus it is a promising approach to balance the low C/N in the reactors. As an example, Ye et al. [65] suggested the co-digestion of RS with kitchen waste and pig manure as a promising approach to balance the low C/N ratio of lignocellulose biomass. Haider et al. [123] assessed the co-digestion of RH with food waste, using fresh cow dung as inoculum pointing out the substrate to inoculum ratio (S/I) as one of the key parameters.

As previously discussed, since the hydrolytic stage is usually considered the bottleneck mostly affecting the conversion rate of RS, many studies were focused on physical, chemical, and biological pretreatments, alone or in combination, aimed to improve hydrolysis (Table 4). Indeed, physical pretreatments such as milling, extrusion, grinding, steam explosion and liquid hot water pretreatments, increase the accessibility of the substrates and reduce the degree of polymerisation and crystallinity of the cellulose. As an example, Chen et al. [88] evaluated the extrusion of RS compared to the milling. The authors demonstrated that the extrusion changed some physical properties of lignocellulose such as bulk density or porosity, thus enhancing the efficiency of bacterial cellulose and hemicellulose degradation. As a consequence, the digestion time of RS was shorter and methane yields increased. Biological pretreatment has great advantages because of fewer energy needs and does not generate toxic compounds [124]. Biological pretreatment mainly involves the use of white, brown and soft rot fungi [125]. A biological approach treating RS with suspensions of *Pleurotus ostreatus* DSM 11191 and *Trichoderma reesei* QM9414 gave interesting outputs [95]. Although moisture content and incubation time affected the efficiency of the treatments, the fungal incubation significantly improved lignin removal as well as biogas and methane yields. In the work of Yan et al. [66], RS was firstly composted to facilitate the biodegradability of complex substrates and, then, treated in a solid-state anaerobic digester with anaerobic sludge as inoculum. After optimization of initial substrate concentration, temperature and C/N ratio, composted RS resulted to be more effectively degraded, thus increasing biogas yields.

Although biological pretreatments have undeniable advantages such as fewer energy requirements, specificity, or generation of fewer toxic compounds, they are expensive and need a long time and complex operating conditions [70]. Thus, to decrease operation time and enhance the biogas conversion efficiency of rice wastes, the utilization of acids or alkali, alone or in combination with physical pretreatments, is preferred. For example, Du et al. [87] reported that the alkaline thermal pretreatment of RS at mild temperature was more efficient than the

Table 4
Biogas production from rice waste: main pretreatments, inocula and yields.

Feedstock	Pretreatments			Inoculum	Temperature (°C)	Biogas Yield[a] mL/g VS	Methane %	Reference
	Physical	Chemical	Enzymatic or microbial					
RH	–	–	–	CRF	30	382	78	[129]
RH and FW	–	–	–	Acclimatized CD	37	584	–	[123]
RH and FW	Milling	–	–	AS and Pig manure	37	674	57	[65]
RS	–	Ozone, aqueous ammonia	Mixed Cellulases	DS	37	396	–	[91]
RS	Hydrothermal	Alkali	–	ADSS	37	411	49	[87]
RS	Milling	-	*Pleurotus ostreatus*	AS	37	353	73	[95]
RS	Autoclaving	Alkali or Acid	–	DS	35	932	–	[90]
RS	Milling	–	–	AS	37	227	–	[88]
RS	–	–	Composting	AS	35.6	447	–	[66]
RS	Milling	–	–	–	39	349	52	[130]
RS	Milling	–	*Pleurotus ostreatus* DSM 11191	AS	37	367	72	[95]
RS	Milling	–	*Trichoderma reesei* QM9414	AS	37	299	72	[95]
RS	Milling	–	–	DM	37	325	55	[127]
RS	Milling	–	–	Acclimatized AS	22 ± 2	340	77	[128]

[a] -Highest values of biogas reported (or calculated from available data) when available. FW- Food waste, DS- Digested sludge, ADSS- Anaerobically digested sewage sludge, AS- Anaerobic sludge, CD- Cow dung, CRF- Cow rumen fluid, DM- Dairy manure.

hydrothermal in terms of lignocellulose decomposition and methane production. Kim and colleagues compared autoclaving the RS after the addition of H_2SO_4, with pretreatment with hot water and alkali [90]. However, although the highest lignocellulose decomposition was obtained by autoclaving after H_2SO_4 addition, the methane production potential was very low probably due to the inhibitory effect of the sulfate ion on methanogenesis, as reported previously [126]. The optimal process parameters for a combined synergistic pretreatment of RS with ammonia hydrochloride and ozone were also defined [91]. The combination of chemical and physical factors enhanced the enzymatic release of fermentable sugar and consequently biogas production.

Gu et al. [127] considered the role of inocula and found that digested manures (from dairy, swine and poultry) were more suitable than digested municipal, granular or paper mill sludges in increasing biogas production from RS. The effect of macro- and micro-nutrients on the performance of anaerobic digestion of RS [128] and RH [129] was also studied. In small scale experiments, using cow rumen liquid and acclimated anaerobic sludge as inoculum, the supplementation with heavy metals, such as Ni^{2+}, Zn^{2+} and Cu^{2+}, improved biogas yield from RH [129], while methane production rate from RS was accelerated by optimizing phosphate levels (465 mg-P/L) [128]. The effect of organic loading rate (OLR) on the conversion of RS to biogas was explored in a 300 m^3 mixed bioreactor [130]. An increase in biogas was observed when OLR was below 2.00 kg $VS_{substrate}/m^3d$ while the maximum production rate was 323 m^3/t dry substrate. The monitoring of prokaryotic community structure in the plant during biogas production confirmed that the hydrogenotrophic and acetoclastic pathways are the most common in the digestion of lignocellulosic wastes to methane [131, 132].

Overall, rice waste and, more specifically RS, have a great potential to generate biogas although it is necessary to adopt the appropriate pretreatments and inocula for the efficient utilization of the substrates and further research is needed to optimize mostly rice lignocellulosic substrates into biogas.

4.3. Biohydrogen

Biohydrogen can be obtained from carbohydrate-rich biomass by anaerobic (dark fermentation) and photoheterotrophic (light fermentation) microbes [133]. In recent years, biohydrogen has gained popularity as a clean fuel to reduce toxic gas releases. Like all other fuels, biohydrogen must be cost-effective as well. Though biohydrogen production can be performed by dark-, photo- and combined (dark- and photo-), to the best of the author's knowledge, only the dark fermentation route was exploited to obtain hydrogen from rice waste streams. Baeyens et al. provided detailed insights of the different pathways adopted by bacteria for the production of biohydrogen [134]. Recent studies on combinative pretreatments of RS have to be considered as an emerging cost-effective, alternative energy technology [97]. The difference in composition of RS, RH, RB and cooked rice leftover waste requires a comparison between the effects of different temperatures on biohydrogen production potential, since for all rice biowaste, except for leftover cooked rice, a significant increase in biohydrogen yields was observed as the temperature increased [135]. Moreover, the concentration and particle size of the substrate were found to represent key parameters for determining the processing time. Similarly, hydrolysis time and concentration of additives were found to play a key role during the biohydrogen production from RS [96].

A further important aspect is concerning the nature and treatment of inocula, which are quite frequently obtained from anaerobic digestors. During anaerobic digestion, hydrogen is produced as an intermediate metabolite with hydrogen-producing and -consuming bacteria working together to obtain methane. To maximize hydrogen yield through dark fermentation, methanogens and hydrogen-consuming bacteria have to be inhibited. Several methods have been proposed to achieve this aim, including heat treatment, acidification, basification, freezing or dehydration [136–139]. Table 5 gives a summary of pretreatments of feedstocks, inocula and the corresponding biohydrogen yields. Along with biohydrogen yield, it is important to monitor the percentage of biohydrogen in the biogas, which ranged between 25 and 70%.

Studies of heat treatment of inoculum were performed on activated sewage sludge and optimal results were obtained at 100 °C for 60 min [140]. However, at a C/N ratio of 25, the use of non-heat treated sewage sludge resulted in a biohydrogen production from RS higher than the yield obtained by heat-treated sewage sludge [141]. On the contrary, other studies suggest the importance of heat treatment of sludge in terms of the selection of hydrogen-producing microflora over methanogenic organisms. As an example, Chen and colleagues explored heat treatments of different sludges and cow dung compost used as inocula for untreated RS [142]. Maximum biohydrogen yields were obtained using heat-treated sludges from municipal waste treatment plants. Moreover, they demonstrated that the heat treatment enriched the inocula in both hydrolytic and fermentative bacteria [142]. This study further highlights the importance of heat treatment of sludge in terms of the selection of hydrogen-producing microflora over methanogenic organisms. Unlike pre-treated mixed inocula, also single cultures approaches have

Table 5
Biohydrogen production from rice waste: main pretreatments, inocula and yields.

Feedstock	Pretreatment	Type of inoculum	Best inoculum treatment	T (°C)	Best H_2 Yield[a]	H_2[a] (%)	Reference
RH	Enzymatic	*Clostridium butyricum* CGS5	–	35	19.15 mmol/g reducing sugar	25	[143]
RS	Milling	ASS	100 °C, 60min	35	14.67 mL/g VS	70	[140]
RS	–	SS	100 °C, 15min	55	0.54 mmol/gVS added	42	[141]
		SS	–	55	0.74 mmol/g VS added	58	
RS	Milling	MWTPS	95 °C, 40min	55	24.80 mL/g TS added	–	[142]
DRB	Acid	*Clostridium acetobutylicum* YM1	–	35	117.24 mL/g consumed sugars	–	[144]
RBDW	–	SHS	100 °C, 60min	57	2.20 mol/mol substrate	42	[148]

[a] -Highest values of hydrogen yield or percentage are reported (or calculated from available data) when available. ASS-Activated Sewage Sludge; DRB- Deoiled rice bran; RBDW- Rice Bran De-oiled wastewater; SHS- Slaughterhouse Sludge; SS- Sewage sludge; MWTPS- Municipal wastewater treatment plant sludge.

been pursued to convert rice waste streams into hydrogen. Cellulolytic bacteria were isolated from soil and it was observed that pure culture of *Clostridium butyricum* CGS5 gave efficient biohydrogen production using enzymatically hydrolysed RH as substrate [143]. A pure culture of *Clostridium acetobutylicum* YM1 was also adopted on an acid-treated starchy waste such as DRB (de-oiled RB) [144].

In concentrated acid-treated RS hydrolysate and wastewater from the food industry, the presence of *Clostridium pasteurianum* was found to support the production of biohydrogen using acetate and butyrate pathway. Also, a 1.5-fold increase in biohydrogen yield was observed with lower substrate utilization in a continuous system as compared to the batch reaction [145]. After confirming the increased biohydrogen production in a continuous system, Liu et al. [146] worked on the optimization of hydraulic retention time (HRT) of a continuously external circulating bioreactor, reporting that the highest hydrogen production rate was observed with an HRT of 4 h. The continuous production process also needs continuous organic loading. Therefore studies on OLR optimization demonstrated that biohydrogen production from RS increased, reaching maximum biohydrogen production of 2.6 L per day when the range of OLR was between 7.1 and 21.4 g COD/L per day [147]. When rice waste was used as a substrate, OLR optimization and the augmentation with pure clostridial cultures, gave a significant increment in biohydrogen production without any inoculum treatment thus making the process more economic. These studies demonstrate the necessity to develop more efficient microbes having the potential to produce higher biohydrogen yields.

4.4. Biodiesel

Biodiesel refers to fatty acid methyl ester (FAME) produced through the transesterification of oils, mainly obtained from specific energy crops such as rapeseed, RB, sunflower, palm and soy, but even from animal fats or waste oils [149,150]. In addition, specific oleaginous microorganisms have been selected and proposed for the sustainable production of lipids as already elegantly reviewed [151,152]. Oleaginous yeast, bacteria, and microalgae are defined as microorganisms with an intracellular lipid content exceeding 20% and reaching up to 70%. Lipids accumulation usually starts when a nitrogen source is limiting but in the presence of an excess of carbon, which will be converted into triacylglycerols [153]. In the perspective of reducing biodiesel costs, residues from rice could be profitable substrates for microbial biomass and lipids production. For this purpose, rice starchy or lignocellulosic wastes have been assessed as feedstocks by few research groups. Since the employed microorganisms are generally lacking specific hydrolytic enzymes, again lignocellulose or starch hydrolysis was found to be necessary as well as the optimization of fermentation conditions. RS and rice food waste were mostly adopted so far as feedstocks for lipids production (Table 6).

Azad et al. [85] optimized pH values of a fermentation broth containing H_2SO_4-hydrolysated RS as a carbon source for *Lipomyces starkeyi,* and found that the yeast accumulated microbial lipids up to 36.14% of cell dry weight (CDW). Diwan et al. [154] developed an effective H_2SO_4 based mild saccharification of RS and successfully employed the crude, non-detoxified hydrolysate for the growth of the yeast *Mortierella alpina* MTCC-6344 that accumulated lipids up to 40% of CDW. A different approach was pursued by using the amylolytic oleaginous yeast *Sporidiobolus pararoseus* KX709872 [155]. This strain produces α-amylase and amyloglucosidase, and was used to directly convert canteen rice residues into biolipids in both flasks and stirred tank bioreactor without previous starch hydrolysis. After broth optimization, lipids reached 56.61% of CDW. Moreover, the produced fatty acids contained high oleic content (60–62%) similar to those of vegetable oil, indicating that these lipids could be a promising alternative to plant fats.

Another methodology was tested by exploiting *Cryptococcus curvatus* ATCC 20509 ability to accumulate lipids from RS. Firstly, RS was treated with NaOH and anaerobically digested using sewage as inoculum. Resulting VFAs were then used by *C. curvatus* ATCC 20509 as building blocks for the synthesis of lipids (up to 26% CDW). The authors also assessed the techno-economical viability of their process, concluding that VFAs broth from anaerobic digestion of RS, compared to synthetic VFAs, appeared the most suitable carbon source for lipids production [153]. Microalgae have also been considered promising for biodiesel production due to their short cell cycle, ability to adapt to harsh environments, and high oil content (up to 80% CDW). Moreover, algae can

Table 6
Biolipids production from rice waste: main pretreatments, inocula and yields.

Feedstock	Pretreatment			Microorganism	T	Lipids	Reference
	Physical	Chemical	Enzymatic/microbial		(°C)	(% CDW)	
RS	Microwave, Autoclaving	4.8% NaOH, 1.5% H_2SO_4	–	*Mortierella alpina* MTCC-6344	25	40	[154]
RS	–	1% Trifluoracetate at 95 °C	Cellulase	*Chlorella pyrenoidosa* MTCC-6344	25	56	[156]
RS	Autoclaving	2% NaOH	Synthesis of VFA by anaerobic digestion	*Cryptococcus curvatus* ATCC 20509	25	28	[153]
RS	Autoclaving	3.5% H_2SO_4	-	*Lipomyces starkeyi*	30	36	[85]
RS	Gamma ray irradiation	1% NaOH	Cellulase	*Chlorella protothecoides* strain 25	–	45	[92]
RRC	–	–	Gluco-amylase & α-amylase	*Sporidiobolus pararoseus* KX9872	22.4	57	[155]

RRC- Rice residues from canteen.

be grown in fermentors without occupying cropped areas. Although algal biodiesel has still a price higher than conventional diesel which makes large-scale industrial applications not economically sustainable, attempts were made to reduce costs, such as using cheap carbon sources. For this purpose, Li et al. [156] used RS hydrolysate to support the fast-growing alga *Chlorella pyrenoidosa* MTCC-6344 which accumulated lipids up to 56.3% CDW. The following *in situ* transesterification obtained promising results with 95% biodiesel yield.

4.5. Biobutanol

Biobutanol is less popular among clean fuels although it represents a good alternative to fossil fuels, due to its unique features such as high energy content, improved heating value, and reduced corrosive action [157]. Moreover, it can be blended with gasoline with a proportion higher than ethanol. Butanol is largely used as an industrial intermediate, particularly for the manufacture of butyl acetate and other industrial chemicals, as a flavour in many food and beverage industries, or as an extractant for various manufactured chemicals and pharmaceuticals. Industrially, butanol is mainly produced via petrochemical synthesis (Oxo process) although biological synthesis is also possible and, for food safety reasons the butanol used in the food industry must be obtained only by microbial fermentation [158]. Biobutanol can be manufactured by the fermentation of glucose by anaerobic clostridia performing the acetone, butanol, ethanol (ABE) metabolism. The ABE catabolism involves a first acetogenic step generating acetic and butyric acids, CO_2, and hydrogen, and a second step (solventogenic) in which acetone, butanol, and ethanol are produced from the acids [159]. Butanol fermentation is much less efficient compared to ethanol fermentation. Therefore, great amounts of energy are necessary for product recovery from the diluted broth. This, together with the substrates cost, makes the entire process non-sustainable [160]. Thus, many efforts have been devoted to improve the efficiency of the process or decrease the costs of the raw material supporting microbial growth.

Rice wastes, especially RS, have a great potential to be efficiently used as a carbon source for butanol. Again, the use of such low-cost feedstock requires pretreatments, subsequent enzymatic hydrolysis to obtain fermentable sugars, and/or butanol-producing strains able to proficiently metabolize the released sugars, such as xylose together with glucose, into butanol (Table 7). The sulfuric or phosphoric acids or alkali pretreatments of RS are reported as cheap and effective, and thus have been extensively evaluated [32,159–164]. Once obtained, the sugars are utilized by specific clostridia to perform the ABE fermentation, with a yield of 2.0–18 g/L. Chen et al. [75] assessed a synthetic non-pretreated enzymatically hydrolysate from RS, under non-sterile conditions minimizing the contaminants interference by increasing the initial cell concentration of *Clostridium sacchaperbutylacetonicum*. Such conditions ensured not only the biobutanol production in a non-sterile environment but demonstrated that the sterilization step of the agricultural wastes used as substrate can be avoided, thus reducing manufacturing cost.

While various research groups focused on the optimization of pretreatment and hydrolysis, others concentrated on fermentation modes. Parameters, such as initial pH, temperature, age and size of the inoculum, and the agitation rate, were optimized for the butanol production from pre-optimized RS hydrolysate [165]. Gottumukkala and coworkers fine-tuned ABE fermentation parameters (i.e., pH, inoculum concentration and calcium carbonate concentration) resulting in enhanced biobutanol yields from a detoxified enzymatic hydrolysate of acid pretreated RS by *Clostridium sporogenes* BE01 [166]. Although not considered as efficient butanol producer in comparison with commercial strains such as *Clostridium acetobutylicum*, *C. sporogenes* BE01 reached a maximum butanol concentration of 5.52 g/L in optimized conditions, one of the highest reported for this species. Moreover this strain produced ethanol and butanol without acetone in the final mixture which is considered an advantage in the industrial bioconversion of biomass to alcoholic fuels [167]. To decrease the cost of the enzymes and increase sugar utilization and biobutanol production, Chi et al. [168] proposed a staged acidogenic/solventogenic fermentation process. In this study, alkaline-pretreated RS was firstly fermented by a microbial consortium of *Clostridium thermocellum* and *Clostridium thermobutyricum* to both hydrolyze lignocellulose and enrich the system with butyric acid. The resulting supernatant was used for ABE fermentation by *Clostridium beijerinckii* NCIMB8052. This strategy resulted in higher butanol production when compared to a conventional SHF (Separated Hydrolysis and Fermentation) process involving the use of commercial cellulases in the lignocellulosic hydrolysis step followed by the fermentation.

The development of a bioprocess for direct butanol production from cellulosic biomass was pursued by Wang et al. [169]. Although strains of clostridia have been reported to produce butanol from various substrates such as agricultural residues, none of them can directly convert cellulose into butanol. For this reason, the authors obtained butanol from filter paper developing a co-culture systems of the efficient butanol producer *C. acetobutylicum* ATCC824 with a newly isolated *Clostridium celevecrescens* N3-2 strain or with a stable undefined consortium. Thus this strategy could be a simplified approach for direct conversion of cellulose to biobutanol and could be efficiently used also with other

Table 7
Biobutanol production from rice waste: main pretreatments, inocula and yields.

Feedstock	Pretreatment			Microorganism	T	Biobutanol Yield[b]	Reference
	Physical	Chemical	Enzymatic/microbial		(°C)	(g/L)	
RS	Autoclaving	4% H_2SO_4, Detoxification	Cellulase	*Clostridium sporogenes* BE01	35	5.52	[166]
RS	Milling, Autoclaving	1% H_2SO_4	-	*Clostridium acetobutylicum* NCIM 2337	37	13.50	[159]
RS	Temperature	1% NaOH	Cellulase, *Clostridium thermocellulm* ATCC 27405, *Clostridium thermobutyricum* ATCC 49875	*Clostridium beijerinckii* NCIMB 8052	37[a] & 55[a]	15.90	[168]
DRB	Autoclaving	1% H_2SO_4, Detoxification	-	*Clostridium acetobutylicum* YM1	30	6.87	[162]
DRB	Autoclaving	1% HCl or H_2SO_4, Detoxification	Cellulase	*Clostridium saccharoperbutylacetonicum* N1-4	30	7.72	[161]
SRSH	–	–	-	*Clostridium saccharoperbutylacetonicum* N1-4	35	6.60	[170]

DRB- Deoiled rice bran, SRSH- Synthetic rice straw hydrolyzate.
[a] Temperature adopted for lignocellulosic hydrolysis by *Clostridium thermocellulm* ATCC 27405 and *Clostridium thermobutyricum* ATCC 49875.
[b] Best biobutanol yield.

lignocellulosic substrates such as rice wastes.

4.6. Bioethanol

Although bioethanol is considered the most promising liquid biofuel potentially obtainable from rice waste streams (Table 2), its commercialization would be possible only if the cost of the entire process, from feedstock collection and treatment to the attainment of the final product, will be sustainable [171]. This would be possible by (i) firstly reducing the number of steps (Fig. 3), i.e. by clubbing them together in a single vessel, (ii) by reducing as much as possible the use of extra reagents such as commercial enzymes, (iii) by shortening the processing time. In addition, fermentation efficiency represents another key factor directly linked to the available microorganisms used in the bioreactor. Further strategies are being applied in which organisms were genetically modified to produce enzymes for saccharification and fermentation, or consortia of different organisms or commercially available enzyme cocktails were used. In terms of fermentation effectiveness, *Saccharomyces cerevisiae* is the main candidate, even if several strains proved not capable of tolerating the inhibitors formed during pretreatments. Hence, detoxification of the resulting hydrolysates is needed or tolerant strains have to be developed [172,173].

This section reviews the following strategies available for the production of bioethanol from rice waste streams:

1. Separate Hydrolysis and Fermentation (SHF)
2. Simultaneous Saccharification and Fermentation (SSF)
3. Consolidated Bioprocessing (CBP)

4.6.1. SHF for bioethanol

Through this method, enzymatic hydrolysis and fermentation are performed in sequence (Fig. 2). Positive aspects are (i) the different optimal temperatures required by the two steps of the process can be optimized separately, (ii) the use of enzyme cocktails demands for different pHs, (iii) the whole design of the equipment, including stirring, can be organized independently [174–176]. Beyond several positive aspects, there are also some negative sides such as (i) this process requires considerable capital investments as more than one vessel must be involved, (ii) it is generally more time-consuming as the two steps are done separately, (iii) the increasing sugar concentration produced by cellulases activity leads to inhibit the enzyme action itself, (iv) in the pretreated biomass slurry several inhibitors are generally present, which may hinder the cellulases. These aspects will increase the final cost of the process [174–176].

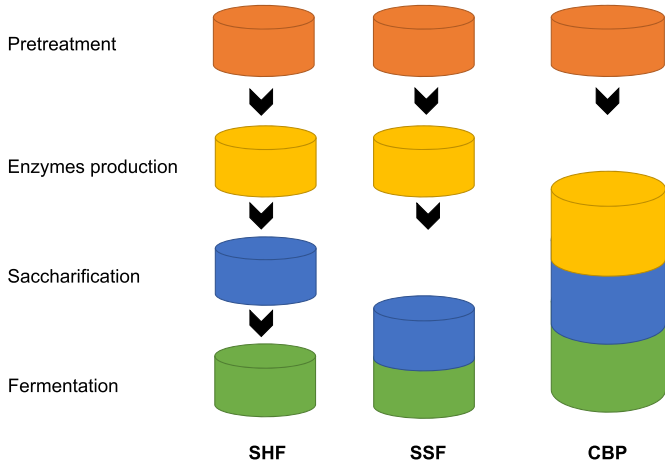

Fig. 3. Graphical representation of process integration in SHF, SSF, CBP approaches to produce biofuels and other valuables products from biomass.

Taken together, the above considerations gave rise to the limited number of SHF applications in the last decade, even if some interesting reports are available on several rice waste substrates (Table 8). For instance, some SHF approaches used enzymatic cocktails containing xylanase and pectinase on pretreated RS using ammonia fiber expansion (AFEX). The combination with *S. cerevisiae* in separate fermentation produced more than 175 g EtOH/kg treated RS. Interestingly, this ethanol yield was achieved even though pretreated biomass was not washed, detoxified, and added with supplemental nutrients. Fermentation of such hydrolysate with two *P. stipitis* strains also gave appreciable results in terms of g ethanol/L [177]. Abedinifar et al. [63] after investigating on optimal pH and temperature for commercial cellulase and β-glucosidase, reported that SHF could be efficiently adopted by using diluted acid pretreated RS. They also reported that the filamentous fungus *M. indicus* can perform at the same level as *S. cerevisiae* in terms of growth and ethanol yield. Moreover, filamentous fungus can convert pentoses into ethanol and produce chitosan, an interesting byproduct.

Saha et al. [178] worked with rice hull (RH) pretreated with alkaline peroxide and hydrolysed with a three enzyme cocktail containing cellulase, β-glucosidase and xylanase. This procedure resulted in a sugar yield of 90%, without the release of any furfural and hydroxymethylfurfural into the medium, increasing up to 96% by separately saccharifying the liquid and solid fractions. In that case, the fermentation step was performed using a recombinant strain of *E. coli* with noticeable ethanol production (Table 8). Biological pretreatments were proposed as promising alternatives to severe thermo-chemical applications on RS by the use of a white-rot fungus coupled to steam at 121 °C [93]. The saccharification efficiencies between the two approaches resulted to be very similar, but in the case of thermo-chemical strategies, the following *S. cerevisiae* fermentation resulted in low ethanol production, thus indicating the presence of inhibitory compounds within the hydrolysates that need to be detoxified.

When the complete process of fermentation is taken into consideration along with all the parameters involved (Table 8), detoxification of pretreated biomass resulted in a significant increase in bioethanol production.

The ethanol production from lime-pretreated and enzyme-hydrolysed RH was reported by Saha et al. [71]. These Authors used a recombinant *E. coli* FBR5 strain for both SHF and SSF and found that the total time to obtain the final product was shorter for SSF as saccharification and fermentation were simultaneous, while the SHF approach worked better in terms of fermentation time as saccharification was already done in the step before fermentation. However, one of the main benefits deriving by the use of lime could be the avoiding of inhibitors, completely absent in the resulting fermentation substrate. Unfortunately, the reported conversion yield seems to be still too low.

4.6.2. SSF for bioethanol

As also reported in 4.6.1, the SHF has evolved and later compared to the SSF approach as an alternative procedure that is generally more effective [176,180]. In SSF, the same vessel is used for both saccharification and fermentation with the original objective to reduce both the equipment costs and the possible contamination of the cell suspension (Fig. 3). The two steps are indeed occurring simultaneously and, as a further resulting advantage, the process time is reduced. In addition, the possibility to select enzymes usually working at room temperature can reduce or completely eliminate heating and cooling costs (Fig. 4). Together with the removal of end-product inhibition of the saccharification process, these are the main reasons leading to devote more and more attention to SSF. Table 9 summarises the organisms, the conditions and the yield obtained by using SSF technology. Overall, substrate loading is a pivotal parameter in SSF setting, with the highest substrate loadings supporting the highest ethanol concentrations.

Some studies indicated that inhibitor-free hydrolysates could be obtained from rice waste streams under specific conditions. For instance, Diwan et al. [154] optimized the hydrolysis process of RS by an

Table 8
Bioethanol production from rice waste streams using SHF technology.

Feedstock	Pretreatment			Organism	Fermentation time	Concentration[a]	Reference
	Physical	Chemical	Enzymatic/microbial		(h)	g/L	
RH	–	Alkali	Cellulase β-glucosidase Xylanase	*Escherichia coli* FBR5[c]	19	9.8	[71]
RH	–	Alkali peroxide	Cellulase β-glucosidase Xylanase	*Escherichia coli* FBR5[c]	24	8.2	[178]
RS	Milling, Autoclaving	Acid	Cellulase β-glucosidase	*Saccharomyces cerevisiae*	25	37	[63]
RS	–	Alkali	Cellulase β-glucosidase	*Clostridium acetobutilicum* NRRL B-591	80	2	[163]
RS	Ultrasound	Acid	*Trichoderma reesei*	*Saccharomyces cerevisiae*	168	11.0	[179]
RS	Milling, Autoclaving	–	Cellulase *Trametes hirsuta*	*Saccharomyces cerevisiae* LN	48	1.1	[93]
RS	Autoclaving	Alkali	Xylanase Pectinase Cellulase	*Saccharomyces cerevisiae 424A(LNH- ST)*	144	37.0	[177]

[c] – GMO, [a]- Highest values of bioethanol are reported (or calculated from available data) when available.

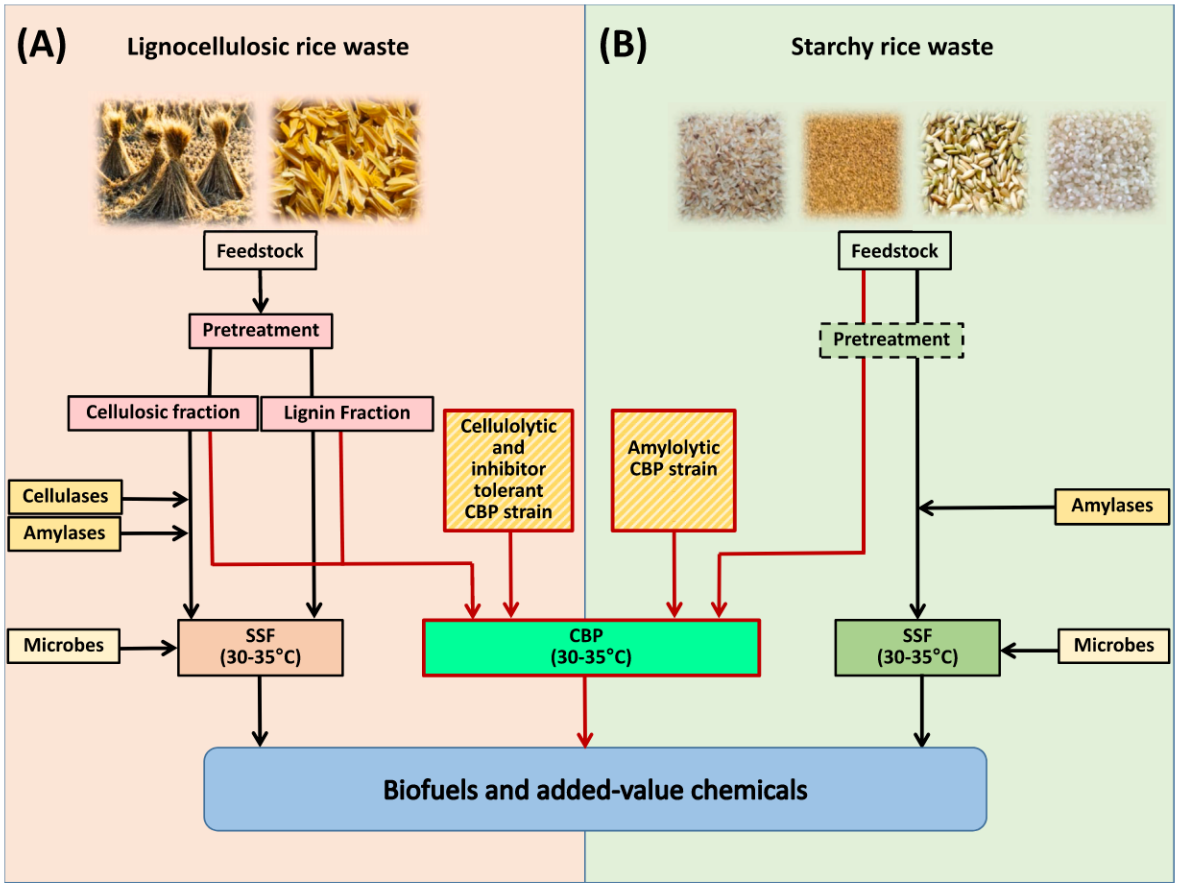

Fig. 4. Detailed representation of the SSF and CBP approaches for the processing of A) lignocellulosic rice feedstocks and B) starchy rice feedstocks for the production of biofuels and other added-value products.

experimental design with variable factors (duration, acid concentration, solid loading percentage, temperature) and found that the non-detoxified hydrolysate did not contain any furfural and hydroxymethylfurfural, thus supporting the growth and the metabolic activities of *M. alpina* much better than the detoxified hydrolysate (Table 6). Although the original objective of this work was the production of lipids, this hydrolysate could be efficiently used for alcoholic fermentation. Another efficient strategy to produce a sugar-rich hydrolysate that does not require a detoxification step, and hence simultaneously suitable as a fermentation medium, has been reported by Castro et al. [72] for RS processing, through SSF by *Kluyveromyces marxianus* NRRL Y-6860. In this case, a dilute acid pretreatment was preceded by biomass deacetylation, with the result to improve the recovery of both pentose and hexose sugars and the consequent ethanol production.

Another interesting attainment, carried out at 38 °C for 48 h, was described for RS by Poornejad et al. [77]. The ethanol production yield was improved if the straw was treated with NMMO and 1-butyl-3-methylimidazolium acetate ([BMIM][OAc]), respectively. The reduction

Table 9
Bioethanol production from rice waste streams using SSF technology.

Feedstock	Chemical Pretreatment	Substrate Loading[a]	Enzymatic/Microbial Saccharification	Organism	Concentration g/L	Reference
RH	Alkali	5% (w/w)	Cellulase	*Mucor hiemalis* CCUG 16148	9	[185]
			β-glucosidase	*Saccharomyces cerevisiae* Thermosacc®	6	
RS	Acid	15% (w/v)	Cellulase	*Rhizopus oryzae*	12	[182]
				Saccharomyces cerevisiae	10	
				Mucor indicus	16	
RH	Acid	5% (w/w)	Cellulase	*Saccharomyces cerevisiae* NCYC2826	4	[183]
RS	Acid	5% (w/w)	Cellulase	*Saccharomyces cerevisiae* NCYC2826	7	[183]
RS	dAT	10% (w/v)	Cellulase	*Kluyveromyces marxianus* NRRL Y-6860	20	[72]
RS	Alkali	5% (w/v)	Cellulase	*Mucor hiemalis*	13	[186]
			β-glucosidase			
RS	NMMO	5% (w/w)	Cellulase	*Saccharomyces cerevisiae* CCUG 53310	14	[77]
			β-glucosidase			
RS	Alkali	10% (w/v)	Cellulase	*Pichia kudriavzevii* HOP-1	24	[184]
			β-glucosidase			
			Pectinase			
RS	Alkali	60% (w/v)	Cellulase	*Saccharomyces cerevisiae* YC-097	18	[74]
BR	–	20% (w/v)	α-amylase glucoamylase	*Saccharomyces cerevisiae* L20	107	[100]
RWC	–	77% (w/w)	*Rhizopus* sp.	*Saccharomyces cerevisiae* KV25	133	[181]

dAT-deacetylationAcid pretreatment; NMMO- *N*-methyl morpholine *N*-oxide.
[a] -for pretreatment. [b]- GMO, RWC- Rice waste cake. [c]- Highest values of bioethanol reported (or calculated from available data).

of crystallinity by these two solvents was the main reason since glucan conversion yield increased from 28% of the untreated straw to 96 and 100%, respectively. Zhu et al. [74] optimized SSF to ethanol for RS pretreated with 1% NaOH or a combination of microwave and 1% NaOH by using cellulases from *T. reesei* and *S. cerevisiae* YC-097 as fermenting yeast. They demonstrated that the microwave application improved the conventional alkali pretreatment. The reduction of high heating energy costs for liquefaction and saccharification was also proposed [181]. They used rice wine cake as feedstock for SSF without cooking and raw-starch-digesting enzyme prepared from *Rhizopus* sp. SSF conditions were optimized for *S. cerevisiae* in terms of incubation temperature, pH, fermentation time, and inoculum size. The effects of several additives such as nitrogen sources, surfactants and metal salts were also studied. The selected optimal SSF conditions resulted in ethanol production improvement within 90 h of fermentation at 30 °C.

A comparison between two filamentous fungi (*Rhizopus oryzae and M. indicus*) and a thermotolerant yeast strain of *S. cerevisiae*, was performed in terms of ethanol production in a SSF of RS [182]. The advantages of using the filamentous fungi are that they can grow at higher temperatures than *S. cerevisiae*, thus approaching the optimum for SSF process, and finally resulting in higher ethanol yield. By quantitative NMR screening methods, Wu et al. [183] investigated the different compositions of the pretreatment liquors deriving from RS and RH, and their consequences on SSF. High-pressure microwave processing was applied in combination with a range of severities, and among a number of different compounds, they found that while fermentation inhibitors, such as hydroxymethylfurfural and furfural, were more present in husk liquor, formic acid was higher in straw liquor. The ethanol production from alkali-treated (NaOH) RS in a SSF process was reported by Oberoi et al. [184]. They used for the first time the recombinant *Pichia kudriavzevii* HOP-1 thermotolerant strain, producing ethanol at amounts comparable to those produced by *S. cerevisiae*. Further interesting investigations by coupling alkali pretreatment of RH with the use of zygomycetes fungi (*M. hiemalis*) for the production of ethanol, was performed [185]. The alkali pretreatment enables to increase the low ethanol yield generally obtainable (around 15%) to more than 85%, as a consequence of lignin removal and cellulose crystallinity decrease. On the other hand, the use of *M. hiemalis* resulted in ethanol yield higher than *S. cerevisiae*, probably due to its high resistance against the inhibitors and to the utilization of pentoses, and also resulted in the production of other value-added proteins and lipids. The same filamentous zygomycetes *M. hiemalis* was used by SSF in combination with sodium carbonate pretreatment [186]. The use of this chemical enabled to remove the high silica content from RS and consequently to enhance enzymatic hydrolysis and ethanol production by the fungus, that proved once more to perform better than *S. cerevisiae*. On BR, Gronchi et al. [100] found a great potential as ethanol producers by newly isolated yeast strains, performing better in a SSF than other well-known benchmark strains. This approach can be followed even with the objective to find superior outperforming phenotypes to be further selected at bioreactor scale for specific feedstocks and also in view of the construction of a recombinant strain for CBP.

4.6.3. CBP for bioethanol

The CBP of biomass into bioethanol is gaining increasing recognition as a potential breakthrough for low-cost biomass processing [187–189] where a single microbe is able to process one-step pre-treated feedstocks (Fig. 3). A four-fold reduction in the cost of biological processing and a two-fold reduction in the overall production cost is projected when a mature CBP yeast will be available [172,188,190]. A CBP approach was proposed also for cellulosic- and starch-rich rice streams (Table 10), using engineered *S. cerevisiae* strain specifically developed for co-expression of efficient cellulases or amylases (Fig. 4). Specific efforts were focused on RS, once pretreated with hot water (80 °C, 16 h), which was converted into ethanol by the *S. cerevisiae* strain MNII/cocδBEC3 co-producing β-glucosidase, endoglucanase and cellobiohydrolase tethered to the cell surface [191]. Although the enzymatic activities of the CPB strain were promising, the ethanol levels obtained from 100 g/L HWP RS were low (with 33% of the theoretical yield), pointing out that both substrate loading optimization and harsher pre-treatment conditions were the most important drivers towards higher ethanol yields. The same group indeed applied heavier pre-treatment on RS (Liquid Hot Water method, 130–300 °C under the pressure of less than 10 Mpa). The resulting hydrolysate was converted into ethanol by the CBP *S. cerevisiae* strain MN8140/XBXX able to hydrolyse hemicellulose by co-displaying the endoxylanase from *T. reesei*, the β-xylosidase from *R. oryzae* and the β-glucosidase from *Aspergillus aculeatus* and to assimilate the released xylose through the expression of *Pichia stipitis* xylose reductase and *S. cerevisiae* xylitol dehydrogenase. The ethanol concentration reached was 8.2 g/L after 72 h fermentation, with an ethanol yield close to 82% of the theoretical [192].

CBP applications were found to be very efficient in the case of starchy rice by-products such as RB, BR, UR and DR (Table 9). Two yeast strains, M2n [TLG1-SFA1] and MEL2 [TLG1-SFA1] co-expressing the glucoamylase TLG1 from *Thermomyces lanuginosus* and the α-amylase SFA1 from *Saccharomycopsis fibuligera,* previously reported for their promise

Table 10
Bioethanol production from rice waste streams using CBP technology.

Feedstock	Physical pretreatment	Substrate loading % (w/v)	*Saccharomyces cerevisiae* strain	Fermentation time (h)	Concentration (g/L)	Reference
RS	Milling, Thermal	100	MNII/cocδBEC3	72	8	[191]
RS	Autoclaving	80	MN8140/XBXX	72	8	[192]
BR	Milling	20	ER T12	168	101	[53]
			M2n T1		100	
BR	Milling	20	M2n [TLG1-SFA1]	144	75	[61]
			MEL2 [TLG1-SFA1]		68	
DR	Milling	20	M2n [TLG1-SFA1]	144	79	[61]
			MEL2 [TLG1-SFA1]		42	
RB	Milling	20	M2n [TLG1-SFA1]	144	39	[61]
			MEL2 [TLG1-SFA1]		68	
UR	Milling	20	M2n [TLG1-SFA1]	144	66	[61]
			MEL2 [TLG1-SFA1]		61	

as raw starch converting microbes [55] were effectively adopted to achieve high ethanol levels (Table 10). The higher the starch content (RB > UR > BR and DR), the higher ethanol concentrations were produced. Noteworthy, even higher ethanol levels were recently obtained by applying efficient amylolytic CBP strains on broken rice (20% w/v). Two strains *S. cerevisiae* ER T12 and *S. cerevisiae* M2n T1, simultaneously secreting an α-amylase and glucoamylase originating from *Talaromyces emersonii*, were adopted in a CBP setting [53]. No substrate pre-treatment was needed, and the final alcohol titers (100 g/L) indicated that this process can be industrially viable.

4.7. Microbial fuel cell

Electricity is one of the most important energy forms that support most of the human activities. Recently, a new, future-promising segment has been added, i.e. electrical vehicles. Many personal cars and public transports are shifting to electricity run vehicles as they are more economical and less polluting. However, the current electricity supply is mostly based on thermal power, generated by coal burning, which unfortunately contributes to environmental pollution. To cope with this excessive demand, it is essential to find a renewable and non-polluting electricity source. Current studies indicate MFC, as a possible future contribution. It is a strategy exploiting bacterial metabolism to generate electricity from a range of bio-wastes. The interest in this technology raised when the possible future use of the high producing bacterial strain *Geobacter sulfurreducens* KN400 was reported in 2009 by Time Magazine as one of the top 50 most important inventions [193].

MFC could be considered as a bioreactor with two chambers, an anode and a cathode separated by a PEM. Electrons, generated at the anode, move to the cathode through an external circuit and protons travel to cathode through PEM, where they combine with oxygen and electrons to form water molecules [194]. Few experiences on MFC exploiting rice by-products are available in the literature (Table 11). The PEMs used in MFC are generally polymeric membranes like Nafion, expensive and susceptible to fouling after repeated usage. Mashkour et al. compared Nafion to polymeric membranes modified with hydrophilic and antibacterial nanoparticles which lead to higher proton conductivity although more expensive [195]. Studies showed that blending of 10% RH ash with soil to fabricate ceramic PEM gave higher volumetric power density as compared to that of control when rice mill wastewater was used as substrate and anaerobic sludge collected from the sediment of a pond was used as inoculum [80].

RH charcoal was also used as anode and cathode electrodes for MFC, showing the potential of RH to be used not only as a carbon source for microbes but also in the construction of MFC [196]. Jiao et al. [197] indicated that the power density is influenced by the surface area of the carbon electrode, i.e. porosity, used in MFC. Rezaei et al. [198] demonstrated for the first time that it was possible to generate electricity using MFC with cellulose as a carbon source and a single strain of *Enterobacter cloacae*. On the other hand, single strain, i.e. pure culture of *S. cerevisiae*, did not give promising results if maximum power density is compared with mixed cultures and consortium [199]. When non-pretreated RS was used as a substrate and the mixed culture of cellulose-degrading bacteria as inoculum, MFC could generate power density up to 145 mW/m^2. When the same MFCs were connected in series, the power density increased more than three times. After an initial lag period of 110 h, the stable power density was maintained for 10 days. The refuelling of the cell was done three times with a medium containing 1 g/L of RS and no lag period was observed, indicating that such MFCs can utilize RS for the production of energy [200].

RB was also used as a carbon source in single-chambered MFC inoculated with paddy field soil. The power density increased drastically when a mineral solution was used as liquid phase instead of pure water along with RB. The amplicon-sequencing showed the presence of *Geobacter* spp. at anode biofilm. The same MFC was continuously adopted for 130 days supplementing the system with RB after 10–20 days [201]. Phylogenetic analysis reveals the presence of a mutualistic behaviour between *Bacteroides, Clostridium* spp. and *Geobacter* spp. in the anode biofilm [202]. On the other hand, when pond bottom sludge was exposed to air, it gave higher volumetric power density as the

Table 11
Production of electricity using microbial fuel cell from rice waste streams.

Feedstock	Pretreatment		Inoculum	Resistance applied	Power Density	Reference
	Physical	Chemical		Ω		
RH	–	Acid, Alkali	AS	1000	318 mW/m^2	[197]
RS	–	–	Consortium	1000	145 mW/m^2	[200]
RS	Milling	–	CDSM	1000	190 mW/m^2	[204]
RB	–	–	PFS	10,000	520 mW/m^2	[201]
RB		HC	PB Mud	510	17 mW/m^2	[202]
RB	–	–	SM	500	477 mW/m^2	[203]
Rice washing water	–	–	*Saccharomyces cerevisiae*	320	1 mW/m^2	[199]
Rice mill wastewater	–	–	PB sludge	100	656 mW/m^{3V}	[205]

AS- Anaerobic sludge, PFS- Paddy field soil, PB- Pond bottom, SM- Swine Manure, HC- Hydrodynamic cavitation, CDSM- Cellulose degrading soil microflora, V-Volumetric power density.

methanogenesis was affected due to aeration. Schievano et al. [203] highlighted that rice waste streams can be usefully exploited in MFC applications. This is of great importance considering that the electricity can be obtained from MFC adopting the biorefinery approach after production of gaseous biofuels, such as biohydrogen and biomethane, from organic waste.

In the case of rice biowaste utilization using microbial fuel cells, pretreatment of the substrate does not improve the production of electric process integration (Table 11). Schievano et al. used rice waste streams for the production of gaseous fuels like biohydrogen as well as biomethane and then utilized the residues for MFC using a microbial consortium. This approach appears as the most efficient way to utilize the rice waste streams, as it depicts the biorefinery approach, also providing 477 mW/m^2 of power density. Moreover, the expensive PEM can be changed by ceramic membrane reinforced with RH to further reduce the cost.

5. Biorefining of rice waste streams into added-value products

To ensure the cost-effective exploitation of rice waste streams, it is essential to recover all the potential co-products together with lower-value products such as bioethanol. As such, the overall process economics will be greatly improved. Once the cellulosic or starchy rice residues are hydrolysed to monomers (i.e. sugars, amino acids, fatty acids, etc.), the latter can serve as a feedstock for biological fermentation or chemical processing to various chemical building blocks. Besides biofuels, potential fermentation products from rice waste could be enzymes [206,207], biopolymers [208], organic acids [209–211] and vitamins [212]. Nevertheless, it is a hallmark to integrate processes for a mixture of products in a biorefinery setting to ensure the economic viability of a specific by-product [213,214]. For example, techno-economic modelling for the integrated waste streams-to-biofuels routes developed by International Energy Agency (IEA) demonstrated a positive outcome when 80% of the hexose sugars were processed to bioethanol and 20% to lactic acid [215]. Furthermore, the efficient integration of biorefineries into existing industrial plants can considerably contribute towards a sustainable bioeconomy [216]. This is particularly true in the case of rice milling residues which could be valorized into biofuels and higher values products nearby the paddy rice processing, thus reducing cost and greenhouse gas emissions related to their transport [54,188].

Few research initiatives, mostly on RS [40], already explored this perspective paving the way for additional and more in-depth research and development efforts. For instance, Zahed et al. [217] developed a continuous co-production of ethanol and xylitol from RS using a membrane reactor. Lignin can be recovered from rice residues and utilized for the production of phenolic compounds which are categories of fragrances. Lignin recovery was indeed successfully pursued from the solid waste of RS after producing relevant quantities of bioethanol in a pilot biorefinery plant [218]. Zheng et al. [219] produced vanillin from ferulic acid present in waste residue of rice bran oil using fungi. The few experiences of biorefining approaches from RS and RB indicated the promise of such substrates in a circular economy landscape relying on microbes as outstanding cell factories. Nevertheless, further research efforts are needed before large scale biorefinery plants can be installed from rice waste. Processes integration and the implementation of new hybrid technologies (i.e. thermo, chemical and biotechnological routes) and life cycle analysis will be useful. Furthermore, towards the future selection of the most efficient biorefining approaches, the holistic sustainability of the different valorization platforms should be assessed using advanced tools which combine life cycle, exergy and techno-economic analysis as recently and elegantly proposed [220] and reviewed [221–223] from a cluster of lignocellulosic materials. These novel and comprehensive research activities will provide reliable insights on process costs, yields, efficiency, and overall sustainability of biofuels and bioproducts production systems from rice waste streams. In this framework, future governmental policies and regulatory legislations can take advantage of these evaluations before implementing carbon credits and tax incentives to support the industrial developments of rice by-products biorefining plants.

6. Conclusions and prospects

The characterization of the residual starchy and lignocellulosic material globally available indicated that rice waste streams have great potential to be converted into energy, thus producing many gigalitres of biofuels, giving an important contribution to meet the world's energy demands and mitigate climate change. Towards their full exploitation, rice by-products can also be co-converted into a cluster of valuable compounds (i.e., organic acids, enzymes, pharmaceutical molecules, biopolymers). Among the biotechnological approaches adopted to convert rice waste into biofuels, ethanol production is one of the most successful applications. The whole process generally requires suitable physical, enzymatic and chemical pre-treatments, the key to extract maximum fermentable sugars. These findings are beneficial for even other liquid biofuels, but also for the most promising gaseous fuels such as biogas and biohydrogen.

As repeatedly emerging from the present review, the main actors of this story are microorganisms, able to metabolize the pretreated raw material into valuable products at the industrial level, thus considered as powerful cell factories. Moreover, microbes can be improved by genetic and/or evolutionary engineering to maximize product(s) yields and can be utilized as microbial consortia or as single bacterial or yeast strain. Biotechnological tools nowadays available and the production strategies extracted from the copious literature and here presented, indicate rice waste biofuels as economically viable. However, despite all these great promises, further research is still required on up-scale and industrial commercialization of the technologies so far developed. Moreover, future process integrations are needed toward biorefinery schemes where rice waste streams can be spun into biofuels and several other added-value products.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by University of Padova [grants GRIC120EG8, DOR1931153/19, DOR1928058/19, DOR2087054/20, DOR2084579/20, DOR2027838/20, DOR2107797/21, BIRD210708, DOR2251254/22].

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Renewing Lost Genetic Variability with a Classical Yeast Genetics Approach

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Abstract: Due to their long domestication time course, many industrial *Saccharomyces cerevisiae* strains are adopted in numerous processes mostly for historical reasons instead of scientific and technological needs. As such, there is still significant room for improvement for industrial yeast strains relying on yeast biodiversity. This paper strives to regenerate biodiversity with the innovative application of classic genetic methods to already available yeast strains. Extensive sporulation was indeed applied to three different yeast strains, specifically selected for their different origins as well as backgrounds, with the aim of clarifying how new variability was generated. A novel and easy method to obtain mono-spore colonies was specifically developed, and, to reveal the extent of the generated variability, no selection after sporulation was introduced. The obtained progenies were then tested for their growth in defined mediums with high stressor levels. A considerable and strain-specific increase in both phenotypic and metabolomic variability was assessed, and a few mono-spore colonies were found to be of great interest for their future exploitation in selected industrial processes.

Keywords: yeast classical genetics; metabolomic fingerprint; sporulation; recombination; stress; glucose; formic acid; copper; FTIR

Citation: Gupte, A.P.; Pierantoni, D.C.; Conti, A.; Donati, L.; Basaglia, M.; Casella, S.; Favaro, L.; Corte, L.; Cardinali, G. Renewing Lost Genetic Variability with a Classical Yeast Genetics Approach. *J. Fungi* **2023**, *9*, 264. https://doi.org/10.3390/jof9020264

Academic Editors: Chang-Hui Shen and Milena Georgieva

Received: 28 December 2022
Revised: 6 February 2023
Accepted: 13 February 2023
Published: 16 February 2023

1. Introduction

Apart from being a powerful model system to answer hallmark biological questions, the species *Saccharomyces cerevisiae* plays a key role in many industrial applications [1,2]. The domestication of *S. cerevisiae* strains independently occurred in many processes even before the first microbes were observed [3–5]. After centuries of continuous growth under favorable conditions, with nutrients readily and abundantly available, many domesticated *S. cerevisiae* strains have partly or entirely lost the ability to reproduce sexually [6], gotten more and more tolerant to specific stressors frequently faced in industrial plants, and metabolized a few sugars more rapidly than natural strains [7]. This is reminiscent of the so-called "domestication syndrome," already described in 1868 by Darwin, where organisms under domestication tend to drop undesirable and/or unselected traits and acquire attributes that make them successful in human-shaped environments [8,9].

In the past centuries, brewers, bakers, and, to some extent, even winemakers were used to perform subsequent fermentations using yeast strains from an old batch [5,10]. The back-sloping procedure was indeed essential to maintain the stability of the final product, thus ensuring the economic sustainability of the process of interest. Both refrigeration and the advent of pure cultures to start the fermentation further enhanced the stability of the final industrial and/or artisanal products [5,11]. Paradoxically, as soon as bakers and brewers recognized the pivotal role of *S. cerevisiae* strains in the fermentation and began to isolate pure cultures, the yeast genetic diversity severely decreased as pure cultures were more and more adopted and clonal batches were maintained by refrigeration [12]. As such,

many yeast strains industrially used today, primarily those adopted in bioethanol, wine, and beer fermentations, are often utilized mostly for historical reasons rather than scientific ones [3,13–15]. Furthermore, since the demand of customers as well as industries has turned and continued to turn, there is still significant scope for improvement of industrial strains despite their long domestication time course. The non-genetically modified organisms (non-GMO) approaches, particularly for food and beverage yeast applications, should be considered the most since they do not suffer from any issues with consumer acceptance and/or specific legislation [16,17].

There are multiple non-GMO strategies to provide suitable yeast strains for specific industrial goals (Figure 1), as elegantly reviewed [18]. A very powerful approach is to look for natural biodiversity by selecting a yeast able to operate best in a specific industrial process [13,18]. Indeed, recent metagenomic surveys underpin the fact that the natural yeast biodiversity is immense and largely unexplored, with the existing industrial strains corresponding to only a small share of the natural biodiversity [19–21]. An alternative route is to regenerate biodiversity with the innovative application of classic genetic methods to already available yeast strains [18]. Both the search for natural biodiversity and the regeneration aim at selecting the best phenotypes.

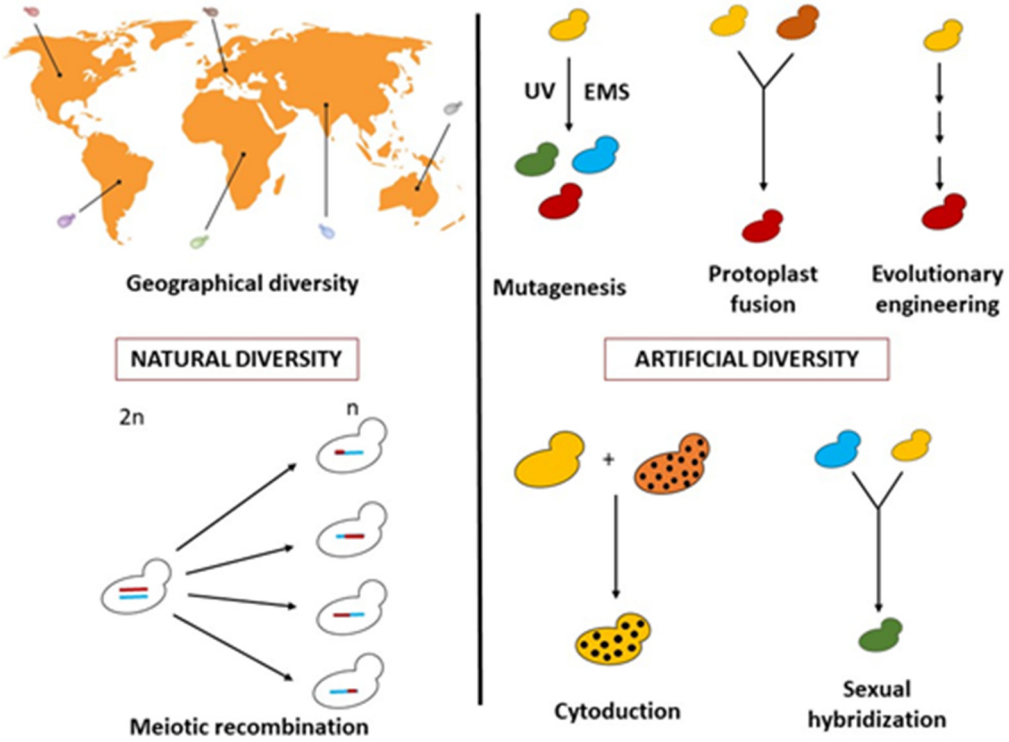

Figure 1. Methods for obtaining genetic variability in yeast adapted from Steensels et al. [18] (UV ultraviolet, EMS, ethyl methane sulfonate).

This work specifically focused on the latter strategy, choosing *S. cerevisiae* as a yeast candidate with a long biotechnological history as well as being a model organism with a homothallic nature. In contrast to higher eukaryotes, yeast gametes enter a haploid life cycle that is substantially similar to the diploid mitosis-based cycle. By taking advantage of this feature, it is possible to produce recombination of important traits by extensive sporulation, obtaining as many combinations as the spores. As an example, *Drosophila* was suggested as a possible niche for sporulation and mating as, different strains of *Schizosaccharomyces japonicus* isolated from Drosophila showed variation for pheromone-related genes [22]. Various sporal cultures can be directly tested or induced to undergo homothallic switching and subsequent diploidization. The output of this route is a collection of diploid cultures that are homozygous at all loci since they are derived from the conjugation of genetically

identical cells. These cultures are theoretically very stable, since mutations would rarely affect the phenotype due to the very low, if any, heterozygosity and would provide the most extreme effects of quantitative trait loci. Whether the genome renewal [23] could reintroduce heterozygosity and to what extent is a matter of obvious importance at both the theoretical and practical levels [24].

This work applied extensive sporulation to three different yeast strains, precisely selected for their different origins as well as backgrounds, with the aim of clarifying how new variability was generated from sporulation. Three different hypotheses were investigated: (i) the isolation of diploid homozygous mono-spore colonies (MSCs) allows to verify the amount of variability produced in the sporulation of each parental genotype, (ii) the quantitative traits analyzed show significant differences from the parental strains; and (iii) the obtained variability strictly depends on the starting parental genotype under sporulation. A new method for obtaining MSC without the use of a micromanipulator has been specifically developed, avoiding the introduction of any form of selection to exclusively focus on the extent of variability generated by recombination under sporification. For this purpose, mono-spore colonies (MSCs) were indeed randomly chosen and sequentially analyzed with increasingly informative tests also considering the presence of specific stressors (i.e., high levels of glucose or formic acid, or copper sulphate).

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions

Three *S. cerevisiae* strains with different backgrounds and geographical origins were specifically selected for this study (Table 1). Strains were maintained in 20% glycerol stocks at −80 °C and usually plated on YPD agar (Yeast extract-10 g L^{-1}, Peptone-20 g L^{-1}, Glucose-20 g L^{-1}, Agar-15 g L^{-1}) and incubated at 30 °C for 48 h. Screening for sporulation was performed at 30 °C for 7–21 days on SM1 (potassium acetate-10 g L^{-1}), McClary's Acetate medium (sodium acetate-8.2 g L^{-1}, glucose-1 g L^{-1}, yeast extract-2.5 g L^{-1}, potassium chloride-1.8 g L^{-1}, Agar-15 g L^{-1}) and modified sporulation medium (MSM) (potassium acetate-10 g L^{-1}, yeast extract-0.5 g L^{-1}, glucose-1 g L^{-1}, Agar-15 g L^{-1}) [25]. All the media were sterilized by autoclaving at 121 °C for 20 min before plating.

Table 1. Strains used in this study: background, origin and tested stressing agents.

Strain	Background	Geographical Location	Genotype	Reference	Stressing Agent	Low Stress	High Stress
TC1517	Grape marcs	Italy	2n, homotallic	[26]	Glucose (g L^{-1})	250	300
YI30	Industrial distillery	South Africa	2n, homotallic	[27]	Formic acid (g L^{-1})	0.3	0.6
YVGC13A	Vineyard, isolated from vine bark	Canada	2n, homotallic	University of Perugia	Copper sulfate (Cu-ppm)	5	7.5

S. cerevisiae TC1517 has been isolated from grape marcs [26] and has shown great promise in terms of fermenting abilities. *S. cerevisiae* YI30 was chosen as a strong candidate for lignocellulosic ethanol because of its high inhibitor and temperature tolerance [27]. The Canadian strain *S. cerevisiae* YVGC13A was chosen to evaluate the variability of a strain directly isolated from vine bark, which is currently considered the main natural reservoir of *S. cerevisiae* strains that could participate in alcoholic fermentation [28].

In addition, stressing experiments and metabolomic studies using Fourier-Transform Infrared Spectroscopy (FTIR), were carried out by inoculating yeast cultures at OD_{600} = 0.1 in 100 mL of filter sterilized (0.22 µm) synthetic defined (SD) medium containing 6.7 g L^{-1} of Yeast Nitrogen Base medium (YNB, Difco Laboratories, Detroit, MI, USA) and 20 g L^{-1} of glucose and grown them for 16–18 h at 30 °C under shaking at 120 rpm.

2.2. Spore Production and Sporulation Efficiency

A fresh single colony of each strain was inoculated into 5 mL YPD broth, and microaerophilic conditions were maintained while shaking at 30 °C for 16 h. The suspension was centrifuged at 3000 rpm for 5 min and the obtained pellet was washed twice with a sterile 9 g L^{-1} NaCl solution. Washed yeast cells were resuspended in 0.5 mL saline solution, and aliquots of 100 µL were plated on MSM. Plates were then incubated at 30 °C for 7–21 days. To avoid moisture loss, plates were sealed with Parafilm® (Bemis Company, Inc., Neenah, WI, USA). Microscopic observation was performed every week to observe spore development. The spores were counted after the addition of methylene blue (MB) to the spore suspension to allow the distinction of the living cells from the dead ones that were excluded. The number of dyads, triads, and tetrads was counted using a counting chamber (Thoma, Germany). Sporulation efficiency, a measurement of the amount of cells that undergo sporulation, was calculated by microscopic observation of the sum of triads and tetrads divided by the total asci. Sporulation efficiency (SE) [29] was then calculated as follows:

$$\% \text{ Sporulation efficiency} = \frac{\text{Number of triads} + \text{Number of tetrads}}{\text{Number of total spores}} \times 100$$

2.3. Screening of Temperature Tolerance of PS and Spores

In order to develop a quick method to produce MSCs, the minimum temperature required to kill vegetative cells of each parental strain (PS) within a population of spores was screened. Each strain was grown in YPD broth for 16 h at 30 °C and centrifuged at 3000 rpm for 5 min. Cells were suspended in sterile saline to a final density of 1×10^7 cells mL^{-1}, and 0.5 mL of cell suspension was transferred to a sterile 1.5 mL tube and exposed for 10 min at different temperatures from 55 to 67 °C at 2 °C intervals.

Each treated suspension was observed microscopically using the MB viability assay [30], and a proper dilution was plated on YPD agar plates in triplicate. The asci of yeast were broken using zymolyase treatment, as explained in Section 2.4 [31], and the related spores' sensitivity was tested at 63, 65, and 67 °C. The quantification protocol was the same as for vegetative cells.

2.4. Production of Mono-Spore Colonies

The ascospore isolation method described by Bahalul et al. [31] was modified to avoid the use of diethyl ether. Briefly, colonies grown on MSM agar were scraped and resuspended in sterile, demineralized water. This high-density suspension of asci was heat-treated at 65 °C for 10 min to kill vegetative cells and then processed with zymolyase treatment (Zymolyase®-100T, ICN; 100 U mL^{-1} in 1M sorbitol) by extending the incubation time to 1 h. Sterile glass beads (400–600 µm) were used to apply shear force on ascus walls. The resultant spore suspension was observed microscopically using MB to check for the presence of asci or viable vegetative cells. Each suspension was then properly diluted and plated on YPD agar plates supplemented with 5% (*w*/*v*) glucose. Thus, obtained colonies were referred to as MSCs. Up to 100 MSCs of each PS were stored in 20% glycerol stock at −80 °C. All MSCs were then grown on YPD 5%, transferred to MSM, and incubated at 30 °C for 7–21 days to test their sporulation ability. Microscopic observation was used to check the occurrence of asci and confirm the homothallic phenotype of the parental strains.

2.5. Phenotypic Variation in MSCs

Thirty MSCs were randomly selected from each PS and grown in SD broth at 30 °C for 16 h. These pre-cultures were inoculated in the same broth to obtain a final OD_{600} of 0.1 in a final volume of 200 µL. The experiment was run in 96-well plates in triplicate (TECAN Spark® 10M, Salzburg, Austria) at 30 °C (flat-bottom cell culture plate with instrument lid; interval time-5min; shaking-60 s; shaking mode-orbital; amplitude-2.5 mm). Growth curves were plotted using the *Pyphe-growthcurves* tool. Growth parameters such

as maximum growth rate (max_slope), time at max_slope (t_max), and lag phase (Lag) were obtained with the same tool [32]. The definition of the growth parameters given by *Pyphe-growthcurves* are as follows: max_slope-maximum slope of growth curve, t_max-time at which maximum growth slope of curve is reached, lag-lag phase.

Principal component analysis (PCA) was performed [33] considering these growth parameters, and the principal component scores and loading vectors were combined in a biplot used for the selection of specific MSCs for further studies. Additionally, a Student's *t*-test was performed to determine if the observed differences were statistically significant. Moreover, at least one MSC with growth parameters similar to those of the parents was also included.

The resulting selected 12 MSCs and their parental strain were then grown under specific stressungconditions to observe growth parameters and, as reported in Section 2.7, metabolomic changes at different stressunglevels. The MSCs and PS were pre-inoculated in SD medium and grown at 30 °C for 16 h. Each PS and respective MCS were inoculated (OD_{600} = 0.1) in SD medium with no stressing agent and in the presence of low and high concentrations of the stressing agent. Each test was performed in triplicate at 30 °C in a 96-well microtiter plate (TECAN Spark® 10M, Austria) with the same protocol described above.

2.6. Metabolomic Fingerprint at the End of Growth

Cell suspensions, prepared as detailed in Section 2.1, were centrifuged (4500× *g*, 5 min), washed twice with distilled sterile water, and re-suspended in 5 mL HPLC (High-Performance Liquid Chromatography) grade water to the final concentration of OD_{600} = 12. From each culture, 105 µL volume were sampled for three independent FTIR readings (35 µL each, according to the technique suggested by Essendoubi and colleagues [34].

2.7. Metabolomic Fingerprint under Stress

The FTIR analysis was also applied to investigate the metabolomic response under the stress of the selected MSCs cultures compared to their respective parental strains. MSCs and parental strain cultures were grown under different concentrations of stressing agents, as detailed in Table 1. However, yeast cultures were pre-inoculated at OD_{600} = 0.1 in 15 mL tubes with 7 mL of SD medium and grown at 30 °C under shaking at 120 rpm. Cell growth was stopped after 15 h. Each cell suspension was adjusted to an OD_{600} = 0.2 in a 2× fresh SD medium. A total of 100 µL of each standardized cell suspension was seeded in each selected well of a flat-bottom 96-well microtiter plate and brought to the final volume of 200 µL by adding 100 µL of a 2× solution of the respective stressing agent. Control (0% stressor concentration) was obtained by re-suspending cells in sterile, distilled water. All tests were carried out in triplicate. The growth was monitored in the TECAN as described above. The samples were collected at the end of the log phase of growth and processed for FTIR analysis [34].

2.8. FTIR Data Analysis

FTIR spectra were recovered from the OPUS software version 6.5 (Bruker Optics GmbH, Ettlingen, Germany) and transferred to MS Excel. Principal Component Analysis (PCA) and Significant Wavelengths Analysis (SWA) were performed in an R environment. SWA was employed to select the FTIR spectral regions with statistically significant differences in the comparison between the spectra of parental and MSCs cultures from the different experimental conditions tested [35]. In addition, pairs of spectra, each with three replicates, were compared using the Student's *t*-test for each wavelength separately. For each wave number, the calculated *p*-value was recorded. Significant wavelengths were selected based on $p < 0.01$. Hierarchical cluster analysis was performed with MetaboAnalyst 5.0 [36]. Data were filtered based on interquartile range, normalized to the sample median, and scaled by Pareto scaling. Hierarchical cluster analysis (HCA) was employed to highlight the metabolic differences under stress between MSCs and PS cultures, using the Euclidean correlation method and the ward.D clustering algorithm. Significant

wavelengths were selected based on these criteria: *t*-test (*p* adjusted < 0.05) and one-way ANOVA (*p*-value < 0.05).

3. Results and Discussion

3.1. Efficiency of Sporulation (SE) and Development of an Easy and Effective Protocol for MSCs Production

The three strains of *S. cerevisiae* were specifically selected for their different geographical origins and phenotypic backgrounds (Table 1). To develop a simple yet efficient protocol for obtaining high numbers of MSCs, the parental strains were first tested for their sporulation efficiency (SE) once plated on different media [25,37,38]. The highest SE was obtained on MSM plates, confirming literature data on the role of nutrient deficiency and non-fermentable carbon sources, such as acetate, in inducing sporulation [39] and on the involvement of the salt acetate cation in promoting the SE of yeast strains [38]. Sporulation media was indeed modified by Petersen et al. [40] to increase the SE. The addition of yeast extract to MSM also improved the SE, as reported by Tremaine et al. [41]. Notably, YI30 showed the highest SE (85.5%), and the other two yeast strains displayed slightly lower values (*S. cerevisiae* TC1517 and YVGC13A, at 68.4, and 64.1% SE, respectively).

Dawes and Hardie proved that vapors of diethyl ether in an agar plate or ether in liquid media kill the vegetative yeast cells, keeping spores alive [42]. This was previously applied once a protocol combining glusulase treatment, sonication, and separation of hydrophobic spores using diethyl ether was developed [43]. The diethyl ether protocol was also adopted after clubbing it with zymolyase and microbead treatment [31].

In the present study, temperature rather than diethyl ether was employed to kill vegetative cells [31], thus avoiding the use of an extremely flammable and volatile chemical solvent. Once exposed to high temperatures, the ascospores displayed greater tolerance than the respective vegetative cells. Separate experiments showed that vegetative cells tolerated temperatures up to 63 ± 0.5 °C. When the temperature was increased to 65 ± 0.5 °C, the parental strain was unable to grow, but the ascospores were able to produce colonies on YPD agar medium. These results are in line with those of Rachon et al. [44], who already observed a significant difference in temperature tolerance for vegetative cells and ascospores at 65 °C.

In order to optimize the ascospore separation protocol step, sporulated parental strains were scraped from MSM agar plates and suspended in sterile demineralized water. The zymolyase treatment, developed by Bahalul et al. [31], was found to be efficient with the extension of the zymolyase treatment to one hour. The combination of zymolyase and glass beads treatment was crucial to separate ascospores from broken asci, followed by heat treatment at 65 °C for 10 min.

Microscopic observation showed that around 60% of the asci were disrupted, releasing circular and refractive spores in suspension. These suspensions gave rise to individual colonies, referred to as MSCs, once plated on YPD agar medium.

Around 100 MSCs from each parental strain were thus obtained, and their homothallic nature was investigated as detailed in the Section 2.4. All parental strains were confirmed to be phenotypically homothallic, since all MSCs tested were able to produce spores.

3.2. Growth of MSCs from Each Parental Strain in SD Broth

Thirty randomly selected MSCs from each parental strain were first screened for their growth at 30 °C in SD medium with 2% glucose. OD_{600} was monitored for 24 h at 30 °C using a 96 well plate reader (TECAN Spark® 10M, Austria). The generated growth curves were processed using the *pyphe-growthcurves* tool to assess specific parameters such as max_slope, t_max, and lag used for the PCA analysis of Figure 2.

The first two principal components explained 97% of the variance between all the MSCs cultures (PC1: 62% and PC2: 35% of the variance). The spatial distribution of the MSCs cultures indicated a clear signature of the respective parental strains, suggesting that both ecological origin and geographical background are of great importance for the phenotypic variation triggered by sporulation. Camarasa et al. [45] observed similar results

when metabolic traits were considered as differentiating parameters to understand the origin of *S. cerevisiae* strains. Interestingly, the growth performances of each parental strain remained outside the confidence ellipse, indicating higher variation between the growth of the parental strain and that of the corresponding MSCs. The highest variability was found within the monosporal progeny of the environmental yeast YVGC13A. Conversely, most of the MSCs from the YI30 and TC1517 strains formed a compact group, except for a few MSCs positioned outside the confidence ellipse.

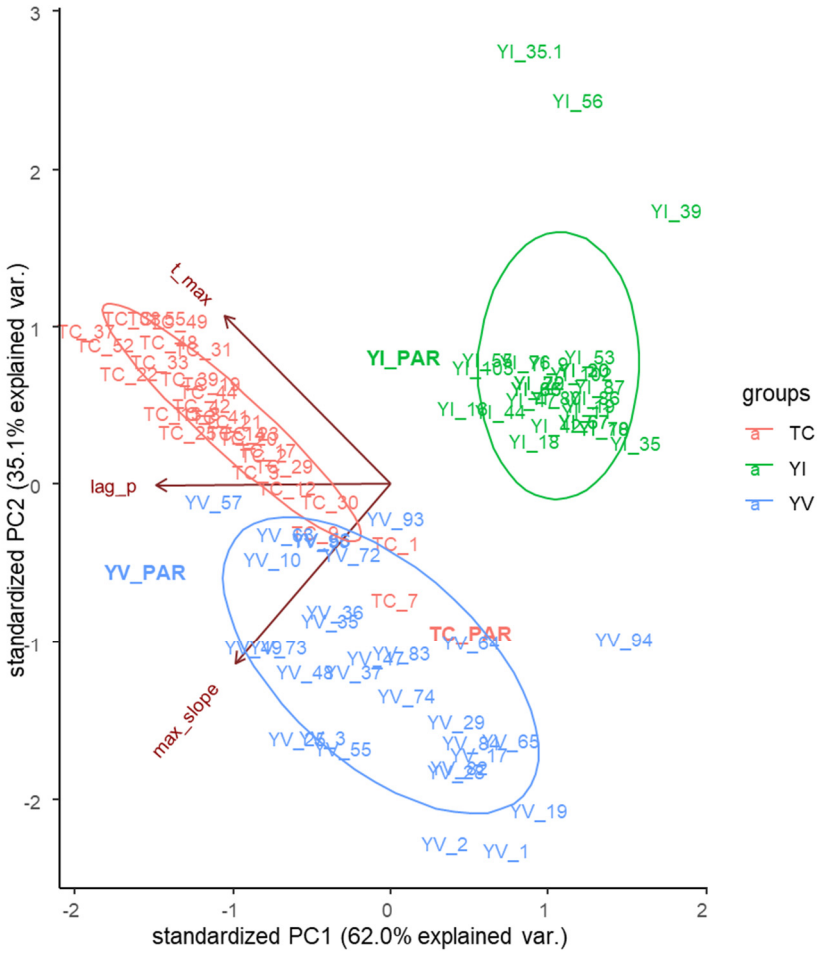

Figure 2. PCA biplot obtained from the growth parameters of all the 90 MSCs selected for the study. Input variables: Lag, t_max and max_slope growth parameters obtained using the *pyphe-growthcurves* tool from the growth curves of YVGC13A (YV, blue), TC1517 (TC, red), and YI30 (YI, green) cultures in SD with 2% glucose.

According to PC1, the Lag Phase parameter was the most differentiating between groups. The other two parameters mainly contributed to the separation of the YVGC13A cluster from those of YI30 and TC1517 along the PC2.

The same analysis was then carried out separately for each tested progeny (Figure 3). In all cases, most of the variance is distributed along the PC1, specifically 62.3, 77.3, and 55.9% for the YVGC13A, TC1517, and YI30 strains, respectively.

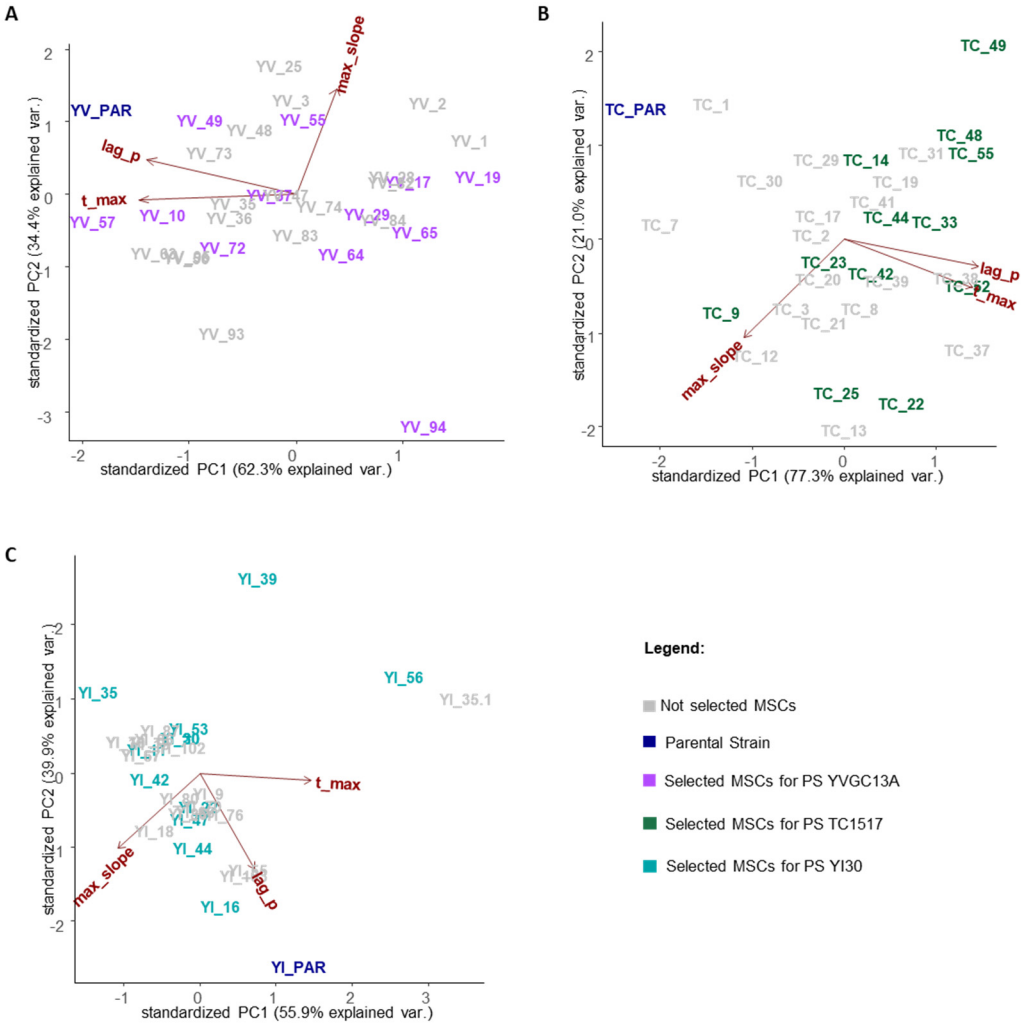

Figure 3. PCA biplot of growth parameters obtained from the 30 MSCs selected from the sporulation of each *S. cerevisiae* parental strain. Input variables: Lag, t_max and max_slope growth parameters obtained using the *pyphe-growthcurves* tool from for 30 MSCs of the parental *S. cerevisiae* strain YVGC13A (**A**), TC1517 (**B**) and YI30 (**C**). Parental strains are reported in blue, not selected MSCs cultures in grey and the twelve MSCs cultures selected for the next step of the analysis in violet (**A**), green (**B**) and light blue (**C**).

Interestingly, as already underlined in Figure 2, the parental strain was not part of the distribution of the variance of the relative MSCs cultures. Moreover, the three parameters differentially shaped the variance within each population, with lag and t_max as the main drivers for PC1 in the YVGC13A and TC1517 populations, while separately contributing along both PC1 and PC2 for the YI30 MSCs cultures.

Overall, these data already suggest that sporulation triggered phenotypic differences during aerobic growth in the presence of glucose. To further assess this evidence, twelve out of the 30 MSCs tested in each group were selected to undergo FTIR fingerprinting. MSCs were selected according to their statistically different growth parameters (p-value < 0.01) with respect to their parental strain (Tables S1–S3). Moreover, at least one MSCs with growth parameters such as those of the parental strain was included in the shortlist.

3.3. Metabolomic Fingerprinting of Selected MSCs

The selected MSCs were grown in SD broth supplemented with 2% glucose, and the cells were harvested at the end of the exponential phase to analyze the metabolomic fingerprint of their primary metabolism. The "R" script for Significant Wavelengths Analysis

(SWA) was then adopted to compare all the statistically relevant differences between the spectra of PS and each related MSC [35]. Significant wavelengths were selected based on the Student's *t*-test ($p < 0.01$, and their number was computed within each spectral region (Figure 4).

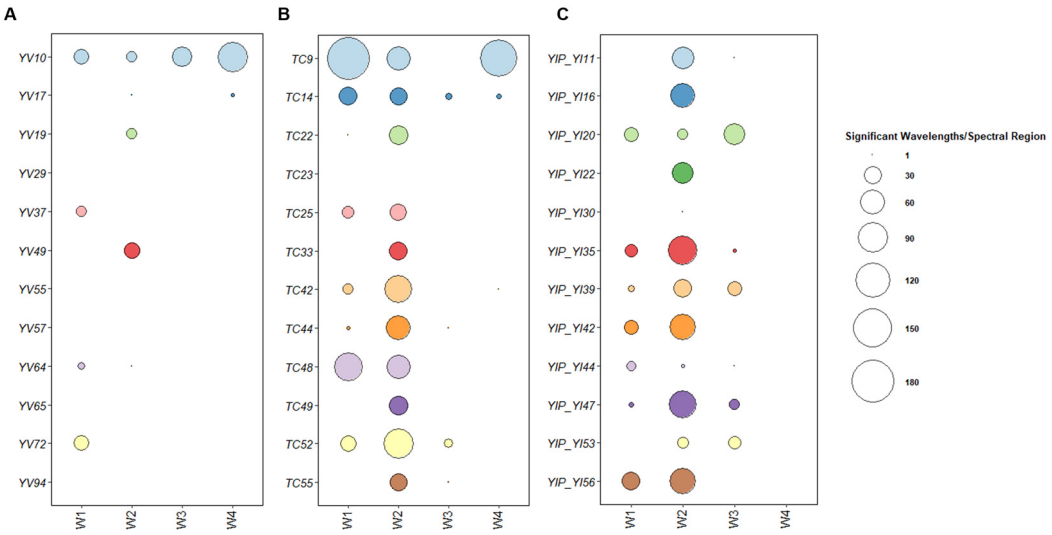

Figure 4. Number of significant different wavelengths detected in the comparison between each *S. cerevisiae* parental strain and the selected 12 MSCs. Spectra were compared using the Student's *t*-test for each wavelength separately. The number of wavelengths with statistically significant difference ($p < 0.01$) was calculated for each specific spectral area separately, namely: fatty acids (W1), amides (W2), mixed region (W3) and carbohydrates (W4) regions. (**A**–**C**): MSCs of YVGC13A, TC1517 and YI30, respectively.

The FTIR fingerprints of monosporal cultures from the parental strain YVGC13A (Table S4, Figure S1) showed little to no variability, except for the MSC YV_10, which displayed significant differences in all the spectral regions tested. Notably, the highest variation was observed in the carbohydrate region of the FTIR spectrum (Figure 4A). Although the five MSCs YV_29, YV_55, YV_57, YV_65, and YV_94 showed significantly different growth kinetics from their parental cells (Table S1), these differences did not induce significant changes in their metabolome.

On the contrary, higher variation of the metabolomic profiles was observed in most MSCs cultures from *S. cerevisiae* TC1517 (Table S5, Figure S2) and YI30 (Table S6, Figure S3). As reported in Figure 4B, within the TC1517 progeny, the greatest variability was focused on the amide (W2) and fatty acid (W1) regions.

Huge variations were observed in TC_9 in the fatty acid, amide, and carbohydrate regions (Figure 4B). Statistical analysis of growth parameters showed significant differences for all the tested MSCs in comparison to the parental strain except TC_9, whose t_max was the only one significantly different ($p < 0.05$) from the parental yeast. TC_23, which displayed a metabolome similar to the parental, was characterized by a t_max statistically divergent from the parental ($p < 0.01$).

Considering YI30, MSCs also showed significant differences in W1 and W2 regions (Figure 4C), with seven out of the twelve selected MSCs carrying metabolomic changes also in the mixed region (W3). No metabolomic alteration was instead detected for the carbohydrate metabolism (W4). The MSC YI_30 shared the metabolome of its parental strain except for a few wavelengths in the W2 region. Of the eleven MSCs exhibiting metabolomic differences in SWA, only four responded differently to the statistical analysis of growth parameters, while YI_16, YI_20, YI_44, and YI_53 showed no significant differences ($p < 0.01$) compared to the parental strain (Table S3).

Overall, FTIR fingerprinting of the tested MSCs clearly indicates a specific progeny signature. The lowest metabolomic changes were detected within the YVGC13A-derived MSC. Conversely, the sporulation of TC1517 and YI30 parental strains pushed the metabolomic variability of MSCs into the amides (W2) region, also triggering a response in the W1 and W3 regions for TC1517 and YI30 MSCs, respectively.

Based on both metabolomic and growth phenotypes, six MSCs for each parental strain were further selected to be representative of the variability produced by sporulation by choosing those with lower, higher, and PS-like growth phenotypes as well as similar or different metabolomic traits.

3.4. Growth and Metabolomic Phenotypes under Stressing Conditions

In order to further assess the phenotypic changes due to the genetic reshuffle mediated by sporulation, the second set of selected MSCs were tested for growth and metabolomic changes (Tables S7–S9; Figures S4–S6) once exposed to stressors specific to the parental strain background (Table 1).

The choice of copper as a stressor for the YVGC13A strain, isolated from vine bark in Canada, is based on the evidence that copper-based fungicides have been used in vineyards for more than 100 years and copper sulphate-based fungicides are the only chemicals allowed under organic standards [46]. High glucose concentrations can damage yeast cells and hamper their normal growth and metabolism [47]. The effect of high glucose levels on altering cell metabolism is therefore particularly interesting for a strain such as TC1517 isolated from grape marc [26]. Finally, since *S. cerevisiae* YI30 has been described as a promising candidate for second-generation bioethanol [13,27,48], formic acid was chosen as one of the most toxic weak acids [26,27] generated during the pre-treatment of lignocellulose wastes and their conversion to ethanol [13].

Overall, when grown under increasing concentrations of stressing agents, both parental strains and related MSCs displayed a dose-dependent response (Figures 5–7).

3.4.1. Phenotypes under Copper Sulphate Stress

S. cerevisiae YVGC13A-progeny were tested for the ability to withstand increasing concentrations of copper sulphate. Growth performances in the benchmark broth SD (Figure 5A) revealed significant differences in lag and max_slope values ($p < 0.05$) for all MSCs tested, except for YV_49 and YV_57. YV_10, YV_64, and, to a lesser extent, YV_94, showed the most interesting phenotypes for the simultaneous increase in the max_slope and decrease in the lag phase, significantly improving the growth kinetics of these MSCs.

Additionally, differences with respect to the parental strain were even more intense once considering the metabolomic reactions (Figures 5D and S4, Table S7). Although with similar growth kinetics, the YV_57 MSC exhibited a metabolomic pattern more divergent from the PS, downregulating bands in mixed and carbohydrate regions (W3 and W4) and up-regulating those in W1. Conversely, YV_72 and YV_94 MSCs, which showed significantly different lag phases, displayed more similar metabolomic patterns, except for an increase in W4 and a decrease in W2 band intensities. The other three MSCs, YV_10, YV_49, and YV_64, had a pattern slightly reversed from that of PS, reducing W4 and increasing W2.

Once exposed to 5 ppm of copper, most MSCs showed higher sensitivities than *S. cerevisiae* YVGC13A, characterized by a longer lag phase and a lower max_slope (Figure 5B). Only the YV_72 displayed a lag phase shorter than PS and can therefore be considered the most tolerant MSC at this copper concentration. The heatmap of the significantly altered FTIR peaks (Figure 5E) highlighted how the increased sensitivity of the MSCs corresponded to a general decrease in intensity of the whole spectra except for the W4_2 bands in the YV_10, YV_57, YV_64, and YV_72 MSCs. Among all MSCs, only the YV_49 clustered together with the PS.

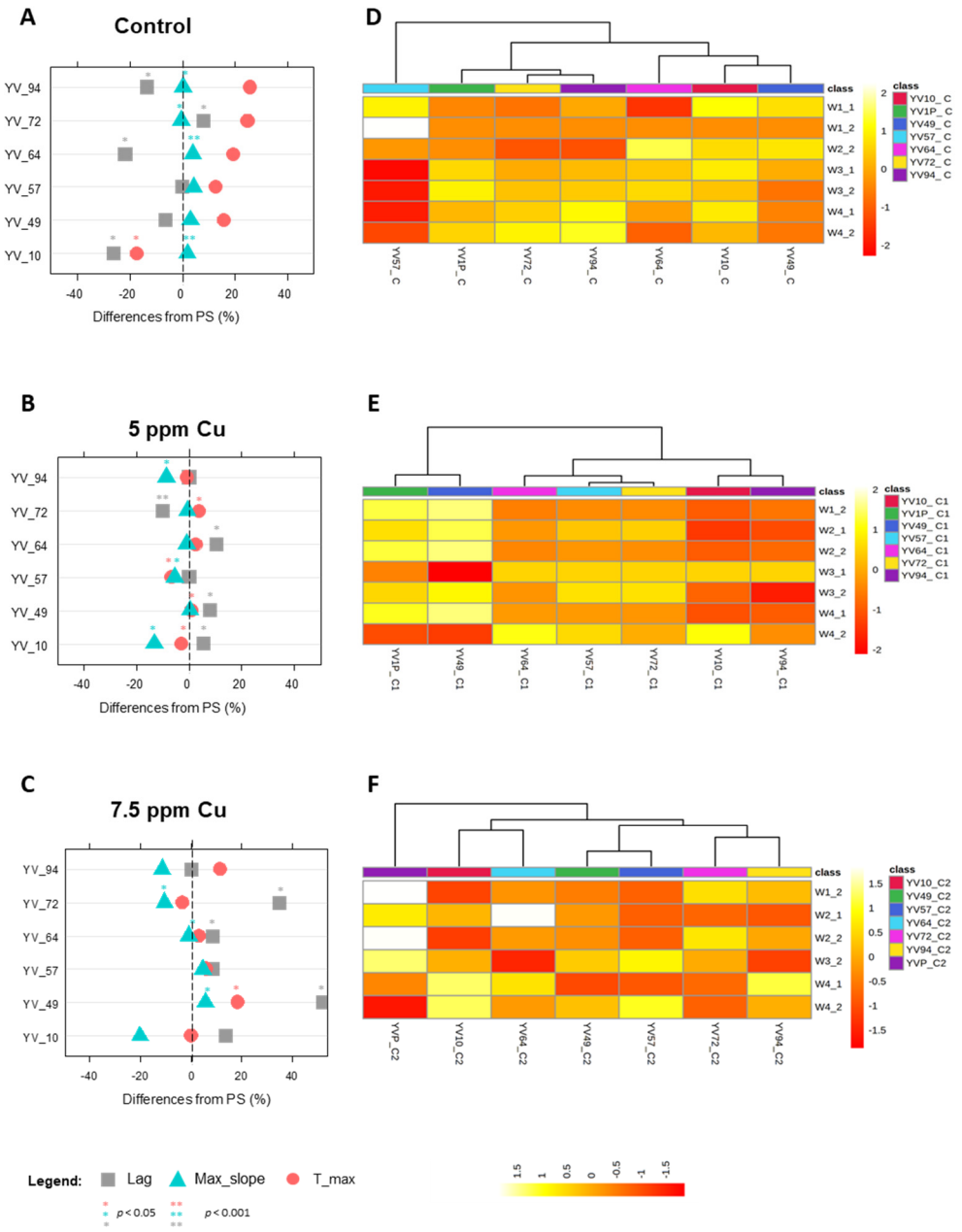

Figure 5. Growth and metabolomic phenotypes of YVGC13A *S. cerevisiae* parental strain and its derived MSCs during growth at increasing concentrations of copper (5–7.5 ppm). Panels (**A**–**C**): Lag time (grey square), max_slope (light blue triangle) and t_max (light red circle) parameters reported as percentage difference respect to the parental strain. Panels (**D**–**F**): Heatmap of the significantly altered FTIR peaks (distance measure using Euclidean, and clustering algorithm using ward.D). The coloured boxes indicate the relative intensities of the mean of peaks in the corresponding spectral region. The colour scale is log2 transformed value and indicates relatively high (yellow) and low (red) peak intensities. Spectral regions have been divided into sub-regions, namely: Fatty acids (W1_1 from 3200 to 3100 cm^{-1}–W1_2 from 3098 to 2801 cm^{-1}); Amides (W2_1 from 1800 to1649 cm^{-1}–W2_2 from 1647 to 1501 cm^{-1}); Mixed region (W3_1 from 1499 to 1352 cm^{-1}–W3_2 from 1350 to 1202 cm^{-1}); Carbohydrates (W4_1 from 1200 to 1053 cm^{-1}–W4_2 from 1051 to 902 cm^{-1}).

As the copper concentration increased (7.5 ppm), a quite different scenario was depicted (Figures 5C and S4, Table S7). YV_10, YV_57, and YV_94 MSCs seem to increase their tolerance, by showing the same phenotype as *S. cerevisiae* YVGC13A. Conversely, YV_64 and YV_72 MSCs displayed increased sensitivity, attributable to the longer lag and

the reduction of the max_slope values. Noteworthy, the YV_49 displayed higher values than the PS for all growth parameters considered. Despite the longer lag phase, this culture was then able to grow more rapidly during the log phase, giving a higher cell density than the parental strain YVGC13A.

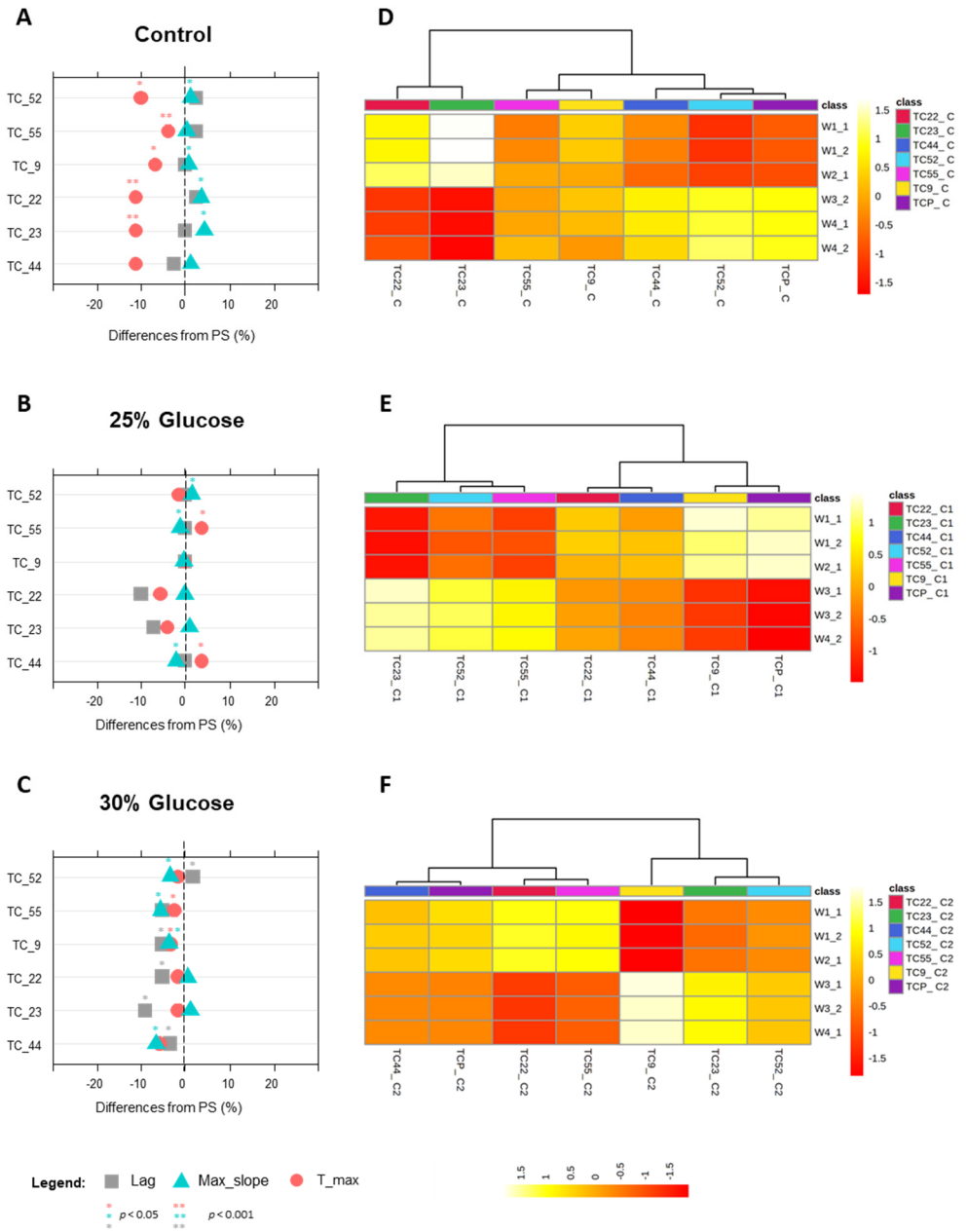

Figure 6. Growth and metabolomic phenotypes of *S. cerevisiae* TC1517 strain and its derived MSCs during growth at increasing concentrations of glucose (25–30%, *w*/*v*). Panels (**A**–**C**): Lag time (grey square), max_slope (light blue triangle) and t_max (light red circle) parameters reported as percentage difference respect to the parental strain. Panels (**D**–**F**): Heatmap of the significantly altered FTIR peaks (distance measure using Euclidean, and clustering algorithm using ward.D). The coloured boxes indicate the relative intensities of the mean of peaks in the corresponding spectral region. The colour scale is log2 transformed value and indicates relatively high (yellow) and low (red) peak intensities. Spectral regions have been divided into sub-regions, namely: Fatty acids (W1_1 from 3200 to 3100 cm^{-1}–W1_2 from 3098 to 2801 cm^{-1}); Amides (W2_1 from 1800 to1649 cm^{-1}–W2_2 from 1647 to 1501 cm^{-1}); Mixed region (W3_1 from 1499 to 1352 cm^{-1}–W3_2 from 1350 to 1202 cm^{-1}); Carbohydrates (W4_1 from 1200 to 1053 cm^{-1}–W4_2 from 1051 to 902 cm^{-1}).

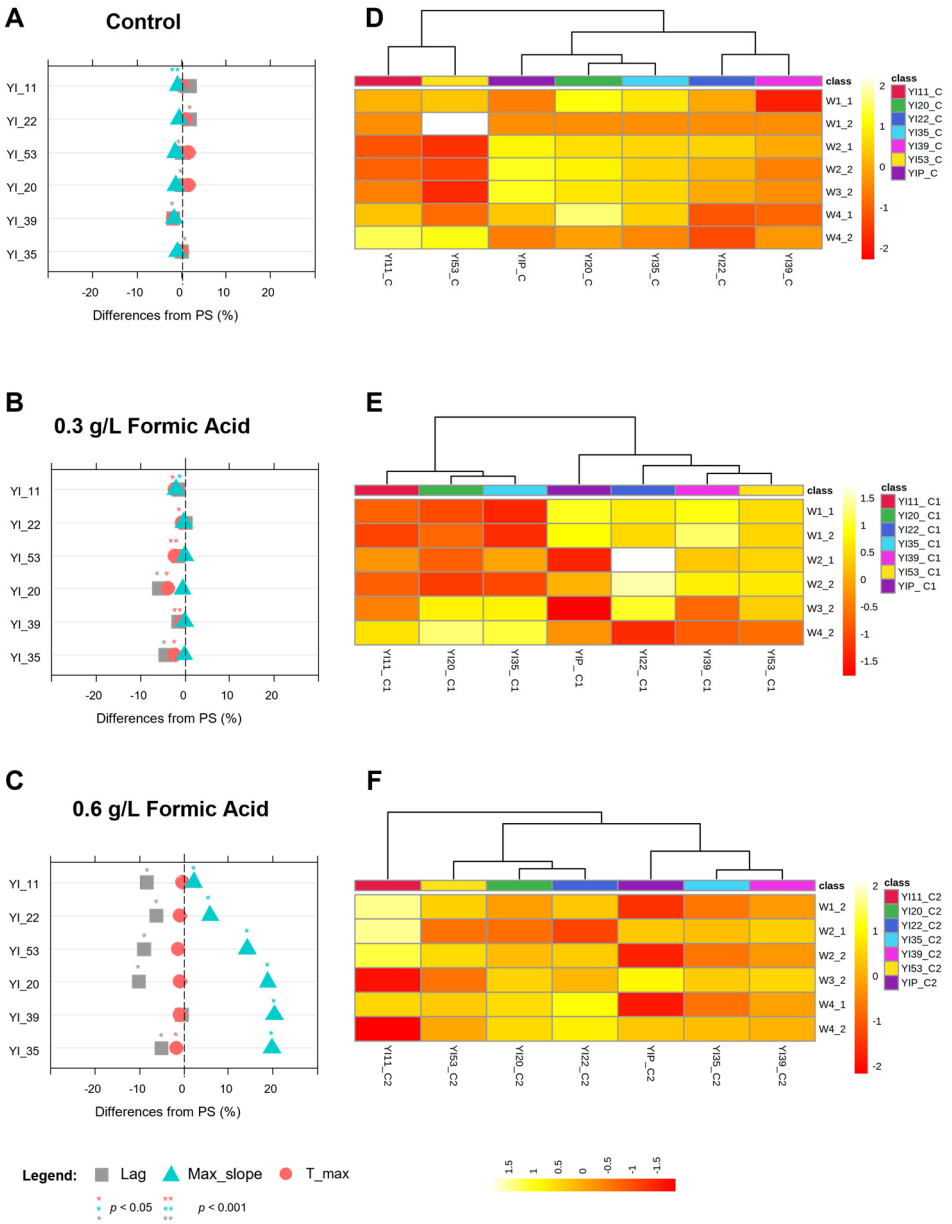

Figure 7. Growth and metabolomic phenotypes of YI30 *S. cerevisiae* parental strain and its derived MSCs during growth at increasing concentrations of formic acid (0.3 and 0.6 g L^{-1}). Panels (**A**–**C**): Lag time (grey square), max_slope (light blue triangle) and t_max (light red circle) parameters reported as percentage difference respect to the parental strain. Panels (**D**–**F**): Heatmap of the significantly altered FTIR peaks (distance measure using Euclidean, and clustering algorithm using ward.D). The coloured boxes indicate the relative intensities of the mean of peaks in the corresponding spectral region. The colour scale is log2 transformed value and indicates relatively high (yellow) and low (red) peak intensities. Spectral regions have been divided into sub-regions, namely: Fatty acids (W1_1 from 3200 to 3100 cm^{-1}–W1_2 from 3098 to 2801 cm^{-1}); Amides (W2_1 from 1800 to1649 cm^{-1}–W2_2 from 1647 to 1501 cm^{-1}); Mixed region (W3_1 from 1499 to 1352 cm^{-1}–W3_2 from 1350 to 1202 cm^{-1}); Carbohydrates (W4_1 from 1200 to 1053 cm^{-1}–W4_2 from 1051 to 902 cm^{-1}).

In reaction to this copper concentration, we observed an increase in the variability of metabolomic profiles, resulting in MSC-dependent signatures (Figure 5F). Overall, the metabolomic patterns of MSCs mirrored those of PS, depicting a general slowing of metabolism to the exclusion, in a few cases, of carbohydrates in the W4_2 region.

With respect to its progeny, *S. cerevisiae* YVGC13A reacted to copper's supplementation by inducing genes responsible for carbohydrates metabolism and protein biosynthesis as a general mechanism of stress response in this species (Figure 5E,F) [45,49]. Conversely, the higher intensities in fatty acids may be the result of higher reactive oxygen species (ROS) accumulation, already described as a specific *S. cerevisiae* response to stress conditions triggered by copper [50,51].

Generally, the sporulation of YVGC13A has produced a significant amount of variability, resulting in some improved phenotypes, both at rest and under stressful conditions. However, the MSCs that performed better in control broth were not the same ones that displayed increased tolerance to copper supplementation. These data supported the hypothesis that the extensive sporulation applied in this study increases the amount of available variability compared to those procedures implying sporulation under selection conditions.

3.4.2. Phenotypes under Glucose Stress

In the case of TC1517-derived progeny, the control in SD broth was useful to confirm and further investigate the differential behaviors of the selected MSCs both in terms of growth parameters (Figure 6A) and alteration of cell metabolomes (Figure 6D).

Only the TC_44 displayed growth parameters such as those of the parents. The other MSCs were able to grow faster than the parental strain, with significantly higher t_max and max_slope values (Figure 6A). Remarkably, the increase in the growth rate of TC_22 and TC_23 was linked to specific metabolomic changes induced by the up-regulation of fatty acids and amides bands (W1 and W2_1) coupled with the down-regulation of mixed and carbohydrates ones (W3_2 and W4) (Table S8, Figure S5). The fingerprints of these MSCs were reversed with respect to those of *S. cerevisiae* TC1517 (Figure 6D). The other four MSCs displayed lower changes and were mainly located in the W1 and W2_1 regions.

At increasing glucose concentrations, significant alterations were evident in both the growth and metabolomic phenotypes of the selected MSCs. In the presence of 25% glucose, TC_52 was the only MSC that significantly increased the growth rate (max_slope) compared to that of the parental, as already revealed in the control condition. No significant differences were detected for the other MSCs, except for TC_44 and TC_55, both affected by a significant increase in t-max values and a reduction in max slope values ($p < 0.05$), resulting in a reduced growth rate (Figure 6B).

Furthermore, once challenged with 25% glucose, the parental strain expressed metabolomic changes opposite those observed in the benchmark broth (Figure 6E). The increase in glucose concentration prompted higher intensities of fatty acid and protein bands (W1 and W2_1) together with a reduction of those in mixed and carbohydrate regions (W3_2 and W4). The same response was observed for TC_9 MSC, which, noteworthily, exhibited growth performances such as those of *S. cerevisiae* TC1517. On the contrary, TC_23, TC_52, and TC_55 MSCs had an antithetical regulation in these spectral regions. Additionally, the last two MSCs, sharing similar metabolomic footprints, showed opposite growth behaviors. Finally, the TC_44 exhibited band intensities around neutrality for all spectral regions.

The glucose supplementation up to 30% significantly affected the growth parameters ($p < 0.05$) and led to a general reduction of all MSCs' growth (Figure 6C). In TC_9, growth reduction was accompanied by a substantial alteration of cell metabolism with the down-regulation of fatty acids (W1) and proteins (W2) and the up-regulation of mixed (W3) and carbohydrate (W4) regions (Figure 6F). A similar response, though of lesser intensity, was displayed by TC_23 and TC_52 MSCs. The TC_22 and TC_55 clustered separately because of the opposite response to that of these three MSCs. At glucose 30%, TC_44 was the only MSC that displayed the same metabolomic alteration as the parental TC1517.

Overall, the sporulation of TC1517 has triggered a renewed level of variability that has impacted both the growth and metabolomic phenotypes of the six MSCs selected. In the absence of glucose stress, the growth phenotypes of most spores improved compared to the parental strain. Conversely, high glucose levels induced a general worsening of growth performance by reducing the growth rate of the MSCs. The only exception was the TC_52

culture, which maintained a growth rate higher than that of the parental cells even under 25% glucose.

Interestingly, TC_22 and TC_23 MSCs, which demonstrated the best performance under control conditions, reacted to glucose addition by significantly shaping their metabolism in the direction of a reduction of the lag phase, a typical response of strains with increased tolerance to a specific stressor [52,53].

The metabolomic fingerprint of these strains, despite having a dose-dependent pattern, showed a peculiarity in the constant clustering of the responses of W1 and W2 in opposition to those of W3 and W4, both under control and in stressed conditions. This evidence could be attributed mainly to the opposite regulation of genes involved in protein and carbohydrate metabolism. The fact that some stressing conditions induce carbohydrate metabolism genes by down-regulating those involved in protein biosynthesis has already been observed in *S. cerevisiae* [54]. It is well documented that *S. cerevisiae* cells accumulate some carbohydrates in response to different types of stress [45,49,55]. In the presence of 15 g L^{-1} of glucose, the production of intracellular glycerol and trehalose was found to be significantly increased [47]. Furthermore, glucose concentration has been reported to have a proportional effect on intracellular ROS, which increases intensity in the W1_1 region [56].

3.4.3. Phenotypes under Formic Acid Stress

When grown in control broth, most of the MSCs of *S. cerevisiae* YI30 were affected by a significant increase in the lag phase ($p < 0.05$) with respect to the parental strain (Figure 7A). The variability induced by sporulation also interested the max slope value in YI_11, which exhibited the worst phenotype together with YI_22.

Based on the metabolomic alterations (Table S9, Figure S6), MCSs were grouped into two main clusters (Figure 7D). The first included four MSCs, of which YI_20 and YI_35 are closest to the PS, whereas YI_22 and YI_39 are in a separate subcluster characterized by a general downregulation of bands in all spectral regions. The second group consisted of YI_11 and YI_53 MSC, which were separated from the PS mainly by reduced intensities in the amide bands and increased signals for carbohydrates.

The phenotypes described for growth in a resting condition significantly changed in response to formic acid, according to the increase in dose (Figure 7B,C). The presence of 0.3 g L^{-1} formic acid modified growth phenotypes except for YI_11, which maintained the same pattern displayed in the control broth (Figure 7B). No significant differences from the parental strain were detected for YI_22, YI_39, and YI_53, while YI_20 and YI_35 were faster thanks to the significant reduction in Lag and t_max ($p < 0.05$).

At the highest tested concentration (0.6 g L^{-1}), formic acid clearly prompted the growth of all MSCs by reducing the lag parameter with respect to the PS ($p < 0.05$), with the only exception of YI_39 MSC (Figure 7C).

The heatmaps of the significantly altered FTIR peaks (Figure 7E,F) showed that the differential ability to withstand formic acid was mediated by a fine tuning of carbohydrates, proteins, and fatty acid pathways. In addition, the improved performance of YI_20 and YI_35 in the presence of 0.3 g L^{-1} formic acid (Figure 7E), supported by the strong down-regulation in W1 and up-regulation in W3_2 and W4_2 regions, could be explained considering that *S. cerevisiae*, under some stressful conditions, induced genes involved in carbohydrate metabolism while down-regulating those involved in protein biosynthesis. In addition, the metabolomic pattern shown by YI_11 at higher concentrations (Figure 7F) suggests that other mechanisms may be involved in the response to formic acid stress. ROS, which are potentially responsible for providing tolerance to toxic formic acid, fall in the fatty acid region (W1_1). The higher intensity in W1_1 bands displayed by YI_11 can possibly be related to the higher accumulation of ROS [56]. This hypothesis is under investigation using a focused LC/MS approach.

Overall, the sporulation of YI30 resulted in MSCs with differential ability to withstand increasing concentrations of formic acid and was useful for the selection of a few candidates with promising phenotypes to be further studied to both shed light on the still poorly

investigated mechanism of formic acid tolerance in *S. cerevisiae* and to develop superior yeast strains with increased resistance to this weak acid [57,58]. Few MSCs, indeed, showed a lower lag phase, thus reacting much faster than the parental strains thanks to strong and strain-specific intracellular metabolomic reactions.

4. Conclusions

The main hypothesis of this paper is that the proposed non-GMO approach was efficient in renewing genetic variability through the extensive sporulation of three *S. cerevisiae* strains with different origins and backgrounds. The procedure involved an initial randomized sampling of the MSCs produced by the extensive sporulation of each strain, without any preliminary selection. In addition, a series of sequential steps focused on the analysis of growth performances and metabolomic reactions allowed the analysis to be restricted to six MSCs for each strain, screened at rest and under specific stress conditions. Overall, data confirmed that *i.* the genome renewal reintroduced a quote of variability, selectable following the approach presented in this study, *ii.* the extensive sporulation generates variability in both growth and metabolomic phenotypes; and *iii.* this variability depends on the starting parental strain, proving that the geographical location and ecological origin of yeast have a major signature on its phenotypic pattern. Although the ongoing whole genome sequencing of selected MSCs will clarify the nature and stability of this variability, this novel procedure looks very promising for renewing yeast genetic variability as a tool to obtain improved organisms with specific phenotypes and industrial fitness. Further, selected MSCs are indeed of great metabolomic interest towards the identification of molecules with deep impact on the yeast resistome against specific stressors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof9020264/s1, Table S1: Probability associated with the Student's *t*-test (*p*-value) of all MSC compared to the parental strain (YVGC13A); Table S2: The probability associated with the Student's *t*-test (*p*-value) of all MSC compared to the parental strain (TC1517); Table S3: Probability associated with the Student's *t*-test (*p*-value) of all MSC compared to the parental strain (YI30); Table S4: FTIR absorbance spectra of the YVGC13A strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose, Table S5: FTIR absorbance spectra of the TC1517 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose, Table S6: FTIR absorbance spectra of the YI30 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose, Table S7: FTIR absorbance spectra of theYVGC13A strain under copper sulphate supplementation; Table S8: FTIR absorbance spectra of the TC1517 strain under glucose supplementation; Table S9: FTIR absorbance spectra of YI30 strain under formic acid supplementation, Figure S1: FTIR absorbance spectra of YVGC13A strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose, Figure S2: FTIR absorbance spectra of TC1517 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose, Figure S3: FTIR absorbance spectra of YI30 strain recorded at the end of the exponential phase of growth in SD broth supplemented with 2% glucose, Figure S4: FTIR absorbance spectra of YVGC13A strain under copper sulphate supplementation, Figure S5: FTIR absorbance spectra of TC1517 strain under glucose supplementation, Figure S6: FTIR absorbance spectra of YI30 strain under formic acid supplementation.

Author Contributions: Conceptualization, L.F., L.C. and G.C.; methodology, L.F. and L.C.; software, A.P.G., D.C.P. and L.C.; formal analysis, A.P.G., D.C.P. and L.C.; investigation, A.P.G., D.C.P., A.C. and L.D.; resources, L.F., L.C., M.B., S.C. and G.C.; data curation, A.P.G. and D.C.P.; writing—original draft preparation, A.P.G., L.F. and L.C.; writing—review and editing, L.F., L.C. and G.C.; visualization, A.P.G., D.C.P. and L.C.; supervision, L.F. and L.C.; funding acquisition, L.F., L.C., M.B., S.C. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially funded by Padova University with the following research projects: DOR2297411/22, DOR2251254/22, and BIRD210708/21. A.P.G. is the recipient of a University of Padova PhD fellowship.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Willem van Zyl (Stellenbosch University) kindly provided *S. cerevisiae* YI30.

Conflicts of Interest: The authors declare no conflict of interest.

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