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Novel Yeast Strains for the Efficient Saccharification and Fermentation of Starchy By-Products to Bioethanol

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Abstract: The use of solid starchy waste streams to produce value-added products, such as fuel ethanol, is a priority for the global bio-based economy. Despite technological advances, bioethanol production from starch is still not economically competitive. Large cost-savings can be achieved through process integration (consolidated bioprocessing, CBP) and new amylolytic microbes that are able to directly convert starchy biomass into fuel in a single bioreactor. Firstly, CBP technology requires efficient fermenting yeast strains to be engineered for amylase(s) production. This study addressed the selection of superior yeast strains with high fermentative performances to be used as recipient for future CBP engineering of fungal amylases. Twenty-one newly isolated wild-type *Saccharomyces cerevisiae* strains were screened at 30 °C in a simultaneous saccharification and fermentation (SSF) set up using starchy substrates at high loading (20% w/v) and the commercial amylases cocktail STARGENT™ 002. The industrial yeast Ethanol Red™ was used as benchmark. A cluster of strains produced ethanol levels (up to 118 g/L) significantly higher than those of Ethanol Red™ (about 109 g/L). In particular, *S. cerevisiae* L20, selected for a scale-up process into a 1-L bioreactor, confirmed the outstanding performance over the industrial benchmark, producing nearly 101 g/L ethanol instead of 94 g/L. As a result, this strain can be a promising CBP host for heterologous expression of fungal amylases towards the design of novel and efficient starch-to-ethanol routes.

Keywords: ethanol; starchy waste; natural yeast strains; consolidated bioprocessing; broken rice

1. Introduction

In the near future, the non-renewable resources such as crude oil, coal and natural gases, that collectively account for about 82% of global energy needs [1], will no longer be viable. Therefore, the global communities are moving toward the search for reducing fossil oil dependence and long-term sustainable forms of energies such as biofuels [2–6].

Among liquid biofuels, bioethanol has emerged as a clean and eco-friendly fuel that could replace gasoline, both as pure ethanol in specially designed engines, or most widely as blends with fossil gasoline ranging from 5 to 20% (also referred to as E5 or E20) [1,7,8]. Although the energy equivalent of ethanol as fuel is 66% than petroleum, its combustion is 15% more efficient because of higher oxygen content and less exhaust emissions, such as sulfur and nitrogen oxides, are produced [9]. It has been estimated that the use of 10% ethanol blends could reduce greenhouse gasses emissions by 12–19% compared to conventional fossil fuel [10].

In Brazil and US, the leading producers, first generation bioethanol is obtained from sugarcane or corn starch, respectively. However, since bioethanol market is expanding further, a good strategy is to look for alternative feedstocks that do not compete with food supplies [11]. In this perspective, biomasses are accounted as the fourth largest source of energy on Earth [1,12] and it is expected that the only foreseeable primary source that could provide adequate fuel supplies for transportation sector is waste biomass [13]. Before being processed to any added-valued compounds, solid waste streams have to be carefully managed through the promotion of safe practices and effective technologies, such as source separation, biological treatment and supply chain development to ensure the overall economic feasibility of the process. A wide variety of waste organic materials, like residues from agricultural, forest and industrial processing as well as the organic fraction of municipal solid waste, actually contains considerable amounts of fermentable glucose, as monomers or polymers (i.e., starch, cellulose), that have been used for biofuels, biopolymers and enzymes production [14–20].

Lignocellulosic biomasses are the most promising raw materials considering their great availability and limited cost [11,21,22]. Along with lignocellulose, starchy waste streams with high starch content could be exploited for bioethanol production [23–27]. Indeed, starch is the most abundant form of energy storage in plants. The structure can vary regarding the botanical species (starch content can range from 50 to 90% in cereals, tubers and roots) and represents a high-yielding ethanol resource [2,9,28].

The current industrial process of starch conversion into bioethanol, involves four steps: (I) liquefaction at high temperatures (80–100 °C), (II) saccharification into glucose by thermostable α -amylases and glucoamylases, (III) microbial fermentation to ethanol and (IV) alcohol distillation and dehydration [29]. However, the global-scale application is hampered by high cost processing. The feedstock (corn) is considered as the main share (60%) followed by processing expenditures (10–20%). To ensure the economic feasibility of the overall process, the substitution of corn with cheaper biomasses together with improved process integrations have been proposed [9,30]. The total cost expenditure to produce bioethanol from starch could be reduced by consolidated bioprocessing (CBP) configuration and by using a single microorganism able to both secrete hydrolytic enzymes and ferment the resulting sugars in a single reactor [31,32]. The advantages include the direct utilization of raw starch without pre-treatment steps, eliminating the cost for energy inputs, exogeneous enzymes, maximizing the ethanol yield and minimizing by-product formation [33].

To date, no natural microorganism is available to perform CBP. Genome engineering, such as heterologous gene expression, is fundamental to create a new microbial biocatalyst to be used at industrial scale for the starch-to-ethanol processing [31]. In *S. cerevisiae*, heterologous gene expression is well established. Several amylase genes have been expressed for starch conversion, mainly in laboratory strains and, lately, also in natural and industrial strains [34–37]. However, the conversion rate of raw feedstocks or high substrate loading was not relevant for industrial scale. Even though good progresses have been done, the major challenge is the co-production of both amylases and glucoamylases at optimal levels to achieve high volumetric activities. The effective conversion of raw starch in a short timeframe could be competitive only if the fermentation abilities of the strain are not affected in terms of ethanol yield and starch utilization [32,35,38,39].

This study looked for novel *S. cerevisiae* strains with superior fermenting abilities to be used as host strains for heterologous expression of novel fungal hydrolytic enzymes with the final aim of developing efficient CBP yeast. A collection of wild-type strains, newly isolated from a winery, has been evaluated for fermentative performances at 30 °C under SSF regime of high substrate loading (20% w/v) of starchy materials. A commercial mix of α -amylase and glucoamylase, STARGEN™ 002, was used at optimized doses to perform starch hydrolysis. The *S. cerevisiae* Ethanol Red™ strain, currently applied in first and second generation bioethanol plants, served as benchmark [37].

Broken rice, where starch accounts up to 87.5% of dry matter, has been adopted as a model of several agricultural and industrial wastes mainly composed by starch [9]. It is one of the most abundant waste streams of rice processing with more than 45 million tons globally produced per

year [40]. Such enormous amount of cheap biomass would be directly accessible and/or collectable at rice processing sites [24]. Raw corn starch (Sigma-Aldrich, Milano, Italy) was used as control feedstock.

2. Results and Discussion

2.1. Isolation, Genetic Characterization and Fermentative Abilities of Novel Yeast Strains in Glucose

With the final aim of isolating and selecting strains with high fermenting performances from glucose, the winery background was chosen since it is related to ethanol production and yeast are expected to produce and tolerate high alcohol concentrations. Yeast strains were isolated from grape marcs and incubated on Wallerstein Laboratory (WL) plates at 30 °C. Twenty-one isolates were classified as *S. cerevisiae* and first screened for their ability to consume glucose at 30 °C in must nutritive synthetic (MNS) minimal medium supplemented with 200 g/L glucose, as such concentration is typical of the first generation ethanol plants after saccharification of corn [38].

Due to their relatively different phenotypic backgrounds, seven control strains of *S. cerevisiae* were included in this research as benchmarks (Table 1). Three of these reference strains, namely *S. cerevisiae* Fm17, M2n and MEL2, have been already exploited for ethanol production from different lignocellulosic and starchy substrates [18,32,41,42], whereas three additional strains (*S. cerevisiae* HR4, WL3, YI30), recently characterized for their inhibitors tolerance and fermenting abilities [43], were included as additional controls. Finally, *S. cerevisiae* Ethanol Red™ was considered as industrial reference strain, widely applied in both first and second generation ethanol applications [37].

Table 1. *S. cerevisiae* strains used in this study.

<i>S. cerevisiae</i> Strains	Relevant Phenotype and Origin	Source/Reference
L1–L21	wild-type	This study
Ethanol Red™	industrial strain	Fermentis
Fm17	wild-type strain with high lignocellulosic inhibitors tolerance	[41]
M2n	distillery strain	[32]
MEL2	wild type strain from grape marcs	[42]
HR4	wild-type strain from wine fermentations	[43]
WL3	wild-type strain from wine fermentations	[43]
YI30	wild-type strain from wine fermentations	[43]

In this project, the ability of the yeast to utilize glucose was determined as fermenting vigour and expressed in terms of grams of glucose consumed per L of MNS medium, as described in Materials and Methods. Overall, both newly selected isolates and control strains exhibited high and comparable level of fermenting vigour (Figure 1). The newly isolated strains *S. cerevisiae* L1, L2, L5, L7, L8, L9 and L21, were not included as their fermenting abilities were lower than those of the benchmark strain M2n, displaying the bottom fermenting vigour.

Once incubated in 200 g/L glucose, few isolates quickly consumed all the sugar within 15 days (Figure 1). Among them, *S. cerevisiae* L6, L14, L15, L16 and L20 demonstrated outstanding fermentative vigour. Interestingly, their ability outperformed those reported for *S. cerevisiae* MEL2 and *S. cerevisiae* Ethanol Red™, which showed the most favorable vigour among the reference yeast strains. Moreover, the selected newly isolated strains disclosed fermentative efficiency higher than those recently described from high glucose concentrations by several *S. cerevisiae* yeast [42,44,45]. As such, the fermenting capabilities of the novel yeast strains are promising considering also the medium adopted to screen for fermenting vigour. MNS broth, when compared with the formulation of other commonly used defined media [46,47], has indeed the lowest levels of components, macro and micro-nutrients. Therefore, yeast able to grow rapidly and efficiently ferment under nutrient limitation should be considered very interesting for bioethanol industrial scale applications. To further assess their aptitude to ferment starchy substrates under SSF configurations, representative of starch-to-ethanol processes, the novel collection of yeast has been adopted to produce ethanol from broken rice and raw corn starch.

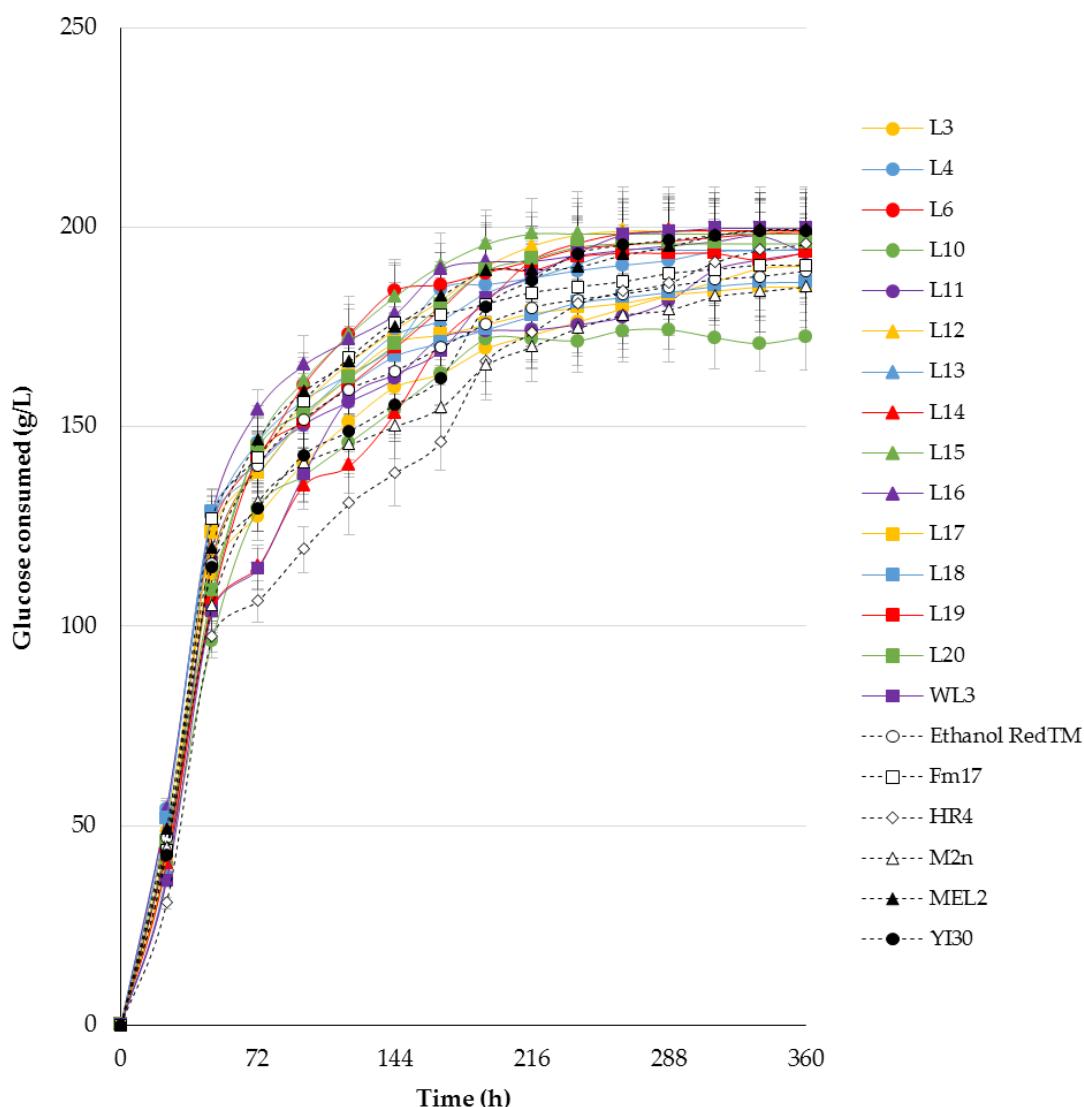


Figure 1. Cumulative sugar utilization (g/L) of *S. cerevisiae* strains in MNS medium with 200 g/L glucose. The experiment was conducted in triplicate (\pm SD).

2.2. Fermentative Abilities under SSF Setting on Starchy Materials

The fermentative vigour of all the strains was then evaluated on broken rice and raw corn starch at high substrate loading (20% w/v) under oxygen-limited conditions at 30 °C. The commercial mixture of amylases STARGEN™ 002 was supplied to support starch saccharification. Based on the assumptions that one gram of starch is equivalent to 1.11 g of glucose [48], the final theoretical glucose concentration achievable by complete saccharification of 20% w/v of starchy materials was 194 and 235 g/L of YNB containing 20% w/v of broken rice and corn starch, respectively.

Owing to the great volume of data generated, total cumulative glucose consumption (Figure 2) and fermentation parameters (Table 2) are reported only for the best ten fermenting strains (namely L11, L12, L13, L15, L16, L17, L18, L19 and L20) as well as for the top performing benchmark yeast *S. cerevisiae* WL3 and Ethanol Red™.

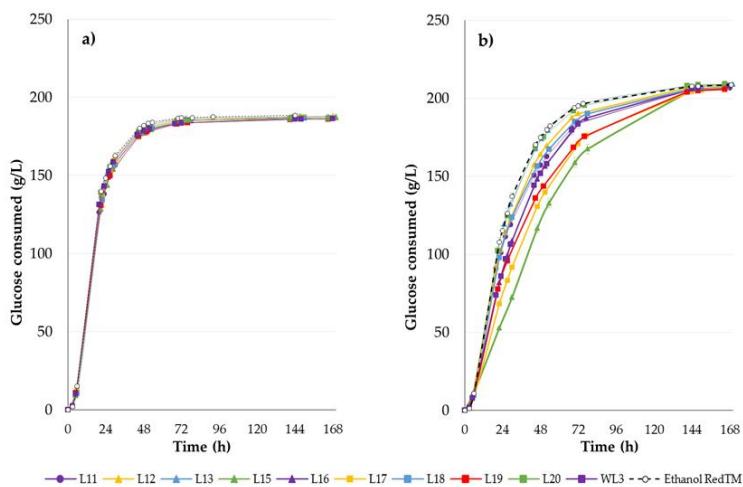


Figure 2. Cumulative sugar utilization (g/L) of *S. cerevisiae* strains at 30 °C in YNB medium with 20% w/v (a) broken rice or (b) raw corn starch in the presence of STARGEN™ 002. The experiment was conducted in triplicate (\pm SD).

In the presence of broken rice, all the selected strains performed comparably and only slight differences in fermenting vigour could be appreciated between the newly isolated and the benchmarks (Figure 1a). The highest glucose consumption on broken rice was approximately 97% of the theoretical content (188 g for L18 and L20 out of 194 g/L). Furthermore, broken rice was confirmed to be a promising material for ethanol production [24] with more than 100 g/L of ethanol produced (Table 2). Nevertheless, this substrate was not suitable for strain selection, since no significant differences, at least in terms of fermenting vigour, were evident among the tested strains. This could be ascribed to the native high amount of simple sugars (mainly glucose, fructose and sucrose) and proteins (8.5% DM) of broken rice (see Materials and Methods), which could have enhanced and supported the fermenting activities.

On the contrary, raw corn starch, with very limited values of protein (0.3% DM, see Table 4) and no free sugars available, demonstrated to be a useful feedstock to screen for the most promising yeast strains. As reported in Figure 1b, indeed, the ten selected novel yeast strains showed comparable but different levels of fermenting vigour. Overall, only about 85% of glucose theoretically obtainable by saccharification of raw corn starch was used (200 g of glucose out of 235 g/L). Noteworthy, a small number of strains, including L13 (up to 208.9 g/L), L20 (up to 206.5 g/L) and WL3 (up to 207.9 g/L), performed very similarly to Ethanol Red™, which clearly reaffirmed its great ability to consume glucose (208.7 g/L) (Figure 2b). The strains demonstrating a glucose consumption significantly lower than Ethanol Red™ were not considered as proficient fermenting yeast.

The ethanol yield, glucose and glycerol content of SSF experiments after 120 h are reported in Table 2. Only minimal amounts of residual glucose were observed in few spent fermentation broths (Table 2), indicating that the majority of the strains were able to completely utilize the sugar. The cluster of selected strains produced ethanol levels significantly greater than Ethanol Red™ from both broken rice and raw corn starch. This means that the industrial yeast *S. cerevisiae* Ethanol Red™, although presenting the fastest glucose consumption rates (Figure 1), was outperformed in terms of ethanol yield by all the selected novel strains as well as by the benchmark *S. cerevisiae* WL3, recently reported for promising fermenting performances [43]. Glycerol was detected as a common secondary fermentation product. According to what is reported for vinification, the concentrations of glycerol were found to be nearly 10 times smaller than the ethanol produced [49]. To increase ethanol production as biofuel, it is fundamental to minimize glycerol formation to better redirect the carbon flux towards ethanol. Interestingly, all newly isolated strains together with the reference *S. cerevisiae* WL3 produced glycerol at comparable levels from both broken rice and raw corn starch confirming their great ability to rapidly convert glucose into ethanol. On the contrary *S. cerevisiae* Ethanol Red™ showed the highest glycerol

production when SSF was conducted on raw starch and high glycerol levels comparatively to ethanol produced also from broken rice (Table 2).

Table 2. Ethanol, glucose and glycerol content of SSF experiment at 30 °C using as substrate broken rice or raw corn starch. The substrate loading for each experiment was 20% (w/v). The experiment was conducted in triplicate (\pm SD). $Y_{E/S}$, % of theoretical maximum ethanol yield per gram of glucose equivalent available.

Strain	Broken Rice				Raw Corn Starch			
	Residual Glucose (g/L)	Glycerol (g/L)	Ethanol Concentration (g/L)	$Y_{E/S}$	Residual Glucose (g/L)	Glycerol (g/L)	Ethanol Concentration (g/L)	$Y_{E/S}$
Ethanol Red™	-	8.83 ± 0.12	101.05 ± 0.54	91	0.30 ± 0.06	10.05 ± 0.17	109.36 ± 0.33	86
L11	-	8.75 ± 0.02	107.70 ± 0.44	97	1.68 ± 0.28	9.19 ± 0.20	116.07 ± 0.06	91
L12	0.58 ± 0.14	9.03 ± 0.03	108.39 ± 1.22	98	0.82 ± 0.65	9.21 ± 0.19	116.22 ± 1.97	91
L13	0.62 ± 0.01	9.45 ± 0.18	107.15 ± 0.28	97	1.55 ± 0.76	9.63 ± 0.04	116.12 ± 0.96	91
L15	-	8.90 ± 0.06	107.43 ± 0.16	97	-	8.24 ± 0.08	117.17 ± 0.08	92
L16	-	8.93 ± 0.04	107.77 ± 0.21	97	-	9.25 ± 0.15	117.93 ± 0.14	92
L17	-	8.36 ± 0.02	107.16 ± 0.66	97	-	8.53 ± 0.01	116.78 ± 0.42	92
L18	-	7.79 ± 0.05	106.73 ± 0.34	96	-	8.45 ± 0.09	117.35 ± 1.06	92
L19	-	8.25 ± 0.08	107.32 ± 0.25	97	-	8.60 ± 0.11	116.44 ± 0.21	91
L20	-	8.17 ± 0.14	107.19 ± 0.15	97	-	7.97 ± 0.09	116.98 ± 1.73	92
WL3	0.30 ± 0.01	9.16 ± 0.12	106.17 ± 0.36	96	0.29 ± 0.04	9.60 ± 0.07	115.05 ± 0.24	90

Taken together the experiments on fermenting vigour from glucose (Figure 1) and SSF configurations with broken rice and raw starch as substrates (Figure 2 and Table 2) indicated that the newly isolated strains have great potential as ethanol producers, with performances even higher than those exhibited by several benchmark yeast strains. More specifically, *S. cerevisiae* L20 displayed one of the most outperforming phenotypes, especially in the early stage of fermentation, and was then selected for further fermentation experiments at bioreactor scale.

2.3. Scale-up in 1-L Bench Fermenter

S. cerevisiae L20 together with the reference Ethanol Red™ have been investigated for SSF in 1-L bench reactor using 20 % w/v of either broken rice or raw corn starch (Figure 3). When broken rice was used as feedstock, the fermentation trend was similar for both strains (Figure 3). Interestingly, the strains produced comparable ethanol levels (about 86 g/L) after 72 h of fermentation. Nevertheless, the novel strain displayed ethanol performances always better than those of Ethanol Red™, particularly after 24 h of SSF (Table 3), with a maximum productivity of 3.10 g/L/h, which was 1.10-fold than that of the industrial benchmark (2.83 g/L/h). Such ethanol productivity values are of great interest and potential application [39]. Moreover, final ethanol yields were also higher, with the novel strain displaying 86% of the maximum theoretical instead of 83% detected for *S. cerevisiae* Ethanol Red™.

On raw starch, *S. cerevisiae* L20 confirmed to be a promising strain (Figure 3a and Table 3), with almost 101 g/L ethanol produced within 72 h of incubation. On the contrary, as reported in Figure 3b and Table 3, the industrial yeast produced lower alcohol values (up to 94 g/L). Fermenting parameters were again much better for *S. cerevisiae* L20 (Table 3), with a maximum productivity of 3.20 g/L/h, which was 1.33-fold higher than that of the industrial benchmark (2.41 g/L/h). Ethanol yields and carbon conversion values confirmed that the novel strain outperformed the industrial yeast further supporting both the saccharification of starch to glucose and then glucose-to-ethanol fermentation.

Overall, as reported in Table 3, ethanol levels and efficiencies obtained by the yeast strains from both substrates were found to be lower than those detected at smaller scale (Table 2). This finding could be due to an increase of viscosity of the medium which was found to limit the ethanol yield in up-scaling of high gravity SSF experiments on sweet potato [50] or to an increase of stress exposure linked to limited transportation and elimination of CO₂, toxic metabolites and additional heat generated by agitation [51]. This calls for further experimental activities in order to optimize the scaling up of the

process and is in agreement with lower ethanol yields recently obtained up-scaling the simultaneous liquefaction, saccharification and fermentation (SLSF) of broken rice at high gravity [52].

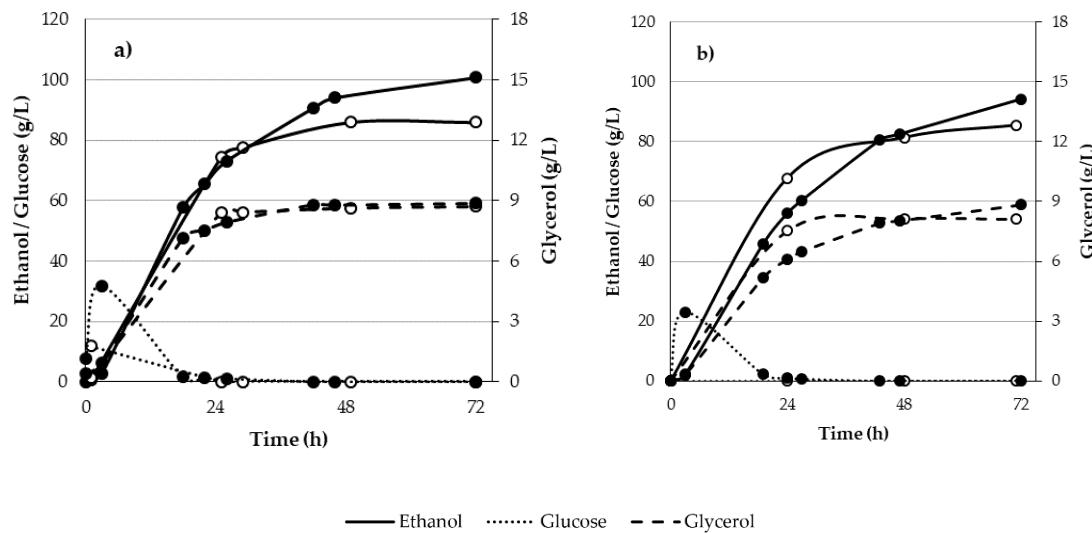


Figure 3. Ethanol, glucose and glycerol production by *S. cerevisiae* wild-type strains (a) L20 and (b) Ethanol Red™ during SSF in 1-L bioreactor with 20% (w/v) of broken rice (○) and raw corn starch (●). The experiment was conducted in duplicate and average values are presented.

Table 3. Conversion of starchy substrates at bioreactor level to ethanol and by-products by *S. cerevisiae* L20 and Ethanol Red™ strains.

Feedstock	<i>S. cerevisiae</i> L20		<i>S. cerevisiae</i> Ethanol Red™	
Broken rice = a glucose equivalent of 198.55 g/L and a total carbon available (mol C) of 6.62				
Product (g/L)	24 h	72 h	24 h	72 h
Glucose	nd	nd	nd	nd
Glycerol	8.40	8.70	7.54	8.12
Ethanol	74.44	87.01	67.86	85.46
CO ₂	71.20	83.23	64.91	81.74
Total carbon	5.13	5.96	4.67	5.84
Carbon conversion (mol C)	77%	90%	71%	88%
$Y_{E/S}$ (% of theoretical)	73%	86%	67%	83%
Q (g/L/h)	3.10	1.21	2.83	1.19
Q_{max} (g/L/h)	3.10 after 24 h		2.83 after 24 h	
Raw corn starch = a glucose equivalent of 238.82 g/L and a total carbon available (mol C) of 7.93				
Product (g/L)	24 h	72 h	24 h	72 h
Glucose	1.04	nd	1.14	nd
Glycerol	7.92	8.86	6.10	8.84
Ethanol	73.10	100.84	56.02	94.20
CO ₂	69.92	96.46	53.58	90.10
Total carbon	5.06	6.87	3.89	6.43
Carbon conversion (mol C)	64%	86%	49%	81%
$Y_{E/S}$ (% of theoretical)	60%	83%	46%	77%
Q (g/L/h)	3.05	1.40	2.33	1.31
Q_{max} (g/L/h)	3.20 after 18 h		2.41 after 18 h	

nd: not detected; $Y_{E/S}$, theoretical maximum ethanol yield per gram of glucose equivalent available.

3. Materials and Methods

3.1. Feedstocks and Commercial Enzymes

Broken rice was obtained from La Pila (Isola della Scala, Verona, Italy), dried in a forced-air oven at 60 °C for 48 h and milled in a hammer mill to pass throughout a 1.25 mm screen. The raw material was stored at room temperature. Starch from corn (Sigma-Aldrich) was used as benchmark substrate for SSF. No pre-treatments were performed, and raw feedstocks were used as such. The composition in terms of starch and protein, determined according to international standard methods [53], is reported in Table 4. The content of fermentable sugars in broken rice, namely glucose, fructose and sucrose, were assessed at 11.5 g/Kg on a total of sugars of 13 g/Kg.

Table 4. Composition of feedstocks used in this study.

Feedstock	Dry Matter, DM (%)	Protein (% DM)	Starch (% DM)
Broken rice	96.0	8.5	84.0
Raw corn starch	90.3	0.3	95.3

The enzyme mix STARGEN™ 002, kindly supplied by Genencor (DuPont-Danisco group, Itasca, IL, USA), is an optimized blend of *Aspergillus kawachii* α -amylase expressed in *Thricoderma reesei* and glucoamylase from *T. reesei* that works synergistically to hydrolyze granular starch to glucose. The enzymatic activity is 570 Glucoamylase Unit (GAU)/g and specific gravity is 1.14 g/mL. STARGEN™ 002 was used following the supplier's instructions (<http://www.dupont.com/content/dam/dupont/products-and-services/industrial-biotechnology/documents/DuPont-STARGEN002-web-EN.pdf>).

3.2. Yeast Strains, Isolation and Genetic Identification

A collection of twenty-one wild-type *S. cerevisiae* strains was isolated in 2013 from grape marcs of a winery in Melara (Rovigo, Italy) (Table 1). After a storage of 30 days at the winery, fifty grams of marcs were dispersed in 500 mL of sterile physiological water (0.85% NaCl), plated, after appropriate decimal dilutions, on WL medium (Wallerstein Laboratory, Oxoid, Milano, Italy; g/L: yeast extract, 4; casein hydrolysate, 5; D-glucose, 50; KH₂PO₄, 0.55; KCl, 0.425; CaCl₂, 0.125; MgSO₄, 0.125; FeCl₃, 0.0025; MnSO₄, 0.0025; bromocresol green, 0.022) containing 200 µg/mL chloramphenicol (Sigma-Aldrich, Italy) to contain bacterial growth and incubated at 30 °C for 72 h. After isolation, yeast colonies were purified by growing on Yeast Peptone Dextrose (YPD; g/L: yeast extract, 10; peptone, 20; glucose, 20) at 30°C for 48 h. Isolates were maintained at –80 °C in YPD containing 20% (v/v) glycerol.

Genetic identification was achieved by D1/D2 region sequence analysis. Amplification of D1/D2 domain was performed using primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') according to the protocol described by Kurtzman and Robnett [54]. Amplification products were checked by 1% agarose gel electrophoresis and then subjected to sequencing. Species identification was performed after BlastN alignment (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the obtained sequences with those present in the Gen-Bank public database. A minimum sequence similarity level of 98% was considered for species identification.

3.3. Fermentative Abilities of *S. cerevisiae* Strains in MNS Broth Supplemented with 200 g/L Glucose

S. cerevisiae strains were assessed for their fermentative ability in MNS minimal medium (g/L: (NH₄)₂SO₄, 0.3; (NH₄)₂HPO₄, 0.3; KH₂PO₄, 1; MgSO₄·7H₂O, 0.5; NaCl, 0.1; malic acid, 2; tartaric acid, 3. mg/L: biotin, 0.02; D-pantothenic acid, 0.4; myo-inositol, 2; nicotinic acid, 0.4; thiamine, 0.4; pyridoxine, 0.4; p-aminobenzoic acid, 0.2; H₃BO₃, 0.5; CuSO₄·5H₂O, 0.04; KI, 0.1; NaMoO₄·2H₂O, 0.2; ZnSO₄·7H₂O, 0.4; FeCl₃·6H₂O, 0.4; CaCl₂·2H₂O, 100) supplemented with 200 g/L glucose according to the method described by Favaro and colleagues [41]. MNS broth was specifically adopted as

it can be considered quite similar to several poor industrial media [47,55] and can resemble the pre-industrial scale composition of bioethanol broth, where mainly $MgSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$ and little amounts of corn steep liquor are generally added during the fermentation step [56,57]. The commercial strain Ethanol RedTM (Fermentis, Marcq-en-Baroeul, France), currently used for large scale bioethanol fermentation, was included as industrial benchmark together with other yeast strains recently reported for their high promise as bioethanol producers (Table 1).

In short, every glass serum bottle was filled with 100 mL of MNS medium and then sealed using rubber stoppers with a needle for the removal of CO_2 produced during fermentation. Pre-cultures of *S. cerevisiae* strains, grown overnight into YPD broth, were collected, centrifuged and washed twice with sterile physiological water (0.85% NaCl). Yeast cells were then inoculated, with an average cell concentration of 7.5×10^4 cells per mL, into each serum bottle containing 100 mL MNS broth. The incubation was performed in static condition at 30 °C. The pH of medium was set at 3.5 using KOH (5 M). Fermentative vigour was daily monitored by measuring weight loss due to CO_2 production. Results were reported as grams of glucose utilized per L of MNS by using a conversion factor of 2.118 [55]. The experiments were carried out in triplicate.

3.4. Fermentative Abilities of *S. cerevisiae* Strains on Starchy Materials

All strains were screened for fermentative abilities in an SSF regime from broken rice and raw corn starch. Pre-cultures were prepared in YNB broth (with amino acids: Yeast Nitrogen Base 6.7 g/L; Sigma-Aldrich) with 2% w/v of glucose in Erlenmeyer flasks and incubated overnight at 30 °C on a rotatory shaker at 600 rpm. Small-scale SSFs were conducted in 120-mL serum bottles with 20% (w/v) dry substrate and appropriate nitrogen source (YNB with amino acids: 6.7 g/L; Sigma-Aldrich) in a total volume of 100 mL of buffered medium (citrate buffer 0.1 M at pH 4). Yeast cells were inoculated at Optical Density (OD) value of 1 and ampicillin (100 mg/L) and streptomycin (75 mg/L) were added to prevent bacterial contamination. STARGENTM 002 was then supplemented at ten times the recommended dosage (11.4 g/kg of substrate). Rubber stoppers were used to set up oxygen-limited conditions and a needle was inserted for CO_2 removal. Serum-bottles were incubated at 30 °C on magnetic stirrer with agitation speed at 700 rpm. The experiments were carried out in triplicate.

The fermentative vigour was daily monitored as described above. Results were reported as grams of glucose utilized per liter of medium by a conversion factor of 2.118 [55]. Samples were withdrawn after 5 days, filtered through 0.22-μm and analyzed for their content by high performance liquid chromatography (HPLC) as indicated in ‘Analytical methods and calculations’.

3.5. Scale-up of SSF in 1-L Bench Fermenter

The most promising strain, exhibiting an outstanding fermentative performance at small scale SSF, together with the benchmark industrial yeast Ethanol RedTM, was up-scaled in a 1-L bioreactor (Applikon Biotechnology, Schiedam, The Netherlands) with a working volume of 900 mL. The BioXpert software version 1.13 (Applikon Biotechnology) was used for data acquisition.

The broth was the same used for small-scale SSF except for pH, which was controlled at 4.0 using automatic titration of 1 M NaOH solution. To maintain oxygen-limited conditions, aeration was not supplied. Yeast cells were inoculated at OD value of 1 and the cultures were stirred at 300 rpm and maintained at 30 °C with a heating blanket. All parameters were controlled by my-Control unit (Applikon Biotechnology). Samples were aseptically collected at regular intervals and kept at –20 °C. Samples were filtered through 0.22-μm and diluted for HPLC analysis performed as described below.

3.6. Analytical Methods and Calculations

Samples from small scale fermentation and bioreactor experiments were analysed for their content in glucose, glycerol and ethanol trough liquid chromatography using a Shimadzu Nexera HPLC system, equipped with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). The chromatographic separations were performed using a Rezex ROA-Organic Acid H+ (8%) column (300 mm × 7.8 mm,

Phenomenex, Torrance, CA, USA). 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 2.5 mM H₂SO₄ as a mobile phase [58].

The ethanol yield, $Y_{E/S}$, (g of ethanol/g of utilized glucose equivalent) was determined considering the amount of glucose equivalent available and compared to the maximum theoretical yield of 0.51 g of ethanol/g of consumed glucose equivalent.

The volumetric productivity (Q) was intended as grams of ethanol per liter of culture per hour (g/L/h) and the maximum volumetric productivity (Q_{max}) was defined as the highest volumetric productivity displayed by the *S. cerevisiae* strains. The theoretical CO₂ yields were determined based on the ethanol produced by each yeast strain, assuming that equimolar ethanol and CO₂ are produced. The percentage of carbon converted to glucose, ethanol, glycerol, and CO₂ was calculated on a mole carbon basis.

Statistical analyses were assessed using the Graphpad Prism 5 package (Graphpad Software, Inc., San Diego, CA, USA). Descriptive statistics mean values and standard deviations were calculated. Data were analysed also by two ways factorial Analysis of Variance (ANOVA) with Duncan test.

4. Conclusions

Developing an amyloytic fermentative organism may overcome important limitations of starch-to-ethanol conversion. The production of alfa-amylases and glucoamylases at high titers still remains a major challenge, despite significant technological advances. By producing efficient amyloytic enzymes, the engineered yeast could reduce bioethanol costs and implement the large-scale biofuel production. Nonetheless, the fermenting yeast must have promising fermenting abilities under SSF configurations.

This paper was successful in selecting and characterizing a cluster of novel yeast strains with fermenting abilities even higher than those of *S. cerevisiae* Ethanol Red™, the most used yeast for first and second generation ethanol. Ethanol yields from glucose under simple fermentation and SSF settings were significantly improved in the case of the newly isolated strains. This finding is of great value considering that, to obtain great additional profits, first generation ethanol plants look for an increase of even 1% in ethanol yield.

Based on the fermentation studies, the collection of novel *S. cerevisiae* strains has great potential for future application in corn-to-ethanol processes. In particular, the selected yeast L20 could be considered as promising for the CBP of different starchy industrial residues and will be engineered for the expression of efficient amyloytic genes.

Author Contributions: N.G. participated in planning of the study, carried out the SSF fermentation experiments, participated in data analysis and interpretation, and drafted the manuscript. L.F. planned the study and the experimental design, carried out the yeast isolation, identification and fermenting vigour characterization, performed data analysis, participated in data interpretation, and revised the manuscript. L.C. participated in the planning of the study and SSF fermentation experiments, composed the graphical abstract, and commented on the manuscript. S.B. participated in fermenting vigour experiments. V.P. participated in SSF fermentation experiments. M.B. participated in data interpretation, and commented on the manuscript. S.C. participated in data interpretation, and commented on the manuscript. All authors read and approved the final manuscript.

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