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Selection and genetic improvement of microorganisms for second generation bioethanol.

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

1st February 2010 Lorenzo Favaro

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to Marta

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LIST OF ABBREVIATIONS.

AFEX	Ammonia fiber explosion
ANOVA	ANalysis Of VAriance
ARDRA	Amplified Ribosomal DNA-Restriction Analysis
ARS	Autonomous Replicating Sequence
ATCC	American Type Culture Collection
BGU	β-Glucosidase Units
CBP	Consolidated BioProcessing
CbU	Cellobiase Units
CellA	Cellulolytic activity
CMC	Carboxymethyl-cellulose
DDGS	Distillers Dried Grains and Solubles
DNS	Dinitro Salicylic Acid
DP	Degree of Polymerisation
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DM	Dry matter
DMM	Defined mineral medium
DW	Dry weight
E10	10% ethanol blend with petroleum
E85	85% ethanol blend with petroleum
ETBE	Ethyl Tertiary Butyl Ether
EU	European Union
FPU	Filter Paper Units
FXU	Fungal Xylanase Units
GHG	Greenhouse Gas
HMF	5-hydroxymethyl-2-furaldehyde
HPLC	High Performance Liquid Chromatography
KNU-S	α-amylase Units
LHW	Liquid Hot Water
LipA	Lipolytic activity

MSW	Municipal Solid Wastes
MW	Molecular weight
MWB	Milled wheat bran
NREL	National Renewable Energy Laboratory
OECD	Organisation for Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PectA	Pectinolytic activity
PrA	Proteolytic activity
RSH	Raw starch hydrolysing
RWB	Raw wheat bran
SD	Standard deviation
SHF	Separated Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and CoFermentation
SSF	Simultaneous Saccharification and Fermentation
StA	Starch-degrading activity
UPGMA	Unweighted Pair Group Method by using Arithmetic Average
USA	United States of America
WB	Wheat bran
XylA	Xylan-degrading activity

ABSTRACT.

Bioethanol produced from biomass is considered an interesting second generation biofuel. To date a cost-effective method for converting biomass into ethanol has not been developed. Consolidated BioProcessing is one of the most attractive strategies aiming to obtain ethanol from biomass by a single microbial phase.

In this study, the selection and genetic improvement of microbial strains started in order to develop a microbe for the one-step bioconversion of biomass into ethanol.

New efficient cellulolytic microrganisms were isolated and genetically identified. Their hydrolytic activities were remarkable and few strains may have also improvable ethanol production properties.

Four hundred wild type yeasts, having optimal fermentative performance, were evaluated for their extracellular enzymatic activities. The yeasts showed interesting hydrolytic activity on pectin, cellulose and starch. In particular, one non-*Saccharomyces* strain produced efficient cellulolytic enzymes and thirteen *S. cerevisiae* strains, able to use starch as the sole carbon source, were selected. Extensive biochemical, physiological and genetic studies on their potentially amylolytic enzyme(s) were performed to look into this possible new starch-hydrolytic mechanism.

In addition, wheat bran was used, as a model substrate of starchy and cellulosic residues, to design a Separated Hydrolysis and Fermentation (SHF) system aiming at low-cost pre-treatments and high yields. The downstream fermentation, carried out with two *Saccharomyces* sp. yeasts, resulted in interesting ethanol yields.

A metabolic engineering program was conducted in order to obtain an efficient amylolytic yeast for large scale ethanol production. Wild type *S. cerevisiae* strains with selected industrial traits were engineered to express a fungal codon-optimised glucoamylase. The stable recombinants produced ethanol from soluble and raw starch and could be considered promising for the Consolidated Bioprocessing of starchy industrial residues.

On the basis of the preliminary results obtained, this multi-disciplinary work represents a first step towards the development of microbes for the single-step conversion of biomass into ethanol.

RIASSUNTO.

Il bioetanolo di seconda generazione rappresenta una delle alternative più promettenti tra i biocarburanti. Dal punto di vista biotecnologico, la definizione di un processo economicamente sostenibile per la produzione di bioetanolo da biomassa lignocellulosica è ancora lontana. Lo sviluppo di un microrganismo CBP (Consolidated BioProcessing) capace di idrolizzare i polimeri complessi della biomassa e di convertirli efficacemente in etanolo è una delle strategie più interessanti nel panorama scientifico internazionale.

Questo studio ha avviato un programma di selezione e miglioramento genetico di ceppi microbici finalizzato allo sviluppo di un microrganismo adatto alla produzione CBP di bioetanolo da biomassa.

Numerosi ceppi microbici cellulosolitici sono stati isolati ed identificati. Le loro attività idrolitiche sono elevate e alcuni isolati hanno dimostrato potenziali capacità fermentative. Inoltre, quattrocento ceppi di lievito wild type sono stati caratterizzati per la loro capacità di produrre enzimi extracellulari. Alcuni ceppi hanno presentato interessanti attività idrolitiche a carico di pectina, cellulosa ed amido. In particolare, un lievito non-*Saccharomyces* si è distinto per la produzione di efficienti cellulasi e tredici isolati di *S. cerevisiae* sono stati selezionati per la capacità di utilizzare amido solubile come unica fonte di carbonio.

Lo studio ha inoltre sviluppato un sistema SHF (Separated Hydrolysis and Fermentation) per la conversione in etanolo di crusca di grano, scelta come substrato modello di residui agro-industriali a basso costo. Le rese in etanolo, ottenute mediante due ceppi di *Saccharomyces* sp. opportunamente selezionati, sono risultate promettenti.

Un programma di ingenieria genetica ha consentito di ottenere alcuni ceppi mutanti per l'integrazione cromosomica multipla di un gene sintetico codificante per una glucoamilasi fungina. I ceppi ricombinanti, capaci di produrre etanolo da amido grezzo, potrebbero essere impiegati efficacemente in processi CBP a partire da residui industriali amidacei.

In base ai risultati preliminari finora conseguiti, questo studio rappresenta un primo passo verso lo sviluppo di microrganismi idonei alla conversione one-step di biomassa in etanolo.

AIMS OF THE WORK.

The aim of this work was to develop a microbe for the one-step conversion of biomass into ethanol. Such microorganism should possess high-level production of hydrolytic enzymes, efficient utilisation of resulting sugars and proper ethanol production performances.

To achieve this goal, two distinct strategies were defined and followed, namely

(1) the isolation of strains having excellent hydrolytic abilities for their future improvement of desired production properties.

(2) the engineering of desired hydrolytic properties in microbes having optimal fermentative abilities, such as *Saccharomyces cerevisiae*.

Experimental activity focused on these perspectives in order to isolate proficient cellulolytic organisms and to screen the depolymerising activities of *S. cerevisiae* and non-*Saccharomyces* strains, isolated from different oenological environments.

Moreover *Saccharomyces* sp. strains, selected for their fermentative vigour and extracellular activities, were used in a Separated Hydrolysis and Fermentation (SHF) process for the conversion of wheat bran into ethanol. Wheat bran was selected as a model of low-cost and abundant agricultural residues.

To obtain an efficient amylolytic yeast, a molecular biology approach was planned for endowing raw starch hydrolytic properties in wild type *S. cerevisiae* strains with promising industrial fermentative traits.

1. INTRODUCTION.

1.1 Bioethanol as alternative to fossil fuels.

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demand.

Campbell and Laherrere (1998), using several different techniques to estimate the current known as yet undiscovered crude oil reserves, concluded that the decline in worldwide crude oil production will begin before 2015. They also predicted that annual global oil production would decline from the current 25 billion barrels to approximately 5 billion barrels in 2050. Because the world economy in depends on oil, the consequences of inadequate oil availability could be severe. Therefore, there is a great interest in exploring alternative energy sources.

During the past 150 years, human activities have caused a dramatic increase in the emission of a number of greenhouse gases which has led to changes in the equilibrium of the atmosphere. The content of CO_2 in the air has increased from 280 ppm to 365 ppm during this period. The OECD (Organisation for Economic Cooperation and Development) countries contribute more than half of the world total emission of CO_2. The United States is one of the countries with the highest rates of discharge, emitting more than 20,000 kg CO_2 per capita per year (Galbe and Zacchi, 2002). The transport sector is responsible for the greatest proportion of CO_2 emission, and it is increasing from year to year.

One way of reducing environmental effects and the dependence on fossil fuels is to use renewable fuels. In addition, the utilisation of biofuels has important economic and social effects. For instance, Sheehan and Himmel (1999) pointed out that the diversification of fuel portfolio would bring money and jobs back into the USA economy. Moreover, the development of energy crops dedicated to the biofuels production would imply a boost to agricultural sector. This analysis is also valid for developing countries, considering the perspective of drastic reduction of proven oil reserves in the mid term.

Bioethanol produced by different feedstosks is considered one of the most promising biofuels from both energetic and environmental points of view. Many countries have implemented or are implementing programs for addition of ethanol to gasoline (Table 1.1).

Country	Feedstock	Ethanol in gasoline (% v/v)	Remarks
Brazil	Sugar cane	24	ProAlcool program; hydrous ethanol is also used as fuel instead of gasoline.
USA	Corn	10	Tax incentives; 85% blends are also available
Canada	Corn, wheat, barley	7.5-10	Tax incentives; provincial programs aimed to meet Kyoto Protocol
Spain	Wheat, barley	-	Ethanol is used for ETBE production; direct gasoline blending is possible
France	Sugar beet, wheat	-	Ethanol is used for ETBE production; direct gasoline blending is possible
Sweden	Wheat	5	85% blends are also available; there is no ETBE production
China	Corn, wheat	-	Trial use of fuel ethanol in central and north-eastern regions
India	Sugar cane	5	Ethanol blends are mandatory in 9 states
Thailand	Cassava, rice	10	Ethanol blends are mandatory
South Africa	Sugar cane, corn	-	Target production: 1.1 billion litres/year till 2016.

Table 1.1. Fuel ethanol programs in some countries (modified from Berg, 2004; Sànchez and Cardona, 2008).

Apart from a very low net emission of CO_2 to the atmosphere, the combustion of bioethanol in general results in the emission of low levels of non-combusted hydrocarbons, carbon monoxide (CO), nitrogen oxides and exhaust volatile organic compounds (Bailey, 1996; Wyman, 1996). However, the enlarged exhaust emission of reactive aldehydes, such as acetaldehyde and formaldehyde is of environmental concern. Therefore, a key factor with respect to the possible effects of ethanol on urban air quality will be the durability and effectiveness of catalyst systems for aldehyde control.

Today, all cars with a catalyst can be run on a mixture of 90% gasoline and 10% ethanol (E10) without adjusting the engine. New cars could even use mixtures containing up to 20% ethanol. There are also new engines available that can run on pure ethanol, and so-called flexible fuel vehicles that are able to use mixtures of 0-85% ethanol in gasoline (E85). Moreover, ethanol can replace diesel fuel in compression-ignition engines using a proper emulsifier.

Fuel ethanol is used in a variety of ways; however, the major application of ethanol today is as an oxygenated fuel additive (Wheals et al., 1999). Mixing ethanol and gasoline has several advantages. The higher octane number of ethanol (96-113) increases the octane number of the mixture, reducing the need for toxic, octane-enhancing additives. Ethanol also provides oxygen for the fuel, which will lead to the reduced emission of CO and non-combusted hydrocarbons. Bailey (1996) has summarised the pros and cons of replacing spark-ignition and compression-ignition engines with optimised ethanol engines. His conclusions were that ethanol has about the same overall transport efficiency as diesel in compression-ignition engines, but is about 15% more efficient than gasoline in optimised spark-ignition engines.

Fuel ethanol production has increased remarkably because many countries look for reducing oil imports, boosting rural economies and improving air quality. In 2008, the world alcohol production has reached about 69.1 billion litres (Renewable Fuels Association, 2009), being the USA and Brazil the first producers (Table 1.2). In average, 73% of produced ethanol worldwide corresponds to fuel ethanol, 17% to beverage ethanol and 10% to industrial ethanol.

In 2008, the United States continued as the leader in global bioethanol production with an output of 34 billion litres in 2008 and an expected 40 billion litres by the end of 2009. As the world's second largest bioethanol producer, Brazil produced about 24.5 billion litres of bioethanol derived from sugar cane in 2008 with a projected 29 billion litres for 2009. Jointly, the United States and Brazil produced almost 90% of the world fuel bioethanol. Production in 2008 for Asia was recorded at about 6.5 billion litres with China and India as sector leaders.

Country	2008	2007
1. USA	34,068	24,600
2. Brazil	24,500	19,000
3. China	3,800	3,777
4. European Union	2,777	2,159
of which		
France	1,000	539
Germany	568	394
Spain	317	348
Sweden	78	120
Italy	60	60
5. India	1,900	1,640
6. Canada	900	800
7. South Africa	386	330
8. Thailand	340	300
9. Colombia	300	284
10. Australia	100	100
11. Other	100	82
Total	69,171	53,072

Table 1.2. World production (mill litres)of fuel ethanol (modified from: Renewable Fuels Association, 2009; GBEP, 2007)

In the EU, France produced nearly 1 billion litres in 2008, followed by Germany at 568 million litres. In Italy, the bioethanol industry is still in its early stages: the bioethanol is produced from national feedstock (wine alcohol, molasses/sugar beets, cereals) in low amounts.

1.2 Bioethanol from different feedstocks: first and second generation.

The fuel ethanol can be obtained from several materials. On the basis of the used feestocks, bioethanol is defined of first or second generation. Generally, the first generation bioethanol is mainly produced from sugars or starchy feedstocks (mainly sugar cane, beet, corn, wheat). The second generation bioethanol is derived from cellulosic biomass.

As the most abundant biological material on Earth, biomass such as wood, tall grasses, and forestry and starchy-cellulosic crop residues is projected to greatly expand the quantity and variety of feedstock available for biofuel production. In comparison to the conventional starch crops that can contribute only a fraction of the plant material, cellulosic energy crops can produce more biomass per hectare of land since the entire crop is available as feedstock for conversion to fuel, and can be grown on land that is not of prime agricultural use.

Second generation liquid biofuels are attractive from a sustainability standpoint. Waste biomass can be processed, which would not require additional land for production as it is readily available from present forestry, agricultural and industrial activities. Additionally, the greatest potential for reducing GHG (Greenhouse Gas) emissions lies in the development of advanced second-generation feedstock and processes. Most studies project that future bioethanol from perennial crops, woody and agricultural residues could dramatically reduce life cycle GHG emissions relative to petroleum fuels (GBEP, 2007). Some options hold the potential for net emissions reductions that exceed 100 percent - meaning that more CO_2 would be sequestered during the production process than the equivalent emissions released during its life cycle - if fertilizer inputs are minimized, and biomass is used for process energy.

Nevertheless, the complexity of the production process depends on the feedstock. In this way, the spectrum of designed and implemented technologies goes from the simple conversion of sugars by fermentation, to the multi-stage conversion of lignocellulosic biomass into ethanol. Among the new research trends in this field, process integration has the key for reducing costs in ethanol industry and increasing bioethanol competitiveness related to gasoline.

1.3 Ethanol from sugars.

Main feedstock for ethanol production is sugar cane in form of either cane juice or molasses (by-product of sugar mills). About 79% of ethanol in Brazil is produced from fresh sugar cane juice and the remaining percentage from cane molasses (Wilkie et al., 2000). Sugar cane molasses is the main feedstock for ethanol production also in India

(Ghosh and Ghose, 2003). Beet molasses are also source of fermentable sugars for ethanologenic fermentation.

The most employed microorganism is *Saccharomyces cerevisiae* due to its capability to hydrolyse cane sucrose into glucose and fructose, two easily assimilable hexoses. Aeration is an important factor for growth and ethanol production by *S. cerevisiae*. Although this microorganism has the ability to grow under anaerobic conditions, small amounts of oxygen are needed for the synthesis of substances like fatty acids and sterols. The oxygen may be supplied through the addition in the medium of some chemicals like urea hydrogen peroxide (carbamide peroxide), which may contribute to the reduction of bacterial contaminants (Narendranath et al., 2000). Other yeasts, as *Schizosaccharomyces pombe*, present the additional advantage of tolerating high osmotic pressures (high amounts of salts) and high solids content (Bullock, 2002). A fermentation process using a wild strain of this yeast has been patented (Carrascosa, 2006).

Among bacteria, the most promising microorganism is *Zymomonas mobilis*, with a low energy efficiency resulting in a higher ethanol yield (up to 97% of theoretical maximum). However, its range of fermentable substrates is narrow and the bacterium could metabolise only glucose, fructose and sucrose (Claassen et al., 1999). Other disadvantage of the use of *Z. mobilis* during the fermentation of sugar cane syrup and other sucrose-based media is the formation of the polysaccharide levan (made up of fructose units), which increases the viscosity of fermentation broth, and of sorbitol, a product of fructose reduction that decreases the efficiency of the conversion of sucrose into ethanol (Lee and Huang, 2000).

The high osmolality of the media based on cane molasses is negative for the fermentation efficiency. The osmolality is related to the concentration of sugars and salts in the medium. Different studies have been carried out in order to obtain *S. cerevisiae* strains with greater salt and temperature tolerance. For example, Morimura et al. (1997) developed by protoplast fusion and manipulating culture conditions, flocculating strains capable of growing at 35°C and at molasses concentration of 22% (w/v). Interesting ethanol concentration of 91 gL^{-1} and productivities of 2.7 $(gL^{-1})\ h^{-1}$ were obtained.

However, the principal approach for avoiding the negative influence of salts and other compounds on the fermentation is through the conditioning of molasses by the supplementation of different compounds neutralizing the inhibitory effects of the medium

components. Moreover, molasses should be supplemented with nutritional factors promoting the yeast growth (Castellar et al., 1998; Ergun et al., 1997).

1.4 Ethanol from starch.

Starch is a high yield feedstock for ethanol production, but its hydrolysis is required to produce ethanol by fermentation. The polysaccharide is made up of individual units of glucose, linked together in chains by α-1,4 and occasional α-1,6-linkages (Paragraph 1.6.2.1).

Starch has to be completely hydrolysed before its conversion into ethanol. To accomplish this conversion, the pH of the mash is adjusted to pH 6.0, followed by the addition of α-amylase. A thermostable α-amylase enzyme is added to begin breaking down the starch polymer to produce soluble dextrins by quickly and randomly hydrolysing α-1,4 bonds. The mash is heated above 100°C using a jet cooker, which provides the high temperature and mechanical shear necessary to cleave and rupture starch molecules.

The product of this first step, called liquefaction, is a starch solution containing dextrines and small amounts of glucose. The liquefied starch is subject to saccharification at lower temperatures (60-70°C) through glucoamylase obtained generally from *Aspergillus niger* or *Rhizopus* species (Pandey et al., 2000).

1.4.1 Ethanol production from corn.

In the USA, ethanol is produced almost exclusively from corn. Corn is milled for extracting starch, which is enzymatically treated to obtain glucose syrup. Then, this syrup is fermented into ethanol.

Today, most fuel ethanol is produced from corn by either dry grind (67%) or the wet mill (33%) process. The key distinction between wet mill and dry grind facilities is the focus on the resourcing. In the case of a dry grind plant, the aim is maximising the capital return per gallon of ethanol. In the case of a wet mill plant, capital investments allow for the separation of other valuable components in the grain before fermentation to ethanol (Bothast and Schlicher, 2005).

The wet milling process is more capital- and energy-intensive, as the grain must first be separated into its components, including starch, fiber, gluten and germ. Starch is converted into ethanol and the remaining components are processed and sold as co-products.

In the dry grind process, the clean corn is ground and mixed with water to form a mash. The mash is cooked and enzymes are added to convert starch to glucose. Then, grains are not fractionated and all their nutrients enter the process and are concentrated into a distillation co-product utilized for animal feed called Distillers Dried Grains and Solubles (DDGS).

In general, the liquefaction, saccharification and fermentation steps are the same for both technologies. Fermentation is performed using *S. cerevisiae* and is carried out at 30-32°C with the addition of ammonium sulfate or urea as nitrogen sources.

Burmaster (2007) has recently patented a method for improving the fermentation of corn mashes and other feedstocks through the control of oxidation reduction potential. This system allows achieving higher yields, shorter cultivation times, and decreased by-product formation. Configurations involving a higher degree of integration as the Simultaneous Saccharification and Fermentation (SSF) have been successfully implemented, especially in the dry-milling process (Cardona and Sánchez, 2007). The SSF performed with a thermotolerant yeast at temperature above 34°C enables the reduction of cooling requirements and the improvement of the conversion process (Otto and Escovar-Kousen, 2004).

New tendencies in corn-to-ethanol industry are aimed at dry-milling processes. For instance, the increase in ethanol production capacity in the USA is mainly represented by corn dry-mill ethanol plants. Other research efforts are oriented to the development of corn hybrids with higher extractable starch or higher fermentable starch content. Genetic engineering can be applied to direct the accumulation of amylases in the endosperm of transgenic corn kernels making possible the utilisation of “self-processing” grains (Bothast and Schlicher, 2005).

1.4.2 Ethanol production from wheat.

Although in France ethanol is mostly produced from beet molasses, wheat is also used as feedstock by a process similar to that of corn. Some efforts have been done for optimising fermentation conditions. For example, Wang et al. (1999) have determined the optimal fermentation temperature and specific gravity of the wheat mash. Soni et al. (2003) have described optimal conditions for starch hydrolysis using α-amylase and glucoamylase obtained by solid-state fermentation of wheat bran.

To enhance fermentation performance, high gravity fermentations have been proposed, particularly for the case of wheat mashes. In this case, the initial dissolved solids concentration exceeds 200 gL^{-1} implying a higher substrate load. Therefore, higher ethanol concentrations are achieved with the requirement of lower amounts of process water. The drawbacks of this technology include longer fermentation periods and incomplete fermentations probably caused by product inhibition, high osmotic pressures and inadequate nutrition (Barber et al., 2002).

To accelerate high gravity fermentations, the controlled addition of small amounts of acetaldehyde during the fermentation has allowed the reduction in cultivation time from 790 h to 585 h without effect on the ethanol yield. It is believed that this positive effect may be caused by the ability of acetaldehyde to replenish the intracellular acetaldehyde pool and restore the cellular redox balance (Barber et al., 2002).

1.4.3 Ethanol production from cassava.

Cassava represents an important alternative source of starch not only for ethanol conversion, but also for production of glucose syrups. This tuber has gained most interest due to its availability in tropical countries. Cassava is one of the most important tropical crops.

Ethanol production can be accomplished using either the whole cassava tuber or the starch extracted from it. Starch extraction can be carried out through a high-yield large-volume industrialized process as the Alfa Laval extraction method (FAO, 2004), or by a

traditional process for mid-scale plants. The extraction method can be considered as the equivalent of the corn wet-milling process.

The production of cassava with high starch content (85-90% dry matter) and less protein and minerals content is relatively simple. Cassava starch has a lower gelatinization temperature and offers a higher solubility for amylases in comparison to corn starch.

However, it is considered that cassava ethanol would have better economic indicators if the whole tuber is used as feedstock. Fuel ethanol production from whole cassava is equivalent to ethanol production from corn by dry-milling technology. For this, cassava should be transported as soon as possible from cropping areas considering its high moisture content (about 70%). Hence, this feedstock should be processed within 3-4 days. One of the solutions to this problem consists in the use of sun-dried cassava chips. The farmers send the cassava roots to small chipping factories where they are peeled and chopped into small pieces. The chips are sun-dried during 2-3 days. The final moisture content is about 14% and the starch content reaches 65%.

The first step of the process in the distillery is the grinding of dried chips or fresh roots. Milled cassava is mixed with water and undergoes cooking followed by the liquefaction enzymatic process. Liquefied slurry is saccharified to obtain the glucose, which will be assimilated by the yeast during the next fermentation step. The process can be intensified through the SSF as in the corn case. If fresh roots are employed, a fibrous material is obtained in the stillage after distillation. This material can be used as an animal feed similarly to the DDGS by-products of the corn-based process.

1.4.4 Ethanol production from other starchy materials.

Besides corn and wheat, ethanol can be produced from rye, barley, triticale and sorghum (Wang et al., 1997; Zhan et al., 2003) as well as from cereal milling by-products such as sorghum or wheat bran (Chen at al., 2007; das Neves et al., 2006; Palmarola-Adrados et al., 2005).

For these feedstocks, some pre-treatments have proven to be useful such as the pearling of wheat, barley, rye and triticale grains for increasing starch content of the feedstock.

It has been reported the ethanol production from other plant sources with high starch concentration. Abd-Aziz (2002) suggested the utilisation of sago palm for ethanol production in the case of Malaysia. The ethanol production from bananas and banana wastes using commercial α-amylase and glucoamylase has been studied by Hammond et al. (1996).

One of the most promising crops for fuel ethanol production is the sweet sorghum, which produces grains with high starch content, stalks with high sucrose content and leaves and bagasse with high lignocellulosic content. In addition, this crop can be cultivated in both temperate and tropical countries requiring only 1/3 of the water needed for cane cropping and half of the water required by corn. Moreover, it is tolerant to the drought, flooding and saline alcalinity (Sànchez and Cardona, 2008).

1.5. Ethanol from lignocellulosic biomass.

It is evident the importance of lignocellulosic biomass as a feedstock for ethanol production. Lignocellulosic complex is the most abundant biopolymer in the Earth: lignocellulosic biomass comprises about 50% of world biomass and its annual production was estimated in 10-50 billion ton (Claassen et al., 1999).

Many lignocellulosic substrates have been tested for bioethanol production. In general, lignocellulosic materials for bioethanol production can be divided 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, thimothy grass), and Municipal Solid Wastes (MSW). The composition of most of these materials is reported in Table 1.3.

Numerous studies for developing large-scale production of ethanol from lignocellulosics have been carried out in the world. However, the main limiting factor is the higher degree of complexity inherent to the processing of the feedstock. This is related to the nature and composition of lignocellulosic biomass. Therefore, the lignocellulose processing to ethanol is still complicated, energy-consuming and non-completely developed.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40-50	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Wheat straw	33-40	20-25	15-20
Wheat bran	10-12	25-35	2-6
Rice straw	40	18	5-7
Cotton seed hairs	80-95	5-20	-
Grasses	25-40	35-50	10-30
Switchgrass	30-50	10-40	5-20
Coastal Bermuda grass	25	35	6-7
Solid cattle manure	2-5	1-3	2-6
Swine waste	6	28	-
Newspaper	40-55	25-40	18-30
Paper	85-99	0	0-15
Waste paper from chemical pulps	60-70	10-20	5-10

Table 1.3. The contents of cellulose, hemicellulose and lignin in common bioethanol feedstocks (modified from Chen at al., 2007; McKendry, 2002; Sun and Chen, 2002).

1.5.1 Pre-treatment of lignocellulosic biomass.

The main processing challenge in the ethanol production from lignocellulosic biomass is the feedstock pre-treatment. The lignocellulosic complex is a matrix of cellulose and lignin bound by hemicellulose chains. During the pre-treatment, this matrix should be broken in order to reduce the crystallinity degree of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack. Additionally, main part of hemicellulose should be hydrolysed and lignin should be released.

The fact that the cellulose hydrolysis is influenced by the porosity (accessible surface area) of lignocellulosic materials should be also considered. The yield of cellulose hydrolysis without proper pre-treatments is less than 20% of the theoretical, whereas the yield after pre-treatment often exceeds 90% of theoretical (Lynd, 1996).

Therefore, the aim of the pre-treatment is the removal of lignin and hemicellulose, the reduction of crystalline cellulose and the increase in the porosity of the materials. In addition, the pre-treatment should improve the release of sugars and avoid the formation of inhibitors for subsequent hydrolysis and fermentation processes. For the pre-treatment of lignocellulosics, several physical, physical-chemical, chemical and biological processes have been proposed and developed (reviewed in Hendriks and Zeeman, 2009; Mosier et al., 2005; Sun and Cheng, 2002).

1.5.1.1 Physical methods.

Feedstocks can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. This reduction facilitates the access of cellulases to the biomass surface increasing the conversion of cellulose. The energy requirements of mechanical comminution of lignocellulosic materials depend on the final particle size and biomass characteristics.

Although mechanical pre-treatment methods increase cellulose reactivity towards enzymatic hydrolysis, they are unattractive due to their high energy and capital costs (Ghosh and Ghose, 2003). Pyrolysis has also been tested as a physical method for pre-treatment of lignocellulosic biomass since cellulose rapidly decomposes when is treated at high temperatures.

1.5.1.2 Physical-chemical methods.

Physical-chemical pre-treatment methods are considerably more effective than physical. Several methods have been developed according to the composition of lignocellulosic feestock.

Steam explosion (autohydrolysis).

Steam explosion is the most commonly used method for pre-treatment of lignocellulosic materials. In this system, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive

decompression. Steam explosion is typically initiated at a temperature of 160-260°C (corresponding pressure 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure.

The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Ninety percent efficiency of enzymatic hydrolysis has been achieved in 24 h for poplar chips pre-treated by steam explosion, compared to only 15% hydrolysis of un-treated chips (Grous et al., 1986). The factors that affect steam explosion pre-treatment are residence time, temperature, chip size and moisture content (Duff and Murray, 1996; Hendriks and Zeeman, 2009; Sun and Chen, 2002).

The advantages of steam explosion pre-treatment include the low energy requirement compared to mechanical treatment and no recycling or environmental costs. The conventional mechanical methods require 70% more energy than steam explosion to achieve the same size reduction (Holtzapple et al., 1989). Steam explosion is recognized as one of the most cost-effective pre-treatment processes for hardwoods and agricultural residues, but it is less effective for softwoods (Clark and Mackie, 1987).

Limitations of steam explosion include destruction of a portion of the xylan fraction and generation of inhibitory compounds for microorganisms used in downstream processes.

Ammonia fiber explosion (AFEX).

AFEX is another type of physico-chemical pre-treatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is quickly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1-2 kg ammonia per kg dry biomass, temperature 90°C and residence time 30 min.

AFEX pre-treatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pre-treatment of many lignocellulosic materials including wheat straw (Mes-Hartree et al., 1988), barley straw, corn stover, rice straw (Vlasenko et al., 1997), MSW, softwood newspaper (Holtzapple et al., 1992), coastal Bermuda grass, switchgrass (Reshamwala et al., 1995), and bagasse (Holtzapple et al., 1991). The AFEX pre-treatment does not significantly solubilise hemicellulose compared

to acid pre-treatment (to be discussed in the following section) and acid-catalyzed steam explosion (Mes-Hartree et al., 1988; Vlasenko et al., 1997).

Liquid Hot Water (LHW).

One of the most promising physical-chemical methods is the pre-treatment with Liquid Hot Water (LHW) or thermo-hydrolysis. In this case liquid hot water is used instead of steam. The objective of the liquid hot water is to solubilise mainly the hemicellulose to make the cellulose better accessible and to avoid the formation of inhibitors (Hendriks and Zeeman, 2009).

Laser et al. (2002) mentioned that under optimal conditions, this procedure is comparable to dilute acid pre-treatment but without addition of acids or production of neutralization wastes. In addition, this technology presents elevated recovery rates of pentoses and does not generate inhibitors (Ogier et al., 1999). Negro et al. (2003) compared steam explosion and LHW pre-treatments for poplar biomass and described best results for the latter at 210°C during 4 min.

1.5.1.3 Chemical methods.

Chemical pre-treatments employ different chemical agents as ozone, acids, alkalis, peroxide and organic solvents.

Acid hydrolysis.

Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995).

Dilute acid hydrolysis has been successfully developed for pre-treatment of lignocellulosic materials. The dilute sulfuric acid pre-treatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997).

Schell et al. (2003) studied the dilute-acid pre-treatment of corn stover at pilot plant level using high solid loads. The pentose sugars yields were interesting, corresponding to the 77% of the maximum theoretical value. This pre-treatment method was evaluated through a kinetic model that allowed the prediction of process conditions in order to maximise the yield. Similar kinetic studies were carried out for cane bagasse pre-treated with nitric acid (Rodríguez-Chong et al., 2004) or without acid addition (Jacobsen and Wyman, 2002).

Dilute acid pre-treatment also can be accomplished in a two-stage way. For this, a first depolymerisation stage of hemicellulose at 140°C during 15 min is carried out in order to avoid the formation of inhibitors (mainly furans and carboxylic acids). In a second stage, the temperature increases at 190°C for 10 min rendering cellulose more accessible to enzymatic hydrolysis (Saha et al., 2005a; Saha et al., 2005b). These authors pointed out that the realization of dilute-acid pre-treatment at low temperatures (121°C) could avoid the degradation of sugars to furfural and 5-hydroxymethyl-2-furaldehyde (HMF), but the sugars yields are lower.

Dilute acid pre-treatment along with steam explosion are the most widely studied methods. The National Renewable Energy Laboratory (NREL) of the US Department of Energy, which currently is developing ethanol production technologies from biomass, has preferred the dilute acid pre-treatment for the design of its process alternatives. The main advantage of this process related to steam explosion is the higher recovery of sugars derived from hemicellulose. For hardwood, this recovery is about 80% for dilute acid pre-treatment, and does not exceed 65% for steam explosion. Ogier et al. (1999) stated that the methods appearing as the most efficient are dilute-acid pre-treatment, steam explosion with catalyst addition and LHW. These methods are also chosen by Hamelinck et al. (2005) as the more perspective in short-, mid- and long-term evaluations.

Alkaline hydrolysis.

Alkaline pre-treatment is based on the effects of the addition of dilute bases on the biomass (NaOH or ammonia). Several works reported an increase of internal surface by swelling, decrease of polymerization degree (DP) and crystallinity and destruction of links

between lignin and other polymers. The effectiveness of this method depends on the lignin content of the biomass (Sun and Cheng, 2002).

In general, the utilisation of bases as sodium hydroxide or solvents such as ethanol or methanol (organosolv process) allows the dissolution of lignin, but their costs are so high that these methods are not competitive for large scale plants (Lynd et al., 1999).

1.5.1.4 Biological methods.

Biological pre-treatment has low energy requirements and mild environmental conditions. However, most of these processes are too slow for efficient industrial applications. Many white-rot fungi degrade the lignin and, for this reason, they have been utilised for ligninases production and lignocellulose degradation.

Lee (1997) reports the main microorganisms producing lignin-degrading enzymes and indicates the fermentation processes for producing them by both submerged culture and solid-state fermentation. For instance, the fungus *Phanerochaete chrysosporium* has been proposed in the patent of Zhang (2006) for degrading the lignin in a biomass-to-ethanol process scheme involving the separate fermentation of pentoses and hexoses.

Kang et al. (2004) highlighted the feasibility of producing cellulases and hemicellulases by solid-state fermentation. However, one of the main problems during the pre-treatment and hydrolysis of biomass is the variability in the content of lignin and hemicellulose. This heterogeneity mainly depends on the type of plant from which the biomass is obtained, crop age, method of harvesting. Therefore, no one of the enzymatic hydrolysis pre-treatment could be applied in a generic way for many different feedstocks (Claassen et al., 1999). The future trends for improving the pre-treatment of lignocellulosic feedstocks also include the production of genetically modified plant materials with higher carbohydrate content or properly modified plant structure. It is estimated that the use of these new materials along with improved conversion technologies, could reduce the ethanol cost from lignocellulosic biomass in US$ 0.11 L^{-1} in the next twenty years (Lynd et al., 2006; Wooley et al., 1999).

1.5.2 Inhibitors and biomass pre-treatment.

Pre-treatment of lignocellulosic biomass with such methods generate a broad range of compounds as indicated in Figure 1.1. D-glucose is mainly obtained from the hydrolysis of cellulose. D-glucose, D-galactose, D-mannose and D-rhamnose (hexoses), as well as D-xylose and L-arabinose (pentoses) are released from the hemicellulose fraction. Uronic acids, such as 4-*O*-methylglucuronic acid, are also produced during hydrolysis of hemicellulose. Lignin is an aromatic polymer non-soluble in water, consisting of phenylpropane subunits.

Hydrolysis treatments may result in further degradation of lignin and monomeric sugars to three major groups of compounds that inhibit the following fermentation step: (I) furan derivatives (2-furaldehyde and 5-hydroxymethyl-2-furaldehyde); (II) weak acids (mainly acetic acid, formic acid and levulinic acid); and (III) phenolic compounds.

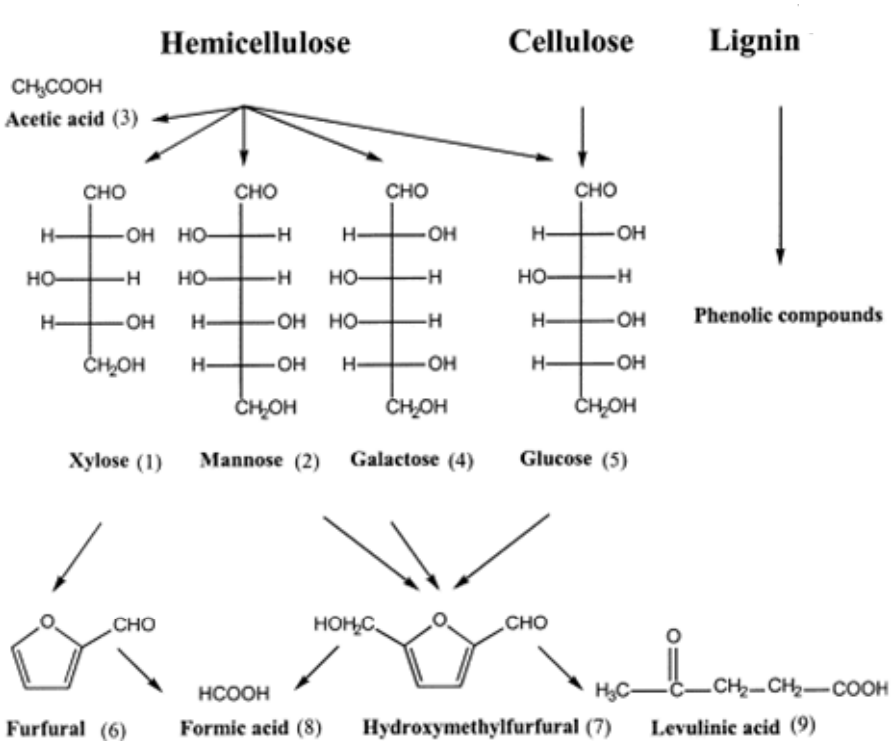


Figure 1.1. Products formed during pre-treatments of lignocellulosic biomass (modified from Palmqvist and Hahn- Hägerdal, 2000).

Furan derivatives.

The furan compounds 5-hydroxymethyl-2-furaldehyde (HMF) and 2-furaldehyde are formed by dehydration of hexoses and pentoses, respectively (Dunlop, 1948; Ulbricht et al., 1984). The level of furans varies according to the type of raw material and the pre-treatment procedure (Table 1.4).

As an example, HMF concentrations in spruce hydrolysate can vary from 2.0 to 5.9 gL^{-1} depending on whether one-step or two-step dilute acid hydrolysis is performed (Almeida et al., 2007; Larsson et al., 1999; Nilvebrant et al., 2003). Inversely, HMF is absent from wet-oxidation treated wheat straw (Klinke et al., 2003). Furfural is usually found in lower levels than HMF. However, it is often still in high enough concentration (around 1 gL^{-1}) to be inhibitory.

Phenolics.

A wide range of phenolic compounds are generated due to lignin breakdown and also carbohydrate degradation during acid hydrolysis. The amount and type of phenolic compounds depend on the biomass source (Table 1.4), since lignin in several raw materials has different internal bonding and association with hemicellulose and cellulose in the plant cell wall (Perez et al., 2002).

Therefore, the aromatic compounds present in hydrolysates are dependent on the type of pre-treatment and the H/G/S ratio of the lignin contained in the biomass material. The most versatile phenols found were 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, and syringic acid. Phenol monomers have been quantified in lignocellulosic hydrolysates from pine, oak, willow, spruce, wheat straw, bagasse, poplar, corn stover and switch grass (Almeida et al., 2007; Clark and Mackie 1984; Klinke et al., 2003; Martin and Jonsson 2003; Tran and Chambers 1986).

The phenols were divided into three groups by their degree of methoxylation (H, G, S) and their functionality (aldehydes, ketones, acids, other). Softwood materials almost exclusively produce G (guaiacyl) phenols, while hardwoods and herbaceous materials produce H, G and S phenols consistent to the biomass composition.

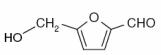
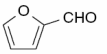
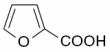
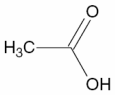
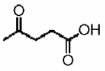
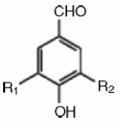
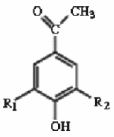
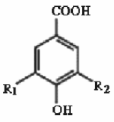
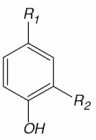
Groups of compounds		Concentration (gL^{-1})				
Furan derivatives		Spruce[a,b]	Willow[c]	Wheat[d]	Sugar cane[e]	Corn stover[f]
	5-hydroxymethyl-2-furaldehyde (HMF)	5.9[a] 2.0[b]	n.q.	n.i	0.6	0.06
	2-Furaldehyde	1.0 0.5	n.q.	n.i	1.9	11
	2-Furoic acid	n.i	n.q.	0.007		
Aliphatic acids						
	Acetic acid	2.4 3.1	n.q.	1.6	4.4	1.6
	Formic acid	1.6	n.q.	1.4	1.4	
	Levulinic acid	0.9 2.6 1.1	n.q.	n.i.		
Phenolic compounds						
	$R_1 = R_2 = H$ 4-hydroxybenzaldehyde	n.i	0.010	0.021		
	$R_1 = H, R_2 = OCH_3$ Vanillin	0.12	0.430	0.032		
	$R_1 = R_2 = OCH_3$ Syringaldehyde	0.107		0.024		
	$R_1 = R_2 = H$ 4-hydroxyacetophenone	n.i	n.i	0.004		
	$R_1 = H, R_2 = OCH_3$ Acetovanillone	n.i	n.i	0.008		
	$R_1 = R_2 = OCH_3$ Acetosyringone	n.i	n.i	0.039		
	$R_1 = R_2 = H$ 4-hydroxybenzoic acid	0.005 0.039	n.q.	0.010		
	$R_1 = H, R_2 = OCH_3$ Vanillic acid	0.034 0.017	n.q.	0.067		
	$R_1 = R_2 = OCH_3$ Syringic acid	n.i	n.q.	0.022		
	$R_1 = R_2 = H$ Phenol	n.i	0.035			
	$R_1 = H\ R_2 = OH$ Cathecol	0.009 0.002	0.440			
	$R_1 = OH\ R_2 = H$ Hydroquinone	0.017	n.i.			

Table 1.4. Common inhibitory compounds present in lignocellulosic hydrolysates from spruce, willow, wheat straw, sugar cane bagasse and corn stover (modified from Almeida et al., 2007).

Biomass source and pre-treatment employed:

[a] upper values; two-step dilute acid spruce (*Picea abies*)

[b] lower values, one-step dilute acid spruce

[c] dilute acid willow (*Salix caprea*)

[d] wet oxidation wheat straw (*Triticum aestivum* L.)

[e] steam pre-treatment sugar cane bagasse

[f] steam pre-treatment corn stover

n.q.: not quantified; n.i.: not identified.

Weak acids.

Acetic, formic and levulinic acid are the most common weak acids present in lignocellulosic pre-treated materials. Acetic acid is ubiquitous in hemicellulose hydrolysates from all lignocellulosics, where the hemicellulose and to some extent lignin is acetylated (Fengel and Wegener 1989; Sarkanen and Ludwig 1971; Torssell, 1997). Formic and levulinic acids are products of HMF breakdown (Almeida et al., 2007). Formic acid can additionally be formed from furfural under acidic conditions at elevated temperatures. Representative amounts of weak acids in lignocellulosic hydrolysates are given in Table 1.4.

1.5.2.1 Inhibitors: effects and mechanisms.

The compounds released during biomass pre-treatment and hydrolysis have the potential of inhibiting microorganism growth and ethanol production. The effect of furans, weak acids and phenolic compounds - as well as their synergistic effect - mainly on *S. cerevisiae* is summarised below.

Furan derivatives.

HMF and furfural decrease the volumetric ethanol yield and productivity and inhibit growth. These effects depend on the furan concentration and on the microbial strain used. Synergistic effects of HMF and furfural have been demonstrated (Taherzadeh et al., 2000). Conversion of HMF and furfural to their less inhibitory alcohol forms was studied by pulse addition of 2 gL^{-1} of each compound to anaerobic batch cultures of *S. cerevisiae*. As long as both furfural and HMF remain in the culture, no growth occurred and the specific uptake rates of HMF and furfural were clearly lower than when 4 gL^{-1} of only one of the components was added. When equimolar concentrations of each compound were compared, HMF had a less severe inhibitory effect than furfural, even though it took longer to be converted by yeast (Taherzadeh et al., 2000).

Several mechanisms may explain the inhibition effects of ethanol fermentation by furans. *In vitro* measurements showed that furfural and HMF directly inhibited alcohol dehydrogenase, pyruvate dehydrogenase and aldehyde dehydrogenase (Modig et al., 2002).

The reduction of furans by yeast may also result in NAD(P)H depletion (Palmqvist et al., 1999).

In *S. cerevisiae* furfural causes reactive oxygen species to accumulate, vacuole and mitochondrial membranes damage, chromatin and actin damage (Almeida et al., 2007). In general, the effects of furans can be explained by a re-direction of yeast energy to fixing the damage caused by furans and by reduced intracellular ATP and NAD(P)H levels, either by enzymatic inhibition or consumption/regeneration of cofactors (Liu, 2006).

Phenolics.

The inhibitory effects of phenols have recently been reviewed (Klinke et al., 2004). As for furans, it was found that biomass yield, growth rate and ethanol productivity are generally more decreased than ethanol yields. Low Molecular Weight (MW) phenolic compounds are more inhibitory to *S. cerevisiae* than high MW phenolics. Also the substituent position, *para*, *ortho*, *meta*, influences the toxicity of the compound (Larsson et al., 2000). The *ortho* position increases the toxicity of vanillins while methoxyl and hydroxyl substituents in *meta* and *para* positions or vice versa do not influence the toxicity (Almeida et al., 2007). The phenolic hydrophobicity was correlated with reduced volumetric ethanol productivity in *S. cerevisiae* for a series of separate functional groups of phenol aldehydes, ketones, and acids (Klinke et al., 2003). Generally, aldehydes and ketones are stronger inhibitors than acids, which in turn are more inhibitory than alcohols both for *S. cerevisiae* and for *E. coli* (Zaldivar et al., 1999; Zaldivar et al., 2000)

Inhibition mechanisms of phenolic compounds on *S. cerevisiae* and other eukaryotic microorganisms have not yet been completely elucidated, largely due to the heterogeneity of the group and the lack of accurate qualitative and quantitative analyses. Phenolic compounds may act on biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Heipieper et al., 1994).

Weak acids.

The weak acids inhibit yeast fermentation by reducing biomass formation and ethanol yields. Addition of acetic, levulinic and formic acids individually or in combination

reduced the ethanol yield with identical levels, indicating the absence of synergistic effects among the three acids (Larsson et al., 1999).

The inhibitory effect of weak acids has been ascribed to uncoupling and intracellular anion accumulation (Russel, 1992). The undissociated form of weak acids can diffuse from the fermentation medium across the plasma membrane and dissociate due to higher intracellular pH, thus decreasing the cytosolic pH. The decrease in intracellular pH is compensated by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis. Consequently, less ATP is available for biomass formation. Low levels of acetic, levulinic or formic acid increase the ethanol yield, whereas ethanol yield decreases at higher concentrations. It is believed that low concentrations of acids stimulate the production of ATP, which is achieved under anaerobic conditions by ethanol production. However, at higher concentrations, the ATP demand would be so high that cells cannot avoid acidification of the cytosol.

According to the intracellular anion accumulation theory (Russel, 1992), the anionic form of the acid is captured inside the cell and the undissociated acid will diffuse into the cell until equilibrium is reached. This is supported by the fact that formic acid is more inhibitory than levulinic acid which, in turn, is more inhibitory than acetic acid (Maiorella et al., 1983). Increased toxicity of formic acid seems to be associated with a smaller molecule size, which may facilitate its diffusion through the plasma membrane and probably higher anion toxicity. The higher toxicity of levulinic acid in comparison with acetic acid may be related to the higher hydrophobicity of levulinic acid, which possibly penetrates more easily into the cell membrane.

Weak acids have also been shown to inhibit yeast growth by reducing the uptake of aromatic amino acids from the medium (Bauer et al., 2003).

1.5.3 Detoxification of lignocellulosic hydrolysates.

During pre-treatment and hydrolysis of lignocellulosic biomass, a great amount of compounds that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars. For this reason and depending on the type of employed pre-treatment, detoxification of the hydrolysates is required.

Biological, physical, and chemical methods have been studied (Klinke et al., 2003; Olsson and Hahn-Hägerdal, 1996; Palmqvist and Hahn-Hägerdal 2000). These methods cannot be directly compared because they vary in the neutralization degree of the inhibitors. In addition, the fermenting microorganisms have different tolerances to the inhibitors. Moreover, several reports on microbial adaptation to inhibiting compounds in lignocellulosic hydrolysates are found in the literature (Amartey and Jeffries, 1996; Buchert et al., 1988; Nishikawa et al., 1988; Tran and Chambers, 1986).

1.5.3.1 Biological detoxification methods.

Treatment with the enzymes peroxidase and laccase, obtained from the ligninolytic fungus *Trametes versicolor*, has been shown to increase two-fold the maximum ethanol productivity in a hemicellulose hydrolysate of willow (Jönsson et al., 1998). The laccase treatment led to selective and virtually complete removal of phenolic monomers and phenolic acids.

The absorbance at 280nm, indicative of the presence of aromatic compounds, did not decrease during the laccase treatment, whereas an increase in absorbance for the large-sized material and a decrease for the small-sized material were observed for all wavelengths tested. Based on these observations, the detoxifying mechanism was suggested to be oxidative polymerisation of low MW phenolic compounds.

The filamentous soft-rot fungus *Trichoderma reesei* has been reported to degrade inhibitors in a hemicellulose hydrolysate obtained after steam pre-treatment of willow, resulting in around three times increased maximum ethanol productivity and four times increased ethanol yield (Palmqvist et al., 1997). In contrast with the results above presented about laccase, treatment with *T. reesei* resulted in a 30% decrease in absorbance at 280 nm, indicating that the mechanisms of detoxification were different. Acetic acid, furfural and benzoic acid derivatives were removed from the hydrolysate by the treatment with *T. reesei*.

1.5.3.2 Physical detoxification methods.

The most volatile fraction (10% (v/v)) of a willow hemicellulose hydrolysate obtained by roto-evaporation has been shown to slightly decrease the ethanol productivity compared to a reference fermentation containing glucose and nutrients (Palmqvist et al., 1996). The non-volatile fraction was found to be considerably more inhibitory.

After continuous overnight extraction of a strongly inhibiting spruce hydrolysate with diethyl ether at pH 2, the ethanol yield (0.40 g g^{-1}) has been reported to be comparable to the value in the reference fermentation (Palmqvist and Hahn-Hägerdal 2000). The ether extract contained acetic, formic, and levulinic acid, furfural, HMF and phenolic compounds. Resuspension of the extracted components in fermentation medium decreased the ethanol yield and productivity to 33% and 16%, respectively, of the values obtained in a reference fermentation. In agreement with this result, ethyl acetate extraction has been reported to increase the ethanol yield in fermentation by *Pichia stipitis* from 0 to 93% of that obtained in a reference fermentation (Wilson et al., 1989) due to removal of acetic acid (56%) and complete depletion of furfural, vanillin, and 4-hydroxybenzoic acid. Ethyl acetate extraction has also been shown to increase the glucose consumption rate in a hydrolysate of pine by a factor of 12 (Clark and Mackie, 1984). The low molecular weight phenolic compounds were suggested to be the most inhibiting compounds in the ethyl acetate extract.

1.5.3.3 Chemical detoxification methods.

Detoxification of lignocellulosic hydrolysates by alkali treatment, i.e., increasing the pH to 9-10 with $Ca(OH)_2$ (overliming) and readjustment to 5.5 with H_2SO_4, has been described as early as 1945 by Leonard and Hajny.

$Ca(OH)_2$ adjustment of pH has been reported to result in better fermentability than NaOH usage due to the precipitation of 'toxic compounds' (van Zyl et al., 1988). In agreement with this, ethanol yield and productivity in a solution of the components which had been extracted with ether from a dilute-acid hydrolysate of spruce has been reported to be considerably higher after adjustment to pH 5.5 with $Ca(OH)_2$ than with NaOH

After an overliming treatment (pH 10), causing the formation of a large precipitate, the ethanol productivity was further increased. The detoxifying effect of overliming is due both to the precipitation of toxic components and to the instability of some inhibitors at high pH.

In the 1940s, treatments with a reducing agent such as sulphite, or a large yeast inoculum were suggested as means to overcome an 'unfavourable reduction potential' in lignocellulosic hydrolysates (Leonard and Hajny, 1945). In more recent studies adding sodium sulphite to a dilute-acid hydrolysates of spruce (Larsson et al., 1999), or using a large cell inoculum (Palmqvist and Hahn-Hägerdal, 1999) have been shown to decrease the concentrations of furfural and HMF.

A combination of sulphite and overliming was detected the most efficient method to detoxify willow hemicellulose hydrolysate prior to fermentation by recombinant *E. coli* (Olsson et al., 1995). Only 24% of the xylose was fermented in 40 h in the un-treated hydrolysate, whereas complete depletion of monosaccharides was obtained in the same time after overliming.

1.5.4 Hydrolysis of cellulose.

For fermentation of lignocellulosic materials, cellulose should be degraded into glucose (saccharification) using acids or enzymes. In the former case, concentrated or dilute acids can be used. If dilute acids (H_2SO_4 and HCl) are employed, temperatures of 200-240°C at 1.5% acid concentrations are required to hydrolyse the crystalline cellulose, but these severe conditions lead to the degradation of glucose into HMF and other non-desired products. Similarly, xylose is degraded into furfural and other compounds.

During two-stage regime, a first stage under mild conditions (190°C, 0.7% acid, 3 min) is carried out to recover pentoses, while in the second stage, the remaining solids undergo harsher conditions (215°C, 0.4% acid, 3 min) to recover hexoses. In this way, 50% glucose yield is obtained (Hamelinck et al., 2005).

One variant of the acid hydrolysis is the employ of extremely low acid and high temperature conditions during batch processes (auto-hydrolysis approach) that has been applied to sawdust (Ojumu and Ogunkunle, 2005). Concentrated acid process using 30-

70% H_2SO_4 has higher glucose yield (90%) and is relatively rapid (10-12 h) but the amount of used acid is a critical economic factor.

However, cellulose hydrolysis is currently performed using microbial cellulolytic enzymes. Enzymatic hydrolysis has demonstrated better results for the subsequent fermentation because no degradation components of glucose are formed.

Most of the commercial cellulases are obtained aerobically from *T. reesei* and a small portion is produced by *A. niger*. *T. reesei* releases a mixture of cellulases: two cellobiohydrolases, five endoglucanases, β-glucosidases and hemicellulases (Zhang and Lynd, 2004). The action of cellobiohydrolases causes a gradual decrease in the polymerization degree. Endoglucanases action results in the rupture of cellulose in smaller chains reducing rapidly the polymerization degree. Endoglucanases especially act on amorphous cellulose, whereas cellobiohydrolases can act on crystalline cellulose as well (Lynd et al., 2002).

Although *T. reesei* produces some β-glucosidases, which hydrolyse cellobiose into two molecules of glucose, their activities are not very high. Unfortunately, cellobiohydrolases are inhibited by the cellobiose. For this reason, β-glucosidase from other microbial source needs to be added. Factorial optimisation techniques have been applied for the design of cellulases mixtures from different sources including β-glucosidase in order to maximise the yield of produced glucose (Kim et al., 1998).

Cellulases should be adsorbed on the surface of substrate particles before hydrolysis of insoluble cellulose takes place. The three-dimensional structure of these particles in combination with their size and shape determines whether β-glucosidic linkages are or are not accessible to enzymatic attack (Zhang and Lynd, 2004). This makes cellulose hydrolysis to be slower compared to the enzymatic degradation of other biopolymers. For instance, the hydrolysis rate of starch by amylases is 100 times faster than hydrolysis rate of cellulose by cellulases under industrial processing conditions.

1.5.5 Fermentation of biomass hydrolysates and process integration.

The classic configuration employed for fermenting biomass hydrolysates involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in

different units. This configuration is known as Separate Hydrolysis and Fermentation (SHF). In the alternative variant, the simultaneous saccharification and fermentation (SSF), the hydrolysis and fermentation are performed in a single vessel. However, when enzymatic hydrolysis is applied, different levels of process integration are possible.

1.5.5.1 Separate Hydrolysis and Fermentation (SHF).

The first application of enzymes to wood hydrolysis in an ethanol process was to simply replace the cellulose acid hydrolysis with a cellulase enzyme hydrolysis step. In the SHF configuration, the joint liquid flow from both hydrolysis reactors first enters the glucose fermentation vessel (Figure 1.2). The mixture is then distilled to remove the ethanol leaving the unconverted xylose behind. In a second reactor, xylose is fermented to ethanol, and the ethanol is again distilled (Grethlein and Dill, 1993). The cellulose hydrolysis and glucose fermentation may also be located parallel to the xylose fermentation.

One of the main features of the SHF process is that each step can be performed at its optimal operating conditions. The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load (Hamelinck et al., 2005).

By testing lignocellulosic material from sugar cane leaves, Hari Krishna et al. (1998) have found the best values of all these parameters. Cellulose conversion of about 65-70% was achieved at 50°C and pH of 4.5. Although enzyme doses of 100 FPU g^{-1} cellulose caused almost a 100% hydrolysis, this amount of enzymes is not economically justifiable. Hence, 40 FPU g^{-1} cellulose dosage was proposed obtaining only 13% reduction in conversion. Regarding the substrate concentration, solids loads of 10% was defined as the most adequate considering arising mixing difficulties and accumulation of inhibitors in the medium.

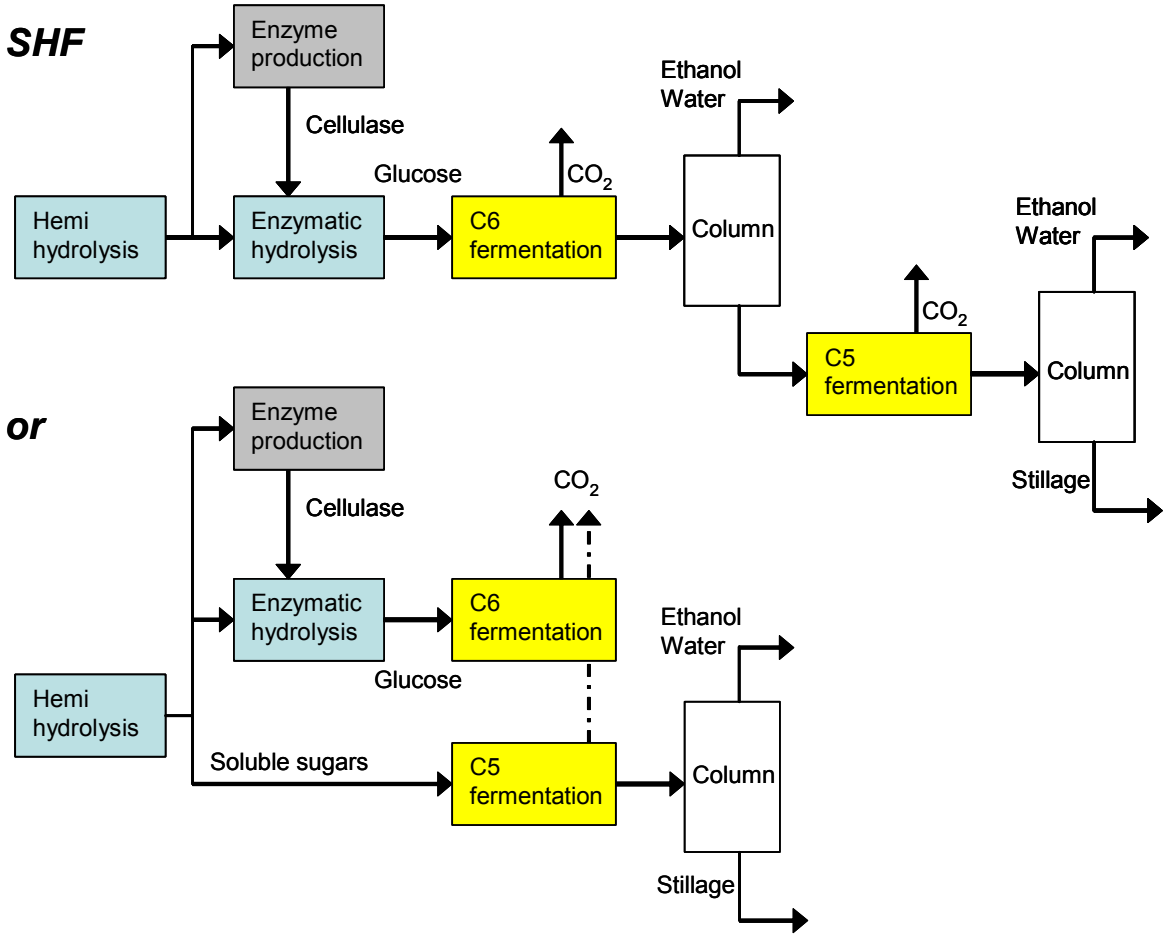


Figure 1.2. Scheme of Separate Hydrolysis and Fermentation (SHF) process for lignocellulosic ethanol (Hemi: Hemicellulose).

The composition of lignocellulosic material has an important influence on the enzyme dosage as described in Foody et al. (2000). In particular, the ratio of arabinan plus xylan to total nonstarch polysaccharides determines its relative cellulase requirement. Therefore, the higher this ratio, the less enzyme is required after the pre-treatment. Feedstocks with values of this ratio over about 0.39 are particularly well suited for a cellulose-to-ethanol process as certain varieties of oat hulls and corn cobs.

Saha and Cotta (2006) obtained 96.7% yield of monomeric sugars using an enzymatic cocktail of cellulase, β-glucosidase and xylanase for saccharification of wheat straw pre-treated by alkaline peroxide method. An ethanol concentration of 18.9 gL^{-1} and a yield of 0.46 g g^{-1} of available sugars were achieved in the subsequent fermentation using a recombinant *E. coli* strain capable of assimilating both hexoses and pentoses.

Park et al. (2001) have studied the hydrolysis of waste paper contained in MSW obtaining significant sugars yield. Bioethanol production from the cellulosic portion of MSW has been already patented (Titmas, 1999). Moreover, some strategies for improving the fermentability of MSW acid hydrolysates have been defined. Nguyen et al. (1999) employed a mixed solids waste for producing ethanol by SHF using yeasts. In this process, a recycling of enzymes was implemented through microfiltration and ultrafiltration achieving 90% cellulose hydrolysis at a net enzyme loading of 10 $FPUg^{-1}$ cellulose.

1.5.5.2 Simultaneous Saccharification and Fermentation (SSF).

The SSF process shows more attractive indexes than the SHF as higher ethanol yields and less energetic consumption. In this case, the cellulases and microorganisms are added to the same process unit (Figure 1.3). Therefore the glucose formed during the cellulose enzymatic hydrolysis should be immediately consumed by the microbial cells converting it into ethanol. Thus, the inhibition effect caused by the sugars over the cellulases is neutralised (Olofsson et al., 2008).

However, the need of employing more dilute media to reach suitable rheological properties makes that final product concentration be low (Sànchez and Cardona, 2008). In addition, this process operates at non-optimal conditions for hydrolysis and requires higher enzyme dosage, which negatively influences on process costs. In SSF there is a trade-off between the cost of cellulase production and the cost of hydrolysis/fermentation. Short hydrolysis reaction times involve higher cellulase and lower hydrolysis fermentation costs than longer reaction times. The optimum is constrained by the cost of cellulase, and is about 3-4 days (Hamelinck et al., 2005).

Considering that enzymes account for an important part of production costs, it is necessary to optimise cellulases dosage. With this aim, surfactants dosage has been proposed. Alkasrawi et al. (2003) showed that the addition of the non-ionic surfactant Tween-20 to the steam exploded wood has some effects: 8% increase in ethanol yield, increase in ethanol productivity and 50% reduction in cellulases dosage (from 44 FPU to 22 FPU g^{-1} cellulose). It is postulated that the surfactant avoids or diminishes the non-

useful adsorption of cellulases to the lignin. However, Saha et al. (2005a) obtained marginal increases (3.5%) in saccharification of rice hulls adding 2.5 gL^{-1} of Tween 20.

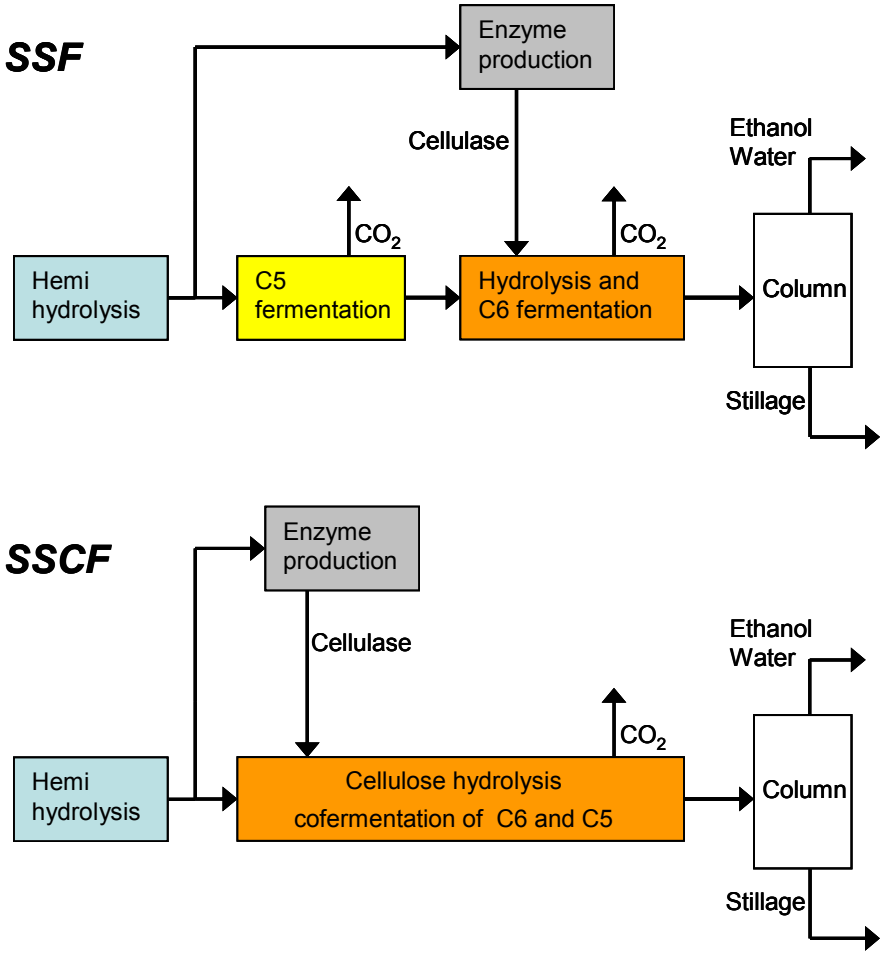


Figure 1.3. Scheme of Simultaneous Saccharification and Fermentation (SSF) and Simultaneous Saccharification and CoFermentation of hexoses and pentoses sugars (SSCF) processes for lignocellulosic ethanol. (Hemi: Hemicellulose).

Hari Krishna et al. (1998) evaluated the optimal conditions of the SSF of sugar cane leaves, as they did for the SHF. These authors defined a temperature of 40°C and pH of 5.1 as the best conditions for 3 day cultivation, achieving 31 gL^{-1} of ethanol. Nevertheless, the enzyme dosage was quite high (100 $FPUg^{-1}$ cellulose).

Varga et al. (2004) proposed a non-isothermal regime for batch SSF process in the case of wet oxidized corn stover: in the first step of the SSF, small amounts of cellulases were added at 50°C to obtain better mixing conditions. In the second step, more cellulases were added along with the yeast *S. cerevisiae* at 30°C. In this way, the final solid concentration in the hydrolysate could be increased up to 17% dry matter concentration achieving 78% ethanol yield.

In general, increased cultivation temperature accelerates metabolic processes and lowers the refrigeration requirements. Yeasts as *K. marxianus* have been tested as potential ethanol producer at temperatures higher than 40°C. Kádár et al. (2004) compared the performance of thermotolerant *K. marxianus* and *S. cerevisiae* during batch SSF of paper sludge. No significant differences between both yeasts were detected at 40°C, although cellulose conversions (55-60%) and ethanol yields (0.30-0.34 g g^{-1} cellulose) were relatively low.

SSF system was further developed in the SSCF process aiming at the Simultaneous Saccharification and Cofermentation of pentoses and hexoses by a single microbe or microbial consortium (Figure 1.3). Lynd (1996) considered the SSCF perspective as a focus for nearterm development, which meanwhile is being tested on pilot scale (Chandrakant and Bisaria, 1998; Lynd et al., 2006; McMillan et al., 1999; Zhang et al., 2009).

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1.5.6 Fermentation of pentoses.

One of the main problems in bioethanol production from lignocellulosics is that *S. cerevisiae* can ferment only certain mono- and disaccharides like glucose, fructose, maltose and sucrose. This microorganism is not able to assimilate cellulose and hemicellulose directly. In addition, pentoses obtained during hemicellulose hydrolysis (mainly xylose and arabinose) cannot be assimilated by this yeast.

Species of bacteria, yeast, and filamentous fungi naturally ferment xylose to ethanol (Jeffries, 1983; Skoog and Hahn-Hägerdal, 1988; Toivola et al., 1984). In the lignocellulosic context and considering modern molecular strain development strategies, each group of microorganisms has its advantages and disadvantages. In Table 1.5, the substrate and product ranges of microorganisms most frequently considered for ethanolic

fermentation of lignocellulosic biomass are summarized. Also, parameters relating to their industrial performance are presented.

Organism	Natural sugar utilisation pathways					Major products		Tolerance			O_2	pH
	Glu	Man	Gal	Xyl	Ara	EtOH	Others	Alcohols	Acids	Hydrolysate		
Anaerobic bacteria	+	+	+	+	+	+	+	−	−	−	−	Neutral
E. coli	+	+	+	+	+	−	+	−	−	−	−	Neutral
Z. mobilis	+	−	−	−	−	+	−	+	−	−	−	Neutral
S. cerevisiae	+	+	+	−	−	+	−	++	++	++	−	Acidic
P. stipitis	+	+	+	+	+	+	−	−	−	−	+	Acidic
Filamentous fungi	+	+	+	+	+	+	−	++	++	++	−	Acidic

Table 1.5. Pros and cons of various natural microorganisms with regard to industrial ethanol production (modified from Hahn-Hägerdal et al., 2007). O_2 indicates the oxygen requirement.

1.5.6.1 Bacteria.

Obligate anaerobic bacteria (Table 1.5) can ferment all lignocellulose-derived sugars, including their oligomers and polymers, to ethanol, other solvents, and acids (Wiegel and Ljungdahl, 1986).

Because these bacteria are more severely inhibited than other bacteria by high sugar concentrations and moderate concentrations of ethanol and acids, efforts are being made to isolate sugar- and ethanol-tolerant variants (Fong et al., 2006; Sommer et al., 2004).

So far their fermentative performance has only been investigated in dilute alkali-treated hydrolysate. Nevertheless, anaerobic bacteria have an established industrial record for the production of acetone and butanol, most recently in the former Soviet Union and in South Africa. However, these processes could not compete in the market economy of the 1990s. Also, the use of obligate anaerobic bacteria is hampered by the lack of simple and efficient molecular biology tools for genetic engineering; however, protocols for thermophilic anaerobes are being developed (Tyurin et al., 2005).

Ethanol-producing bacteria generally display mixed acid product formation where ethanol is a minor product. Furthermore, their optimal pH around 6-7 makes bacterial fermentation susceptible to infection and their low tolerance to lignocellulose-derived inhibitors requires a detoxification step to be included in the fermentation process (Hahn-Hägerdal et al., 1994). Nevertheless, the presently most efficient microorganisms for fermentation of detoxified lignocellulose hydrolysates are recombinant strains of *E. coli* (Bothast et al., 1999; Dien et al., 2003; Hespell et al., 1996; Ingram et al., 1987).

In contrast to other bacteria, *Z. mobilis* (Table 1.5) produces ethanol with stoichiometric yields. It also displays high specific ethanol productivity (Lee et al., 1979; Rogers et al., 1979). Despite intensive efforts over the past 20 years, the industrial exploitation of *Z. mobilis* has so far not achieved.

In relation to the variety of sugars present in lignocellulosic raw materials, the substrate range of *Z. mobilis* is limited. Recombinant xylose- and arabinose-fermenting strains, capable to ferment these sugars in detoxified lignocellulose hydrolysates, have been constructed (Deanda et al., 1996; Mohagheghi et al., 2002; Zhang et al., 1995). However, *Z. mobilis* would also need pathways for the metabolism of mannose and galactose, which constitute a considerable fraction of some lignocellulosic raw materials (Hayn et al., 1993).

1.5.6.2 Yeasts.

Although a large number of yeast species metabolise xylose and arabinose and display fermentative capacity (Barnett, 2000), only approximately 1% of them are capable of fermenting xylose to ethanol. No arabinose-fermenting yeast was found in an early screening study (McMillan and Boynton, 1994), while a subsequent work identified four yeast species able to ferment arabinose to ethanol (Dien et al., 1996). The discrepancy between these studies is most likely due to that the latter screen used a complex (YP) medium containing yeast extract and peptone, which have compounds that may act as electron acceptors and thus aid conversion of arabinose to ethanol.

The requirement for electron acceptors translates to very low, carefully controlled, levels of oxygen required for maximum ethanol production from arabinose and xylose by these yeasts (Fonseca et al., 2007). However, such precise oxygenation is technically impossible

to maintain in large-scale industrial conditions, with concomitant reduced product yield. Also, the naturally pentose-fermenting yeasts are generally inhibited by industrial substrates (Hahn-Hägerdal et al., 2007; Hahn-Hägerdal and Pamment, 2004; Olsson et al., 1992) and do not grow under anaerobic conditions even on hexose sugars (Visser et al., 1990).

S. cerevisiae.

S. cerevisiae has traditionally been used in large-scale fermentation of sugar- and starch-based raw materials and it is therefore well adapted to the industrial context. It produces ethanol with stoichiometric yields and tolerates a wide spectrum of inhibitors and elevated osmotic pressure. Its superiority in fermenting non detoxified lignocellulose hydrolysates has been demonstrated (Hahn-Hägerdal et al., 1994, Hahn-Hägerdal and Pamment, 2004; Olsson et al., 1992;). Moreover, *S. cerevisiae* usage in lignocellulosic hydrolysates has the potential of integrating large-scale lignocellulosic ethanol processes into the existing sugar cane- and starch-based ethanol plants already using this yeast. The only, but major, inconvenience to use *S. cerevisiae* for lignocellulosic fermentation is its inability to metabolise and ferment the pentose sugars xylose and arabinose to ethanol.

To develop industrial hexose- and pentose-fermenting strains, genetic engineering of *S. cerevisiae* has been applied with several approaches (reviewed by Hahn-Hägerdal et al., 2007). Based on current knowledge of pentose metabolism, numerous metabolic engineering strategies have been explored in laboratory strains of *S. cerevisiae* to determine their effect on fermentation of xylose and arabinose.

Only a limited number of industrial pentose-fermenting strains has been described in literature. All genetic engineering has been limited to the introduction of the initial xylose and arabinose utilisation pathways. Further improvement of the recombinant strains was achieved by adaptation strategies, including random mutagenesis (Wahlbom et al., 2003) as well as evolutionary engineering and breeding (Sonderegger et al., 2004a; Sonderegger et al., 2004b). Industrial xylose-fermenting strains of *S. cerevisiae* are now reaching levels of fermentation performance that approach economically feasible ethanol production from lignocellulose. For instance, the yeast TMB3400 showed interesting ethanol yields in a fed-batch SSF fermentation setup of non detoxified corn stover hydrolysate (Öhgren et al.,

2006). Only recently has the development of industrial arabinose-fermenting *S. cerevisiae* strains been initiated (Karhumaa et al., 2006). Moreover, the simultaneous cofermentation of hexose and pentose sugars constitutes the major strain engineering challenge.

1.6 Consolidated BioProcessing (CBP) for bioethanol production.

Biomass is the only foreseeable renewable feedstock for sustainable production of biofuels. The main technological impediment to more widespread utilisation of this resource is the lack of low-cost technologies to overcome the recalcitrance of the cellulosic structure (Lynd et al., 2002).

As discussed above, four biological steps occur during conversion of lignocellulose to ethanol: production of saccharolytic enzyme (cellulases and hemicellulases), hydrolysis of the polysaccharides present in pre-treated biomass, fermentation of hexose sugars, and fermentation of pentose sugars (van Zyl et al., 2007). The hydrolysis and fermentation steps have been combined in Simultaneous Saccharification and Fermentation (SSF) of hexoses and Simultaneous Saccharification and Cofermentation (SSCF) of both hexoses and pentoses schemes (Figure 1.4).

The ultimate objective would be a one-step "consolidated" bioprocessing (CBP) of lignocellulose to bioethanol, where all four of these events occur in one reactor and are mediated by a single microorganism or microbial consortium able to ferment pre-treated biomass without added saccharolytic enzymes (Figure 1.4).

CBP is gaining increasing recognition as a potential breakthrough for low-cost biomass processing. A four-fold reduction in the cost of biological processing and a two-fold reduction in the cost of processing overall is projected when a mature CBP process is substituted for an advanced SSCF process featuring cellulase costing US $0.10 per gallon ethanol (Lynd et al., 2006).

The detailed analysis of mature biomass conversion processes by Greene et al. (2004) found CBP to be responsible for the largest cost reduction of all R&D-driven improvements. Moreover, a recent report entitled *Breaking the Biological Barriers to Cellulosic Ethanol* states: "CBP is widely considered to be the ultimate low-cost configuration for cellulose hydrolysis and fermentation" (US DOE, 2006).

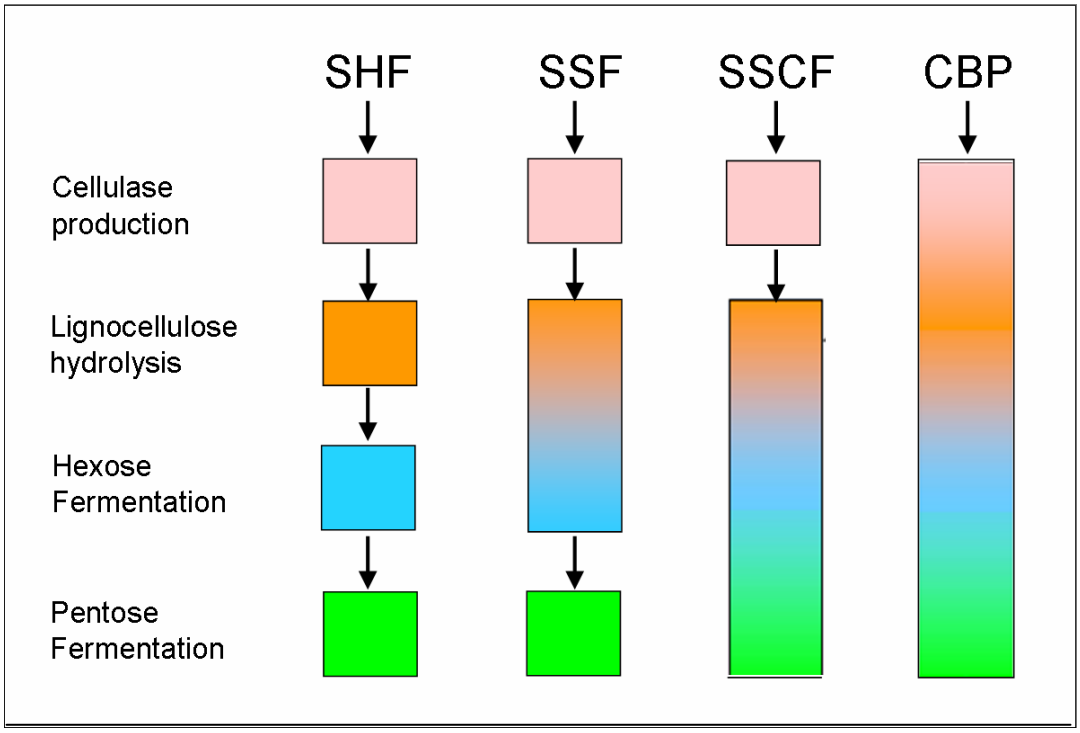

Figure 1.4. Scheme of Consolidated Bioprocessing (CBP) for ethanol production as integration of the other systems developed for lignocellulosic biomass: SHF: Separate Hydrolysis and Fermentation, SSF: Simultaneous Saccharification and Fermentation and SSCF: Simultaneous Saccharification and CoFermentation of hexoses and pentoses sugars. (modified from Lynd, 1996).

Recent studies of naturally occurring cellulolytic microorganisms provide increasing indications that CBP is feasible. Lu et al. (2006) showed that cellulase-specific cellulose hydrolysis rates exhibited by growing cultures of *Clostridium thermocellum* were 20-fold higher than the specific rates exhibited by the *T. reesei* cellulase system. The substantial part of this difference resulted from "enzyme-microbe synergy".

Although no natural microorganism exhibits all the features desired for CBP, a number of microorganisms, both bacteria and fungi, possess some of the desirable properties. These microorganisms can be divided into two groups: (1) native cellulolytic microorganisms having superior saccharolytic capabilities, but not necessarily product formation, and (2) recombinant cellulolytic microorganisms that naturally give high product yields, but into which saccharolytic systems need to be engineered.

Examples of native cellulolytic microorganisms include anaerobic bacteria with highly efficient complexed saccharolytic systems, such as mesophilic and thermophilic

Clostridium species (Demain et al., 2005; Lynd et al., 2005). Moreover there are also fungi that naturally produce a large repertoire of saccharolytic enzymes, such as *Fusarium oxysporum* (Panagiotou et al., 2005; Singh and Kumar, 1991).

However, the anaerobic bacteria produce a variety of fermentation products (Table 1.5), limiting the ethanol yield, whereas the filamentous fungi are slow cellulose degraders with low ethanol yields. Candidates considered as potential recombinant cellulolytic microorganisms into which saccharolytic systems have been engineered include the bacteria *Z. mobilis*, *E. coli* and *Klebsiella oxytoca*, and the yeast *S. cerevisiae* and xylose-fermenting yeasts *Pachysolen tannophilus*, *Pichia stipitis* and *Candida shehatae*.

Significant advances related to recombinant enzyme expression support the great potential for *S. cerevisiae* as a CBP host (Lynd et al., 2005; van Zyl et al., 2007). However, the challenge of integrating all the different aspects of enzymatic hydrolysis and subsequent fermentation of the released sugars to ethanol in a single reactor with a CBP should not be underestimated. A pertinent question often asked by critics is, "Would *S. cerevisiae* be able to simultaneously express multiple genes, while producing and secreting the different cellulases, hemicellulases, and pentose utilizing enzymes required?" (van Zyl et al., 2007). Several studies demonstrate coexpression of multiple genes in *S. cerevisiae*, for example in the case of the expression of tethered cellulolytic and xylanolytic enzymes (Fujita et al., 2004; Katahira et al., 2004), xylose and arabinose utilizing enzymes (Becker and Boles, 2003), as well as xylose and cellooligosaccharide utilizing enzymes (Katahira et al., 2006). The expression and secretion of a variety of cellulases, amylases, and pectinase has also been demonstrated without adversely affecting yeast growth (Petersen et al., 1998; Van Rensburg et al., 1998)

However, the number of genes expressed is probably not as important a challenge as the need for high-level expression as well as the stress responses that may accompany such high-level expression. Main factors that could impose unnecessary stress on the host cell are:

1. sequestering of transcription factors at highly expressed promoters used for heterologous gene expression;

2. impact of unfavorable codon bias on the translation of heterologous protein (can be overcome by the use of codon-optimized synthetic genes);
3. improper folding of foreign proteins.

Therefore the proper strategy would not be the sole overexpression of all the required genes to ensure a functional CBP yeast with the desirable enzymatic activities. More attention should also be devoted to the careful manipulation of the enzyme activities and producing them at the right concentration.

Essentially all work aimed to efficient heterologous expression of saccharolytic enzymes in yeast has involved laboratory strains. Much of this work has to be transferred to industrial strains that provide the fermentation capacity and robustness desired for industrial processes.

Different strategies have been used for the overexpression of multiple genes in industrial *S. cerevisiae* strains. High copy-number episomal YEp vectors, often using the two-micron Autonomous Replicating Sequence (ARS), have been very helpful in demonstrating proof of concept in laboratory strains of *S. cerevisiae* (Den Haan et al., 2006; La Grange et al., 2001; Van Rooyen et al., 2005). However, these constructs are usually mitotically unstable and require selection for the episomal plasmid, which often means using a defined medium that is not applicable to industrial uses (Romanos et al., 1992).

The preferred route taken for industrial strains has been the use of integrative YIp vectors that facilitate direct integration of foreign expression cassettes into a target gene on the yeast genome or recycling dominant selectable markers for multiple integration. Although these methods provide stable expression from the yeast genome and are amendable to industrial strains, the major drawback has been low expression levels.

Different approaches have been pursued in order to combine the advantages of overexpression from multicopy plasmids with the stability of chromosomal integration, which is also applicable to industrial strains when dominant selectable markers are used. These include the use of repetitive chromosomal DNA sequences such as rDNA and δ-sequences (Lee and Silva, 1997). There are approximately 140-200 copies of rDNA existing in the haploid yeast genome; however, rDNA is located in the nucleolus, which may affect the accessibility to RNA polymerase II transcription. Also, the size of pMIRY

(multiple integration into ribosomal DNA in yeast) vectors could determine the mitotic stability of these multiple integrations (Lopes et al., 1996).

The δ-sequences are the long terminal repeats of *S. cerevisiae* retrotransposon Ty. More than 400 copies of δ-sequences can exist either Ty associated or as sole sites in the haploid yeast genome (Dujon, 1996). δ-Integration thus makes it possible to integrate more copies of a gene into the yeast genome than the conventional integration systems. Host strains and integrated gene size can significantly affect the transformation efficiency at δ-sequences; however, the transformation frequency can be 10- to 100-fold those obtained when transforming with vectors that target a single gene on the yeast genome.

A more strategic approach would be required to design a yeast that produces the proper enzyme activities, yet retains the competence to still perform well under industrial conditions. Such a strategy will most probably start by building on a platform industrial yeast that cometabolises hexoses and pentoses, and subsequently finding the right combination and level of expression for saccharolytic enzymes (van Zyl et al., 2007).

This approach will use reiterated metabolic engineering and flux analysis, selection and mutagenesis strategies, and strain breeding to allow the microorganism itself to overcome rate-limiting hurdles toward developing an efficient CBP yeast. Examples of such approaches in the past have been performed to enhance xylose fermentation in laboratory and industrial strains (Kuyper et al., 2005; Wahlbom et al., 2003).

1.6.1 Reasons for developing a CBP microbe for starch conversion.

The industrial process of converting starchy feedstocks (mainly corn as crop and cereal bran as by-products) into ethanol involves four steps: the extraction of starch from biomass, the conversion of the starch to glucose, the fermentation to ethanol and the alcohol distillation.

Enzymatic hydrolysis is initiated when starch is pre-treated to yield a viscous slurry, which is then liquefied by heat treatment and α-amylase (Figure 1.5). The starch is cooked and undergoes saccharification after addition of glucoamylase. Yeast is added after cooling the mixture for fermentation of sugars to ethanol. The process includes large temperature changes (30-120°C) using large amounts of heating energy (Kelsall and Lyons, 2003).

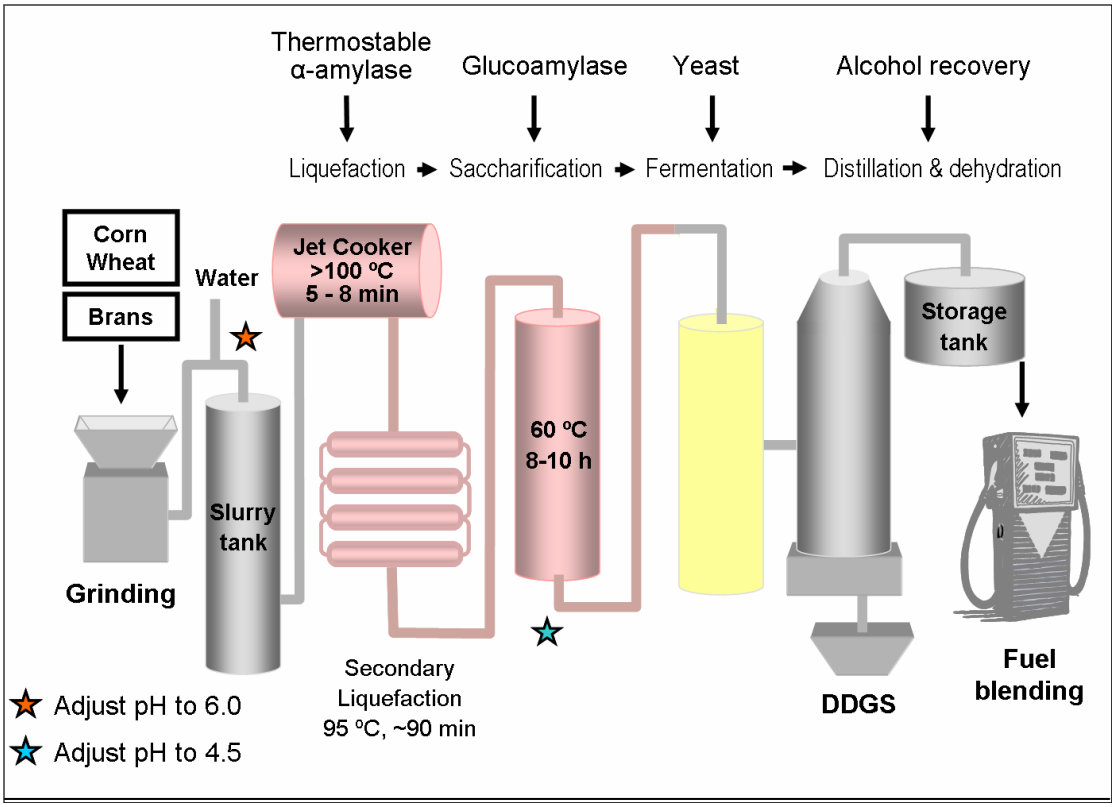

Figure 1.5. Conventional ethanol production process using starchy materials as feedstock (modified from: de Villiers, 2008; Genencor website).

Addition of caustic soda, lime, and sulphuric acid to maintain pH levels suitable for the enzymes, as well as urea as nitrogen source for the yeast, results in high product cost (McAloon et al., 2000).

The energy balance of corn to ethanol has raised some concern in the industry. Reports have however indicated that the balance is positive, even before subtracting energy which is allocated to co-products. This was indicated by an energy output/input ratio of 1.3 (Farrell et al., 2006). A comparison of six studies reporting on the net energy balance has indicated a positive net energy of 4-9 MJ L^{-1} ethanol. Yet another study comparing six starch to ethanol scenarios, and four cellulose to ethanol scenarios reads: "It is safe to say that corn ethanol reduces fossil fuel and oil consumption when used to displace gasoline" (Hammerschlag, 2006).

In order to design a more energy-efficient ethanol production process, the enzymes used for biomass hydrolysis should be more proficient and less expensive (Gray et al., 2006; Nigam and Singh, 1995).

With the intention to increase net energy yield, the hydrolysis temperature required to generate glucose could be lowered to that of the fermentation step in a SSF process (Devantier et al., 2005; Lynd et al., 1999). Lowering the temperature also adds the benefit of decreasing the viscosity of the generated slurry (Kelsall and Lyons, 2003). Thermal treated slurries complicate pumping and stirring of the material. An additional benefit would be that lower temperatures minimise the formation of unwanted Maillard reaction co-products, which could reduce glucose yield for fermentation.

A raw starch hydrolysing (RSH) enzyme cocktail, Stargen 001 (Genencor) was developed, which converts starch into dextrins at low temperatures (<48°C) and hydrolyses dextrins into sugars during SSF. The cocktail contains an acid-stable α-amylase from *Aspergillus kawachi* and glucoamylase from *A. niger*. Comparable ethanol conversion efficiencies, ethanol yields, and Distillers Dried Grains and Solubles (DDGS) yields were reached using the RSH enzyme (Wang et al., 2007). The RSH application could save heating energy as jet cooking is eliminated and less water and fewer chemicals are needed for the process. One disadvantage in converting raw starch to ethanol at a lower temperature is the risk of contamination of fermentation broth. Contamination is usually controlled in the jet cooking stage of a conventional starch-to-ethanol plant (Shigechi et al., 2004a).

To eliminate commercial enzyme costs, SSF has been performed effectively with mixed cultures, where one organism is amylolytic, and the other responsible for ethanol production (Han and Steinberg, 1987; Kurosawa et al., 1989; Tanaka et al., 1986). The amylolytic organism acts as the saccharifying agent, therefore replacing the addition of commercial enzymes. Up to 9.7 g L^{-1} ethanol was recorded during SSF with *Saccharomycopsis fibuligera* and *Z. mobilis* after 25 hours of cultivation with an initial soluble starch concentration of 30 g L^{-1} (Dostalek and Haggstrom, 1983). The volumetric productivity of ethanol was 0.54 (g L^{-1}) h^{-1} and the ethanol yield was calculated as 0.48 gram ethanol per gram available sugar from starch (g g^{-1}), which corresponds 86% of the

theoretical maximum from starch. A mixed culture of *A. awamori* and *Z. mobilis* produced up to 25 g L^{-1} ethanol with initial soluble starch concentration of 100 g L^{-1} (Tanaka et al., 1986). The ethanol yield was of 0.38 g g^{-1} (68% of theoretical maximum yield). However, the main drawback in these systems is that the amylolytic organism utilises most of the soluble starch for growth. Therefore, low sugar amounts are left for the fermentative organism to convert to ethanol.

Developing an amylolytic fermentative organism may solve this limitation. The resulting starch-to-ethanol process could be more cost-effective by using an organism that produces sufficient amounts of amylolytic enzymes to sustain growth on raw unmodified starch and that convert glucose into ethanol as main product (Figure 1.6). The engineered organism producing amylolytic enzymes and ethanol would be suitable for a Consolidated Bioprocessing application.

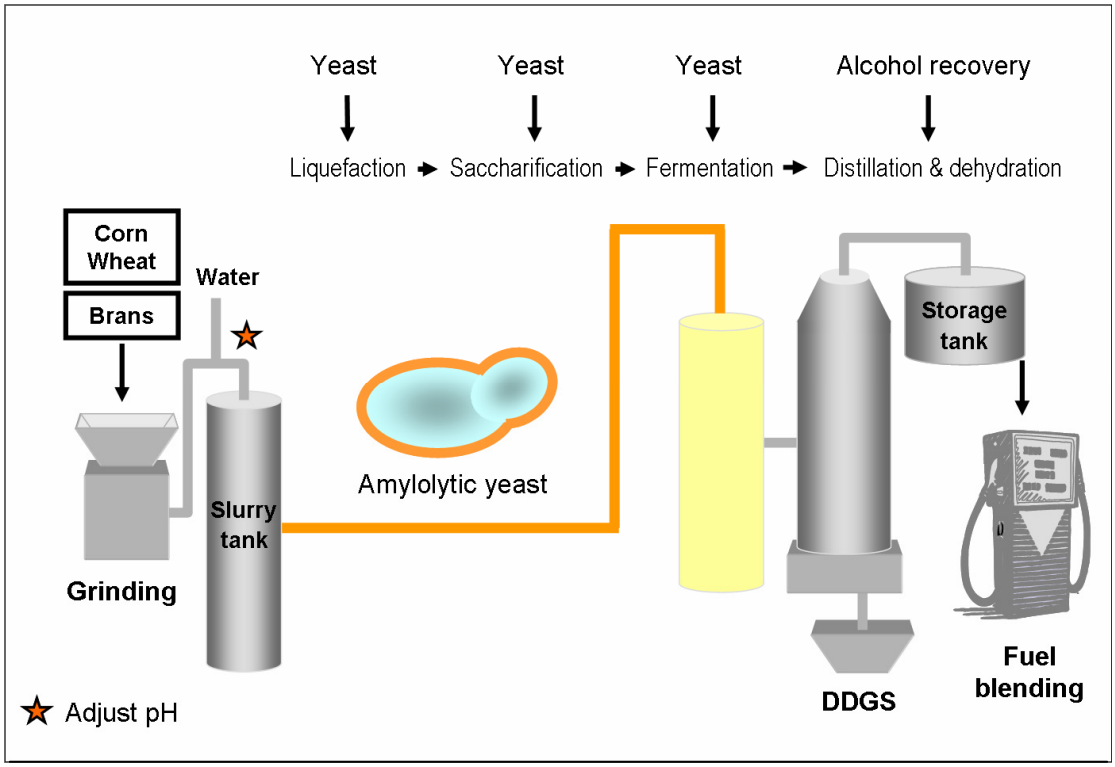

Figure 1.6. Consolidated BioProcessing of starchy feedstocks into ethanol. Amylolytic yeast is introduced to liquefy, saccharify and ferment raw starch to ethanol in one-step process (modified from: de Villiers, 2008; Genencor website).

1.6.2 Bioconversion of starch.

1.6.2.1 Starch composition.

Starch is abundant in various higher plants, and as the primary source of carbohydrate may account for 20-70% of the dry weight of some plants (Solomon, 1978). Synthesis of the α-1,4 glucan-linked D-glucopyranose chains is localised in chloroplasts of green photosynthetic tissues, or in amyloplasts of non-green storage tissues (Thomas and Atwell, 1999). Polymerisation of glucose results in amylose and amylopectin polymers.

Linear amylose chains (MW of 10^5-10^6 Da; DP 500-5000) are composed of α-1,4-linked D-glucopyranose units. A very small portion of α-1,6-linked branches have however been identified on the amylose polymer. Amylose chains are organised in helixes (Figure 1.7).

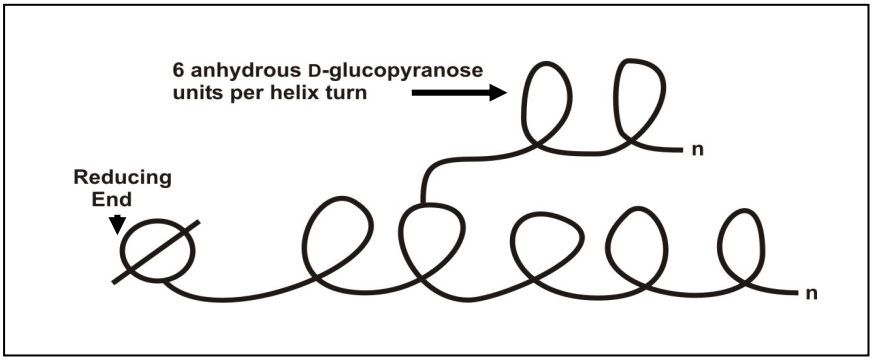

Figure 1.7. Simplified representation of an amylose helix chain (Thomas and Atwell, 1999).

Amylopectin (10^7-10^9 Da) is more complex than amylose as α-1,4 glucan chains are added onto existing α-1,4 glucan-linked chains via α-1,6-linkages at branching points. As shown in Figure 1.8, the chains are highly branched with a tumbleweed-like structure and include helixes, double helixes, and packed clusters (Buléon et al., 1998; Whistler and BeMiller, 1997).

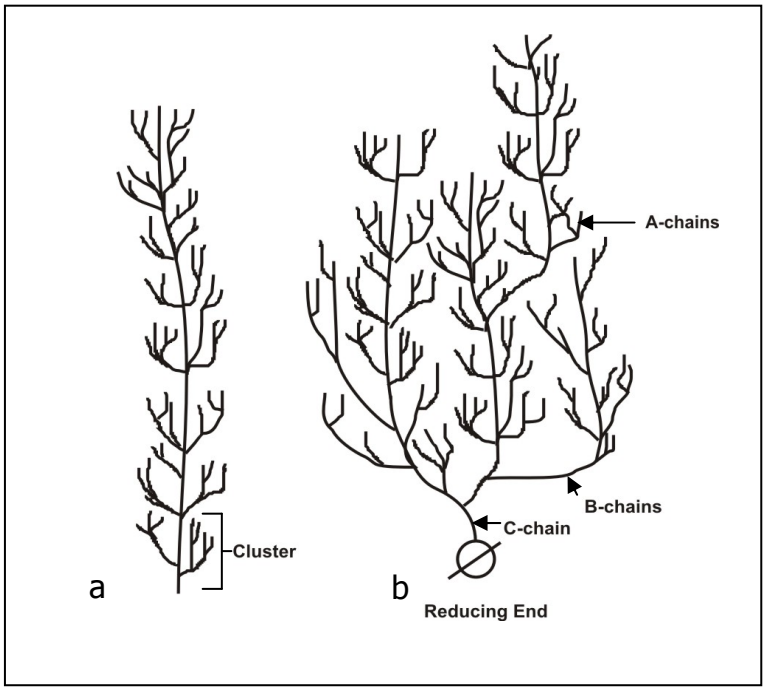

Figure 1.8. Simplified representation of a portion of: (a) amylopectin molecule and (b) the typical packed clusters of amylopectin (adapted from Buléon et al., 1998; Thomas and Atwell, 1999).

1.6.2.2 Enzymes involved in starch degradation.

Starch-hydrolysing enzymes are widely distributed in the animal, microbial and plant kingdoms. Efficient starch hydrolysis needs the activities of both α-1,4 and α-1,6-hydrolysing enzymes. Four groups of starch converting enzymes confer this activity and include endo-amylases, exo-amylases, debranching enzymes and transferases. As reported in Paragraph 1.4, α-amylases and glucoamylases play the most important role in starch bioconversion to ethanol in the industry, and these enzymes will be described in more detail below. Endo-amylases display α-1,4-cleaving activity and include the α-amylases (EC 3.2.1.1). Exo-amylases such as β-amylases (EC 3.2.1.2) cleave α-1,4 glycosidic bonds only, whereas glucoamylases (EC 3.2.1.3) as well as α-glucosidases display both α-1,4- and α-1,6-debranching activities (Nigam and Singh, 1995; Vihinen and Mantsiila, 1989).

α-Amylase.

α-Amylases are endo-hydrolases that hydrolyse the α-1,4-D-glucosidic linkages in polysaccharides containing three or more α-1,4-linked glucose units. The endo-action occurs in random manner to liberate reducing groups with the α-configuration. The term 'α' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed. Hydrolysis reduces the molecular size of starch and therefore the viscosity of the starch solution (Solomon, 1978). Hydrolysis of amylose liberates maltose and maltotriose, but as maltotriose is a poor substrate for α-amylase, the second stage of hydrolysis of maltotriose to maltose and D-glucose is very slow, and only takes place if large amounts of enzyme are available (Walker and Whelan, 1960).

Microbial α-amylases are not able to hydrolyse 1,6-linked units, and therefore a number of α-limit dextrins containing at least one 1,6-linkage are also generated when starch is hydrolysed (Kennedy et al., 1987).

All α-amylases are dependent on at least one calcium ion per mole enzyme for enzyme activity and conformational stability (Hsiu et al., 1964; Imanishi, 1966; Saboury, 2002). Amylases display a typical bell-shaped curve when activity at different pHs is plotted. The maximum activities of the enzymes seem to be in the acidic range of pH 4.5-7.0.

Glucoamylase.

Glucoamylases (1,4-α-D-glucan glucohydrolase EC 3.2.1.3) are inverting enzymes and hydrolyse the terminal 1,4-linked α-D-glucopyranosyl residues successively from non-reducing ends of starch chains. The resulting product is D-glucose. The enzyme acts more rapidly on substrates as the degree of polymerisation increases (Belshaw and Williamson, 1993; Reese et al., 1968).

Most forms of the enzyme can hydrolyse α-1,6-D-glucosidic bonds when the next bond in the sequence is 1,4-linked (Fierobe et al., 1998). The rate of hydrolysis between linkages depends on the nature of the linkage in the molecule adjacent to that of the linkage being hydrolysed. The specific activity towards the 1,6-linkage is however only 0.2% of that for the 1,4-linkage (Fierobe et al., 1996; Frandsen et al., 1995; Hiromi et al., 1966; Kennedy et al., 1987).

Complete conversion to D-glucose is unfeasible when high concentrations of α-limit dextrins are hydrolysed with glucoamylase. The D-glucose yield reaches a maximum and then decreases, as glucoamylases are capable of reforming 1,3-, 1,4-, and 1,6-linkages between α-D-glucopyranosyl residues in the presence of high D-glucose concentrations (Kennedy et al., 1987).

Glucoamylases from *Aspergillus* strains tend to have an optimum activity in pH range 4.5-5.0, and *Rhizopus* glucoamylases in the range of 4.5-5.5. Glucoamylases are relatively stable at higher temperatures, with *Aspergillus* enzymes having greater thermal stability than *Rhizopus* enzymes, and both *Aspergillus* and *Rhizopus* enzymes being more stable than *Endomyces* species (Kennedy et al., 1987).

1.6.3 Development of amylolytic *S. cerevisiae* strains.

The amylase genes from certain microbes have been expressed in *S. cerevisiae* to develop amylolytic yeasts (de Moraes et al., 1995; Eksteen et al., 2003a; Knox et al., 2004; Kondo et al., 2002; Ma et al., 2000). These include secretion of the heterologous enzymes, and/or anchoring the enzymes on the cell wall of the yeast. When the enzymes are displayed on the cell wall, a true biocatalyst organism is generated, as the yeast can be re-used for consecutive fermentations. Secretion of enzymes ensures that the enzyme moves freely in the fermentation. Displaying the enzymes on the cell wall is disadvantaged in this way, as the cells need to be in close proximity to the substrate in order for it to be hydrolysed. Therefore, mixing plays an important role during fermentation. The whole-cell biocatalysts however have the advantage of lowering the risk of contamination during fermentation, as glucose is generated near to the cell wall and is utilised immediately by the yeast.

1.6.3.1 Soluble starch fermenting yeasts.

Several strains have been engineered in the last 20 years for the conversion of soluble or cooked starch to ethanol. Ethanol yields, volumetric productivities or specific productivities

of the most promising amylolytic yeasts are reported in Table 1.5. Values were calculated from available data reported in the different studies.

With the aim of develop novel whole-cell biocatalysts, yeasts for cell surface display of glucoamylase were constructed (Murai et al., 1998; Murai et al., 1999; Ueda and Tanaka, 2000). The *R. oryzae* glucoamylase was displayed on the surface of *S. cerevisiae*, which enabled the yeast to grow on soluble starch during aerobic cultivation.

The *R. oryzae* glucoamylase was also inserted on the cell wall of a flocculent yeast strain, which produced ethanol very effectively in soluble starch medium with a yield of about 0.53 gram ethanol per gram fermentable sugar (Kondo et al., 2002).

The strain was further improved by the addition of the *Bacillus stearothermophilus* α-amylase. An ethanol concentration of 60 g L^{-1} was reached after a 100 h fed-batch soluble starch fermentation (Shigechi et al., 2002). It has to be noted however that a very high cell load was added to yield these results (about 30 g L^{-1} dw cells). These yeast also do not have the ability to utilise raw corn starch (Shigechi et al., 2004b).

Two α-amylase genes from *Lipomyces kononenkoae* were integrated into the *S. cerevisiae* genome (Eksteen et al., 2003b). The strain produced 6.1 gL^{-1} ethanol after 156 hours of fermentation in a 2% starch medium with an ethanol yield of 0.38 g g^{-1}. The strain was improved by the Knox et al. (2004) group: the *sfg1* glucoamylase from *S. fibuligera* and *lka1* α-amylase from *L. kononenkoae* were integrated into yeast genome for secretion. Up to 21 g L^{-1} ethanol was produced after 120 hours of fermentation from soluble starch with a volumetric productivity of 0.175(g L^{-1}) h^{-1} and yield of 0.40 g g^{-1} (Knox et al., 2004).

1.6.3.2 Raw starch fermenting yeast strains.

Very few groups have reported results on yeasts able to utilise raw starch as carbon source. A summary of the strains grown in raw starch is presented in Table 1.6. All approaches to date utilise the *R. oryzae* glucoamylase, which is secreted or displayed on the surface of the yeast. Several strains have been engineered where different α-amylases have been combined with the *R. oryzae* glucoamylase with the aim of improving the yeast amylolytic activity and therefore the ethanol productivity.

Source of gene/s [a]	Inoculum and media [b]	Ethanol[c]	Ethanol productivity (Q)[d] or specific productivity (q)[e]	Ethanol yield [f]	Reference
R. oryzae glucoamylase gene fused to α-agglutinin for cell surface display on flocculent yeast strain.	13 g L^{-1} dw cells in 40 g L^{-1} soluble starch and 5 g L^{-1} glucose	25 g L^{-1} after 30 h	0.71 g L^{-1} h^{-1} 0.190 g (g dw cells)$^{-1}$ h^{-1}	0.53	(Kondo et al., 2002)
R. oryzae glucoamylase displayed on yeast surface.	30 g L^{-1} dw cells in 50 g L^{-1} cooked corn starch	13 g L^{-1} after 48 h	0.42 g L^{-1} h^{-1}	0.50	(Murai et al., 1997; Shigechi et al., 2004a)
R. oryzae glucoamylase and *B. stearothermophilus* α-amylase displayed on yeast surface.	30 g L^{-1} dw cells in 50 g L^{-1} cooked corn starch	18 g L^{-1} after 36 h	1.25 g L^{-1} h^{-1}	0.51	(Shigechi et al., 2004a)
R. oryzae glucoamylase displayed on yeast surface and *B. stearothermophilus* α-amylase secreted.	30 g L^{-1} dw cells in 50 g L^{-1} cooked corn starch	17 g L^{-1} after 48 h	0.64 g L^{-1} h^{-1}	0.49	(Shigechi et al., 2004a)
lka1 and *lka2* α-amylases from *Lipomyces kononenkoae* integrated for secretion.	2 g L^{-1} dw cells in 20 g L^{-1} soluble starch	6.1 g L^{1} after 156 h		0.38	(Eksteen et al., 2003b)
sfg1 glucoamylase from *S. fibuligera* and *lka1* α-amylase from *L. kononenkoae* integrated for secretion.	5% v/v inoculum in 55 g L^{-1} soluble starch	21 g l^{-1} after 120 h and	0.175 g L^{-1} h^{-1} 0.042 g (g dw cells)$^{-1}$ h^{-1}	0.40	(Knox et al., 2004)

Table 1.5. Ethanol concentration, production and yield from amylolytic yeasts cultivated in soluble or cooked corn starch.

[a] Recombinant host was *S. cerevisiae* in all cases, [b] batch fermentation, [c] ethanol concentration (g L^{-1}), [d] Q: ethanol volumetric productivity (g L^{-1})h^{-1},

[e] q: ethanol specific productivity (g (g dw cells)$^{-1}$ h^{-1}, [f] ethanol yield as g (g consumed sugar)$^{-1}$.A blank space indicates that not enough data was presented to determine the value.

Source of gene/s [a]	Inoculum and media [b]	Ethanol[c]	Ethanol productivity (Q)[d] or specific productivity (q)[e]	Ethanol yield [f]	Reference
S. diastaticus glucoamylase secreted from yeast.	50 g L^{-1} soluble starch	14.3 g L^{-1} after 140 h	0.16 (g L^{-1})h^{-1}	0.53	(Nakamura et al., 1997)
A. awamori glucoamylase secreted from yeast.	1% v/v inoculum in 100 g L^{-1} soluble starch	44.8 g L^{-1}	0.030 g (g dw cells)$^{-1}$ h^{-1}	0.48	(Inlow et al., 1987)
A. awamori glucoamylase and *B. subtilis* α-amylase secreted as separate polypeptides from yeast.	10% v/v inoculum in 100 g L^{-1} or	43.8 g L^{-1} after 120 h	0.045 g (g dw cells)$^{-1}$ h^{-1}	0.44	(Birol et al., 1998; de Moraes et al., 1995)
	50 g L^{-1} soluble starch	18.8 gL^{-1} after 120 h	0.0429g (g dw cells)$^{-1}$ h^{-1}	0.38	
A. awamori glucoamylase and *B. subtilis* α-amylase secreted as separate polypeptides from *S. cerevisiae.*	10% v/v inoculum in 40 g L^{-1} soluble starch with 4 g L^{-1} glucose	21.5 g L^{-1} after 70 h	0.31 (g L^{-1})h^{-1}	0.54	(Ülgen et al., 2002)

Table 1.5. Ethanol concentration, production and yield from amylolytic yeasts cultivated in soluble or cooked corn starch (continued).

[a] Recombinant host was *S. cerevisiae* in all cases, [b] batch fermentation, [c] ethanol concentration (g L^{-1}), [d] Q: ethanol volumetric productivity (g L^{-1})h^{-1},

[e] q: ethanol specific productivity g (g dw cells)$^{-1}$ h^{-1}, [f] ethanol yield as g (g consumed sugar)$^{-1}$.A blank space indicates that not enough data was presented to determine the value.

The first work described a raw starch fermenting yeast which secreted the *Rhizopus* glucoamylase (Ashikari et al., 1989a; Ashikari et al., 1989b). The recombinant yeast was grown in ground corn starch for 120 hours. The ethanol production was consistent (up to 51 gL^{-1}) with a yield of 0.20 g g^{-1}.

In order to increase the fermentative ability of strains, α-amylase genes were introduced to assist the glucoamylase. The yeast developed to display glucoamylase from the cell surface by Murai et al. (1997) showed interesting fermentation performance. The engineered strain was able to grow on ground raw corn and produce ethanol at a concentration of 23.4 gL^{-1} with a yield of 0.13 g g^{-1}. The fermentation efficiency of this strain was however lower than that of strains secreting the glucoamylase. The fermentation yield was increased with the addition of a α-amylase preparation from *Bacillus licheniformis* to liquefy the starch material before fermentation (Murai et al., 1998).

Shigechi et al. (2004b) replaced the *B. stearothermophilus* α-amylase from a previously engineered strain with the α-amylase of *Streptococcus bovis*. The new recombinant strain displayed the α-amylase together with the *R. oryzae* glucoamylase on the surface of the yeast and produced up to 61.8 gL^{-1} ethanol after 72 hours of fermentation in raw starch medium. The ethanol yield was of 0.44 g g^{-1} sugar consumed. However, in these experiments a very high cell load was used as inoculum to reach these ethanol yields, as 100 g of wet weight cells were added per litre medium (corresponding to 15 g L^{-1} dw cells).

Khaw et al. (2006) developed a non-flocculent strain displaying the *R. oryzae* glucoamylase and secreting a *Streptococcus bovis* α-amylase. The engineered yeast produced up to 51 g L^{-1} ethanol in media containing 10% raw corn starch and 1% glucose.

The specific ethanol productivity was about 0.18 g (g dw cells)$^{-1}$ h^{-1}, which was three-fold higher than the flocculent strain displaying the glucoamylase and secreting the α-amylase (Khaw et al., 2006).

Recenlty, efficient ethanol production was achieved using a diploid yeast, bred by mating two kinds of amylase gene integrated haploid strains that expressed *S. bovis* α-amylase and *R. oryzae* glucoamylase, respectively (Yamada et al., 2009). The recombinant yeast showed interesting fermentative abilities producing 39 g L^{-1} ethanol after growth for 84 h in raw starch (100 g L^{-1}). The ethanol yield was about 0.44 g g^{-1} sugar consumed.

Glucoamylase	α-amylase	Sugar equivalent in medium	Ethanol [a]	Ethanol productivity (Q)[b] or specific productivity (q)[c]	Ethanol yield [d]	Reference
R. oryzae enzyme secreted.	None	250 g L^{-1}	51 g L^{-1} after 120 h	0.675 (g L^{-1})h^{-1}	0.20	(Ashikari et al., 1989b)
R.oryzae enzyme displayed on surface.	None	230 g L^{-1}	23 g L^{-1} after 168 h		0.13	(Murai et al., 1998)
R. oryzae enzyme displayed on surface.	*S. bovis* enzyme displayed on surface	200 g L^{-1}	62 g L^{-1} after 72 h	0.858 (g L^{-1})h^{-1} 0.069 g (g dw cells)$^{-1}$ h^{-1}	0.44	(Shigechi et al., 2004b)
R. oryzae enzyme displayed on surface of nonflocculent strain.	*S. bovis* enzyme secreted	120 g L^{-1}	52 g L^{-1} after 60 h	0.866 (g L^{-1})h^{-1} 0.18 g (g dw cells)$^{-1}$ h^{-1}	0.46	(Khaw et al., 2006)
R. oryzae enzyme displayed on surface of flocculent strain.	*S. bovis* enzyme secreted	120 g L^{-1}	24 g L^{-1} after 60 h	0.4 (g L^{-1})h^{-1} 0.06 g (g dw cells)$^{-1}$ h^{-1}	0.45	(Khaw et al., 2006)
R. oryzae enzyme displayed on surface	*S. bovis* enzyme secreted	110 g L^{-1}	39 g L^{-1} after 84h	0.46 (g $L^{-1)}$ h^{-1}	0.44	(Yamada et al., 2009)

Table 1.6. Ethanol concentration, production and yield from recombinant amylolytic *S. cerevisiae* strains grown in raw corn starch.

[a] ethanol concentration (g L^{-1}), [b] Q: ethanol volumetric productivity (g L^{-1})h^{-1}, [c] q: ethanol specific productivity g (g dw cells)$^{-1}h^{-1}$, [d] ethanol yield as g (g consumed sugar)$^{-1}$.

A blank space indicates that not enough data was presented to determine the value.

1.6.3.3 Expression of *Aspergillus* amylases in *S. cerevisiae*.

The glucoamylases from *A. awamori* and *A. oryzae*, as well as the α-amylase from *A. awamori* hydrolyse raw starch (Matsubara et al., 2004; Queiroz et al., 1997). These enzymes are industrially used in the production of saké and miso. The fungal strains produce both α-amylase and glucoamylase which have exhibited a synergistic effect during raw starch degradation (Abe et al., 1988; Ueda, 1981).

However, all yeasts to date constructed for raw starch fermentation were engineered for the expression of the *R. oryzae* glucoamylase and *S. bovis* α-amylase genes. Utilising the amylase genes from *Aspergillus* for raw starch conversion in *S. cerevisiae* could contribute to developing a novel CBP microbe.

The glucoamylase and α-amylase genes from *A. oryzae* and the glucoamylase from *A. awamori* have been expressed in *S. cerevisiae* (Hata et al., 1991; Lin et al., 1998). Some groups created polyploid strains and used the δ-integration system to increase heterologous protein production of the host strain (Ekino et al., 2002; Saito et al., 1996). The *A. awamori* α-amylase shown to hydrolyse raw starch has not been expressed in *S. cerevisiae* yet.

Moreover, no recombinant industrial yeast have been reported to date for the conversion of starch to ethanol by secreting *Aspergillus* glucoamylase and-or α-amylase. Developing of such a CBP strain could pave the way for new cost-effective conversion systems of starchy feedstocks into ethanol and other co-products.

2. MATERIAL AND METHODS.

2.1 Media and strains.

The media used in this work are reported in Table 2.1. All chemicals, media components and supplements were of analytical grade standard.

Medium	Reference or supplier
Edimburgh Minimal Medium (EMM)	Favaro et al., 2008
Glucose Fermentation Medium (GFM)	This work
Hankin-Anagnostakis medium (HA)	Hankin and Anagnostakis, 1975
Luria-Bertani (LB)	DIFCO
Minimal Medium Yeast (MMY)	Favaro et al., 2008
Must Nutrient Synthetic (MNS)	Delfini, 1995
Nutrient Agar (NA)	DIFCO
Nutrient Broth (NB)	DIFCO
Protease Medium (PRM)	Ogrydziak and Mortimer, 1977
Poligalacturonase Medium (PGM)	Strauss et al., 2001
Raw starch agar	This work
Raw Starch Fermentation Medium (RSFM)	This work
Synthetic Complete medium (SC)	DIFCO
Soluble starch agar	This work
Starch Fermentation Medium (SFM)	This work
Tansey medium	Tansey, 1971
Terrific Broth (TB)	DIFCO
Tributyrin Agar Medium (TAM)	FLUKA
Trypticase soy yeast extract medium	DSMZ (medium 92)
Yeast-Mould (YM)	OXOID
Yeast Peptone Dextrose (YPD)	OXOID
Yeast Peptone Starch (YPS)	Verma et al., 2000
Wollum medium	Wollum et al., 1982

Table 2.1. Summary of media used in this study.

The genotypes, phenotypes and sources of yeast and bacterial strains used in this work are summarised in Table 2.2.

Strain	Relevant genotype or phenotype	Source or Reference
C. persica DSM 14784	Strain with high cellulolytic activity	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
E. coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F'proAB lacq ZΔM15 Tn10(tet)]*	Stratagene (USA)
Non-*Saccharomyces*	180 *wild type* strains isolated from different oenological environments.	Padova Univ.
S. cerevisiae	220 wild type strains isolated from grape marcs for their high fermentative vigour.	Padova Univ.
of which:		
s1	Potentially amylolytic strain	This work
s2	Potentially amylolytic strain	This work
s3	Potentially amylolytic strain	This work
s4	Potentially amylolytic strain	This work
s5	Potentially amylolytic strain	This work
F6	Control strain	Padova Univ.
F9	Control strain	Padova Univ.
S. cerevisiae DSM 70449	Type strain	DSMZ
S. cerevisiae EC1118	Industrial wine strain	Padova Univ.
S. cerevisiae H1	Industrial distillery strain	Stellenbosch Univ.
S. cerevisiae S228c	*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6, MIP1[S]*	American Type Culture Collection (ATCC)
S. cerevisiae Y294	*α leu2-3, 112 ura3-52 his3 trp1-289*	ATCC
S. diastaticus ATCC 13007	Extracellular glucoamylase producer	ATCC

Table 2.2. Summary of microbial strains used in this study.

Yeast strains pre-cultures were grown in YPD medium (gL^{-1}: yeast extract, 10; peptone, 20 and glucose, 20) at 30°C on a rotary shaker set at 130 rpm unless otherwise stated.

C. persica and *E. coli* were cultured on Trypticase soy yeast extract medium and Luria-Bertani medium, respectively.

2.2 Isolation and characterization of proficient cellulolytic microbial species.

2.2.1 Isolation of cellulolytic microbial strains.

Three biological matrices (forest litter, decayed *Abies* sp. tree and soil from brook bed) were collected from a forest located at Lamen (BL) inside Parco Nazionale delle Dolomiti Bellunesi in the southern section of the Province of Belluno (Italy). All samples were withdrawn during March 2007 and were utilised immediately (i.e. within 24 h) after transportation to the laboratory.

Biological samples were vortexed in sterile 0.9% NaCl solution for 5 minutes. The suspensions were serially diluted and plated on both Tansey and HA selective media. For each dilution, two replicates were incubated at 30°C under aerobic and anaerobic conditions for 14 and 28 days, respectively.

The two media had the following composition:

Tansey medium (gL^{-1}): Avicel (Merck), 5; $NH_4H_2PO_4$, 2; KH_2PO_4, 0.6; K_2HPO_4, 0.4; $MgSO_4*7H_2O$, 0.8; yeast extract, 0.5 and agar, 17;

HA medium (gL^{-1}): carboxymethyl-cellulose (CMC), 5; $(NH_4)_2SO4$, 1; KH_2PO_4, 2; Na_2HPO_4, 3; $FeSO_4*7H_2O$, 0.1; $CaCl_2$, 0.005; yeast extract, 1; trace elements and agar 10.

On HA medium, cellulase activity of the obtained colonies was screened, according to Kluepfel method (Kluepfel, 1988), by observing cellulose degradation halos after staining with Congo Red. In Tansey medium cellulolytic isolates were selected on the basis of the diameter of the clearing hydrolysis zone surrounding the colonies (Tansey, 1971).

Cellulolytic strains were picked from plates, purified by streaking twice on fresh NA plates and stored as stock culture in 20% (v/v) glycerol at -80°C for further examinations.

2.2.2 Enzymatic activity of the cellulose-degrading isolates.

The cellulolytic activity (CellA) of the isolates was compared to the activity of *Cellulomonas persica* DSM 14784, used as reference strain since is well known for the production of proficient xylanase and cellulase. New bacterial isolates and *C. persica* DSM 14784 were grown at 30°C for 72 hours on HA medium plates and on LB agar supplemented with 0.25% CMC. Cellulase activity was monitored according to Kluepfel method.

2.2.3 Genetic characterization of the most proficient cellulose-degrading isolates.

The most efficient cellulolytic isolates were characterized by ARDRA technique (Amplified Ribosomal DNA-Restriction Analysis). DNA was extracted as follows: a small colony of each strain, grown for 24 h on NA plates, were picked up with a sterile toothpick and resuspended in 50 μL of lysis solution (0.05 M NaOH, 0.25% SDS). After vortexing for two minutes, the suspension was heated at 95°C (15 min) and then centrifuged (13000 rpm, 10 min). Obtained lysates were diluted with sterile deionized water and used for PCR (Polymerase Chain Reaction) amplification.

Prokaryotic small rDNA subunits were amplified using bacterial universal primers 1389r and 63F (Hongoh et al., 2003). PCR amplification was performed using a PTC200 thermal cycler (MJ Research Inc., MA) in a total volume of 25 μL into 0.2 mL tubes with the following reagent concentrations: 200 mM dNTPs (Amersham Biosciences AB, Uppsala, Sweden), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.02 UmL^{-1} *Taq* polymerase (Amersham), 2 μM (each) primers (MWG-Biotech, Ebersberg, Germany; HPSF purified).

The thermal protocol was designed as follows: initial denaturation 95°C for 2 min, followed by 35 cycles composed of denaturation at 94°C for 60s, annealing at 54°C for 30s and extension at 72°C for 30s. A final extension step was added at 72°C for 5 min.

To verify the successful amplification, PCR products were run in agarose gel electrophoresis (1% agarose). The presence of a clear 1 Kb band in each PCR product was considered as the proof of correct amplification.

Amplification products were subsequently digested with the restriction endonuclease *Hinf*I and *Hin6*I. The fragments were separated in 1.8% agarose gel at 100 V, 3 h, visualised and photographed under UV light. The resulting profiles were analysed with the pattern analysis software package GelComparII (Applied Maths, Sint-Martens-Latern, Belgium), using the Dice coefficient (Dice, 1945). Dendrograms were constructed from the similarity matrices by means of the UPGMA (Unweighted Pair Group Method by using Arithmetic Average) clustering algorithm (Vauterin and Vauterin, 1992).

Amplification products were also subjected to sequencing (BMR Genomics, University of Padova). Species identification was completed after BLASTN alignment (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequences with those present in the GenBank database. A minimum sequence similarity level of 97% was considered for taxonomic attribution.

2.3 Screening for the production of extracellular hydrolytic enzymes by *Saccharomyces cerevisiae* and non-*Saccharomyces* strains.

2.3.1 Yeast strains.

Two hundred and twenty *S. cerevisiae* strains and 180 non-*Saccharomyces* isolates were screened for their amilolytic, cellulolytic, hemicellulolytic, lipolytic, pectinolytic and proteolytic activities. *S. cerevisiae* DSM 70449 was used as reference negative strain.

All *S. cerevisiae* strains were previously selected by Prof. Viviana Corich and Prof. Alessio Giacomini (Dipartimento di Biotecnologie Agrarie, University of Padova) from grape marcs on the basis of their fermentative vigour (Delfini, 1995) while non-*Saccharomyces* were isolated from different oenological environments. All cultures were identified by means of conventional morphological, physiological and biochemical procedures according to the latest taxonomic guidelines (Yarrow, 1998).

2.3.2 Media and screening procedure.

Calibrated suspensions (A_{600nm}=0.8, corresponding to an average cell concentration of 10^6 mL^{-1}) of 24 h yeast cells were used to inoculate purified agar plates. Petri dishes were checked for the presence of enzymatic activity after incubation at 30°C for 6-14 days.

Cellulolytic activity (CellA).

Cellulase production was detected on HA medium containing 5 gL^{-1} carboxymethyl-cellulose (CMC). After cell growth, the presence of cellulase activity (CellA) was detected by Congo red method. The colonies were rinsed off the plates with distilled water before staining the plates with 0.05% Congo Red, followed by destaining with 1 M NaCl (Teather and Wood 1982). The growth of potential positive strains for CellA was tested also on the Minimal Medium Yeast (MMY) medium, supplemented with (gL^{-1}) yeast nitrogen base (Difco), 0.5; $(NH_4)_2SO_4$, 5; CMC, 5 and agar 10.

Lipolytic activity (LipA).

Strains were tested on TAM medium containing (gL^{-1}): peptone, 5; yeast extract, 3; tributyrin, 10 and agar, 15, pH 6.0 (Atlas and Parks 1993). Lipase activity (LipA) of the strains were indicated by a clear halo around the colony in an otherwise opaque medium as described by Charoenchai et al. (1997).

Pectinolytic activity (PectA).

The secretion of extracellular pectic enzymes was tested on PGM medium (g L^{-1}): yeast nitrogen base, 6.7; glucose, 1 and polygalacturonic acid (Fluka), 7.5, pH 7.0 (Strauss et al., 2001). After cell growth, plates were flooded with HCl (6N) solution. The appearance of a degradation halo around yeast colony, after HCl staining, was considered an indication of the polygalacturonic acid hydrolysis.

Proteolytic activity (PrA).

Extracellular protease production was determined on PRM medium with skim milk (Difco), pH 6.5 (Ogrydziak and Mortimer, 1977). A clear zone around the colony indicated

protease activity (PrA) as described in literature (Charoenchai et al., 1997; Dizy and Bisson, 2000).

Starch-degrading activity (StA).

Yeast strains were screened for their ability to hydrolyse soluble potato starch (Sigma and BDH) on NA medium supplemented with 0.2% starch and on Wollum medium containing (gL^{-1}): Yeast Extract (Difco), 1; Na_2NO_3, 1; KCl, 0.5; $MgSO_4$, 0.5; starch, 10 and agar, 17.

S. diastaticus ATCC 13007, having glucoamylolytic activity, was used as positive control strain.

After incubation, Petri dishes were flooded with iodine solution (Wollum, 1982). A pale yellow zone around colonies in a blue medium indicated starch degrading activity (StA).

Positive strains for StA were grown also on agar plates of YPS (gL^{-1}: yeast extract, 10; peptone, 10 and starch, 20) and EMM containing gL^{-1}: $KHC_8H_4O_4$, 3; Na_2HPO_4, 2.2; NH_4Cl, 5; starch, 20 and agar, 17. Cultures were aerobically incubated at 30°C for 6 days and then monitored for the production of starch degradation halos after iodine solution staining.

Xylan-degrading activity (XylA).

Cultures were screened for hemicellulose degrading activity by growth on modified HA medium containing 0.5% xylan from oat-spelt (Fluka). Colonies showing xylan degrading activity (XylA) were identified by a clear hydrolysis zone around the colony after treatment with Congo Red.

2.3.3 Determination of optimal pH and temperature for extracellular enzymes of a non-*Saccharomyces* yeast.

The enzymatic activity of a non-*Saccharomyces* yeast, selected on the basis of cellulose degradation haloes observed in Petri dishes, was assessed in liquid assay at different pH and temperature values.

Yeast was aerobically grown for 168 h at 30°C in 100 mL Erlenmeyer flasks containing 25 mL YPD broth (0.5% glucose). Samples were periodically taken and the cultures were centrifuged (5000 rpm for 5 min). Pellets were used for the determination of dry biomass whereas the supernatant fluids were used for the enzymatic assay. For dry biomass determination, cell pellets were washed several times with deionised sterile water and dried in an oven (80°C) to constant weight.

Two experiments were carried out in triplicate and each enzymatic assay was repeated three times.

Aliquots (50 µL) of culture supernatant were mixed in 1% CMC citrate-phosphate buffer (0.05 M) at different pH values (4.5-5.0-5.5-6.0-7.5). After incubation for 10 min at 40-50-60°C, the reaction was stopped by boiling the mixture for 15 min. After cooling on ice, the concentration of reducing groups was determined by the DiNitroSalicylic acid (DNS) method described by Miller et al. (1959). Enzymatic activities were expressed as nanokatals per gram dry weight biomass (nkat (g dw cells)$^{-1}$), which is defined as the enzyme activity needed to produce 1 nmol of glucose per second per gram dry cell weight.

2.3.4 Genetic identification of the cellulolytic non-*Saccharomyces* strain.

One non-*Saccharomyces* strain, selected for its proficient hydrolytic activitiy, was grown on YPD plate for 24 h. A single colony was picked up with a sterile toothpick and resuspended in 20 µL of sterile deionized water in 0.5 mL tubes. Five µL of the suspension were used for PCR amplification. The primers ITS1 and ITS4 (Guillamon et al., 1998) were used to amplify a region of the rDNA repeat unit which includes two non-coding regions, designated as the internal transcribed spacers (ITS1 and ITS2), the 3' part of the 18S, the 5' portion of the 26S and the entire 5.8S rDNA genes. A 5-µl aliquot of cell suspension, prepared as described above, was heated at 94°C for 2 min and then subjected to PCR amplification using 30 cycles with initial denaturation at 94°C for 30s, annealing at 56°C for 30s and extension at 72°C for 30s.

Amplification product was checked by agarose gel electrophoresis and then subjected to sequencing. Genetic identification was performed after BLASTN alignment of the obtained

sequences with those present in the GenBank public database. A minimum sequence similarity level of 97% was considered for taxonomic attribution.

2.3.5 Evaluation of technologically related properties of the cellulolytic non-*Saccharomyces* strain.

The non-*Saccharomyces* strain was evaluated for several technologically related properties.

Fermentative vigour evaluation.

The followed method was described by Delfini (1995). Every glass serum bottle was filled with 100 mL of MNS medium supplemented with 20% glucose. Pre-culture of the non-*Saccharomyces* yeast, grown overnight in YPD, was inoculated with an average cell concentration of 7.5 x 10^6 cells per serum bottle and incubated in static condition at 25°C.

Ethanol resistance tests.

To evaluate the alcohol resistance of the non-*Saccharomyces* strain was cultured on YPD plates and in YPD broth supplemented with increasing concentrations of ethanol (0% control, 1.25, 2.5, 5, 7.5, 10, 12.5 %, v/v). Each plate was sealed with plastic film to prevent ethanol volatilization.

Three wild type *S. cerevisiae* isolates (s3, H1, ECC1118) and two laboratory strains (*S. cerevisiae* s288C, *S. cerevisiae* Y294) were used as control strains. Yeasts, grown overnight in YPD, were diluted in cell suspensions at increasing cell densities (3 x 10^2, 10^3, 10^4, 10^5 cells mL^{-1}). A 10 µL sample of each suspension was spotted on YPD plates and incubated at 30°C for 48 h.

Yeast cells were also inoculated in YPD broth at an initial cell density of 1 x 10^5 cells mL^{-1}. Cell growth was monitored by counting cells using a Thoma chamber (depth, 0.02 mm) and/or by determining the optical density at 600nm with a spectrophotometer (Ultrospec 2000, Pharmacia Biotech).

Study on the putative antimicrobial activity.

In order to detect a potential yeast killer activity, the strain was tested for inhibitory activity using both well diffusion assay and spot test. Briefly, cells of the target strain, *S. cerevisiae* s2, were incorporated into soft agar (1% w/v) YPD plates at two cell concentrations: 1 x 10^6 and 1 x 10^7 cells mL^{-1}. The yeast was incubated overnight in YPD broth and then centrifuged (10000 rpm, 30 min). All assays were conducted with YPD agar plates unbuffered (pH 6.4) and buffered at pH 4.5. Supernatant was used for inhibitory activity tests.

Supernatant aliquots (50 µL) were transferred in holes (5 mm diameter) drilled into the agar. Alternatively, 10 µl of supernatant and overnight culture of the yeast were spotted on the surface of the inoculated agar plates. YPD broth was used as control.

The plates were incubated at 20, 30 and 37 °C for 48 h and the antimicrobial activity was recorded as growth-free inhibition zones (diameter) around the well or the spot.

2.4 Extensive biochemical, physiological and genetic study on the starch-hydrolytic mechanism showed by *S. cerevisiae* strains.

2.4.1 Determination of amylolytic activity in liquid media.

The starch-degrading activity of the thirteen *S. cerevisiae* strains selected was assessed in different liquid broths: YPS, YP (YPS without soluble starch) as complete media and EMM and MMY as minimal media.

S. cerevisiae DSM 70449 and *S. diastaticus* ATCC 13007 were used as negative and positive control strain, respectively. Yeast cells, grown to stationary phase in YPD broth, were inoculated to an A_{600nm} of 0.06 to 0.09. Cell growth was monitored by measuring absorbance (A_{600nm}) at 12 h intervals. EMM was chosen for investigating the growth on- and the utilisation of- starch. The exhausted EMM broth after 6 days incubation was determined for starch concentration. Residual starch was estimated by UV-method (Boehringer Mannheim/R-Biopharm). Every experiment was carried out in triplicate.

2.4.2 Glucoamylase production and enzymatic assays.

Glucoamylase production and activity was estimated according to a method recently described for *S. diastaticus* (Verma et al., 2000). All thirteen *S. cerevisiae* strains selected for their potential amylolytic activity were tested together with *S. cerevisiae* DSM 70449 and *S. diastaticus* ATCC 13007, used as negative and positive control strain, respectively.

For glucoamylase production, yeast cells were grown in rich medium (YPD) for 24 h at 30°C. Culture broth was centrifuged (5000 rpm for 5 min) and cell pellet was inoculated to 25 mL of YPS containing 0.5% starch and re-cultivated for 24 h. This culture was inoculated at 10% v/v concentration into YPS medium supplemented with starch 2% and incubated for glucoamylase production. Sample were withdrawn at 24 h intervals and centrifuged.

The supernatant was used for glucoamylase activity estimation by measuring the glucose released from soluble starch using UV-method (Boehringer Mannheim/R-Biopharm). The assay solution contained 0.5 mL starch (1% in an acetate buffer) and 0.1 mL of culture supernatant. The reaction was carried out at 30°C for 20 min and then stopped by boiling. Enzyme activity was expressed in units (UmL^{-1}: nanomoles glucose released per mL per min).

2.4.3 Genetic study on putative glucoamylolytic sequence(s) of *S. cerevisiae* strains.

Yeast colonies (1-2 mm diameter), grown for 24 h on YPD plates, were picked up with a sterile toothpick and resuspended in 20 µL of sterile deionized water in 0.5 mL tubes. Three µL of the suspension were used for PCR amplification.

Primers, listed in Table 2.3, were designed from alignments of DNA sequences of *sga* in *S. cerevisiae* and *sta* genes in *S. diastaticus.* Gene sequences were obtained from Gen-Bank and aligned using the CLUSTAL W software (Thompson *et al.*, 1994). Primers STA1F and STA2R are derived from the 5' region of *sta1* gene of *S. diastaticus.* Primers STA2F and STA2R were designed within the 5' region of *S. diastaticus sta2* gene. Both regions are highly conserved in several other extracellular glucoamylase sequences.

The amplification reaction was performed in a total volume of 25 μL into 0.2 mL tubes using a PTC200 thermal cycler (MJ Research Inc., MA), with the following reagent concentrations: 200 mM dNTPs (Amersham Biosciences AB, Uppsala, Sweden), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.02 UmL^{-1} *Taq* polymerase (Amersham), 0.2 mM (each) primers (MWG-Biotech, Ebersberg, Germany; HPSF purified).

Name	Sequence (5'-3')	T_m	Position	PCR product size (bp)
STA1F	CCGCTGGTAAGACGACAACT	59.4°C	937-957*	700
STA1R	GAACCTCAGGTCCCAACGTA	59.4°C	1646-1686*	
STA2F	TGGAACAGGCACTTTTAGGG	57.3°C	1534-1554°	388
STA2R	TTTCTTTGTTGCAGCAGTGG	56.3°C	1921-1941°	

Table 2.3. Primer used in this work (*Relative to *S. diastaticus sta1* gene sequence: GenBank no. X02649; °Relative to *S. diastaticus sta2* gene sequence: GenBank no. M60650).

The thermal protocol was designed as follows: initial incubation 94°C for 3 min to allow cell lysis and DNA denaturation, followed by 45 cycles composed of denaturation at 94°C for 30s, annealing at 56.5°C for 60s and extension at 72°C for 120s. A final extension step was added at 72°C for 5 min.

Amplified samples were run on 1.2% agarose gels and the bands were visualized after ethidium bromide staining. Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

2.5 Study of a Separated Hydrolysis and Fermentation (SHF) process for the conversion of wheat bran into ethanol.

2.5.1 Wheat bran.

Wheat bran was obtained from Promolog SRL (Marghera, Italy). The material had a dry matter (DM) content of 88.71% and was stored in plastic bags at 4°C. The wheat bran composition was analysed by LAZ laboratories (Department of Animal Science, University

of Padova) according to Martillotti et al. (1987).

2.5.2 Pre-treatment.

Pre-treatment experiments were carried out using wheat bran and on milled wheat bran as substrate. Dry matter was adjusted to 8.7% with deionized water and pretreament flask (volume 120 mL) was filled with 100 mL of the resulting slurry. Bran was autoclaved at 121°C for 30 min. Pre-treatments with the addition of 0.1 and 0.3% H_2SO_4 (w/w) were also investigated and are described in Table 2.4.

Wheat bran	Thermal treatment	Chemical treatment
Raw	121° C, 30 min, 1 atm	-
Milled	121° C, 30 min, 1 atm	-
Raw	121° C, 30 min, 1 atm	H_2SO_4 0.1% (w/w)
Milled	121° C, 30 min, 1 atm	H_2SO_4 0.1% (w/w)
Raw	121° C, 30 min, 1 atm	H_2SO_4 0.3% (w/w)
Milled	121° C, 30 min, 1 atm	H_2SO_4 0.3% (w/w)

Table 2.4. Pre-treatment studies for SHF process of wheat bran.

2.5.3 Enzymatic hydrolysis.

The pre-treated wheat bran slurry was enzimatically hydrolysed to determinate the maximum obtainable sugar yield. Operating conditions and amount of each enzyme (Novozyme) are listed in Table 2.5. Enzymatic hydrolysis was carried out in a stirred waterbath. After pre-treatment, the pH was adjusted to optimal value with NaOH (1 M) or HCl (1 M) and the cocktail of cellulolytic and hemicellulolytic enzymes was added. In order to improve the amylolytic activity of the *Saccharomyces* sp strains used in the following fermentation phase, a small dose of α-amylase enzyme was added after cellulose and hemicellulose hydrolysis.

Enzyme	Activity	pH	Dose (% w/w)	Temperature (°C)	Incubation (h)
NS50013 (cellulase complex)	70 FPU/g	5.5	0.5	50	48
NS50010 (β-glucosidase)	250 CbU/g	5.5	1.1	50	48
NS50030 (xylanase)	500 FXU/g	5.5	0.3	50	48
NS50014 (hemicellulase)	750 FXU/g	5.5	0.3	50	48
NS50029 (β-glucosidase)	200 BGU/g	5.5	0.6	50	48
Liquozyme SC DS (α-amylase)	240 KNU-S/g	5.8	0.035	85	4

Table 2.5. Enzyme activity, pH, temperature, incubation and dosage used for wheat bran hydrolysis (FPU Filter Paper Units, CbU Cellobiase Units, FXU Fungal Xylanase Units, BGU β-Glucosidase Units, KNU-S α-amylase Units).

2.5.4 Fermentation studies on wheat-bran hydrolysates.

The hydrolysates resulting from pre-treatment and enzymatic hydrolysis were fermented by *S. cerevisiae* s1 and *S. diastaticus* ATCC 13007. The fermentation was also conducted with *S. diastaticus* ATCC 13007 once induced for the production of glucoamylase as described by Verma et al. (2000). The experimental scheme is reported in Table 2.6.

The pH value was adjusted to 5.5 with KOH (1M); nutrients were added to final concentrations of gL^{-1}: yeast extract 1; $(NH_4)_2HPO_4$ 1.5; $MgSO_4*7H_2O$ 0.025 and NaH_2PO_4 0.1 M.

Pre-cultures of yeast strains grown to stationary phase in YPD broth were used as inoculum. The fermentation hydrolysates were inoculated to a cell concentration of 0.75 g dw L^{-1}. Pre-treatment flasks were sealed with rubber stoppers with a cannula for the removal of CO_2 produced during fermentation (Figure 2.1). The fermentation was conducted in duplicate under static conditions at 25°C.

Samples were withdrawn at regular intervals and analyzed for arabinose, galactose, glucose, xylose, mannose, furfural, HMF and ethanol by HPLC (High Performance Liquid Chromatography) as described below.

Hydrolysates	H_2SO_4	*S. cerevisiae* s1	*S. diastaticus* ATCC 13007	*S. diastaticus* ATCC 13007 induced
Raw	-	+	+	+
Milled	-	+	+	+
Raw	0.1% (w/w)	+	+	+
Milled	0.1% (w/w)	+	+	+
Raw	0.3% (w/w)	+	+	+
Milled	0.3% (w/w)	+	+	+

Table 2.5. Fermentation studies on wheat bran hydrolysates by *Saccharomyces* sp. strains.

Figure 2.1. Fermentation flasks used for the SHF system proposed in this study.

2.5.5 Analysis.

Exausted hydrolysates were analysed after fermentation phase for their composition in protein, starch, hemicellulose, cellulose and lignin by LAZ laboratories (Dipartimento di Scienze Animali, University of Padova).

The samples from pre-treatment, enzymatic hydrolysis and fermentation were analyzed for arabinose, galactose, glucose, xylose, mannose, furfural and HMF; hydrolysates were characterized also for their content in acetic and lactic acids.

Before analyses samples were filtered through 0.22-μm filters and diluted prior to HPLC analysis. Monosaccharide analysis was performed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The system was equipped with a PA1 column and auto-sampler (Dionex Corporation, Sunnyvale, CA, USA). The mobile phase used was 1 mM NaOH at a flow rate of 1 mL min^{-1} at room temperature.

Organic acids, ethanol, furfural and HMF were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 65°C with 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.5 mL min^{-1}. The system (Shimadzu, Kyoto, Japan) was equipped with refractive index detector (Shimadzu) and cation-H refill cartridge (Bio-Rad, Hercules, USA) prior to the column.

2.5.6 Data analysis.

All treatments were conducted in quintuplicate. Data of pre-treatments were analysed by three ways factorial ANOVA using Duncan test *post hoc* means differentiation. Fermentation kinetics were evaluated by means of chi-square test.

2.6 Development of an efficient amylolytic yeast strain for industrial ethanol production.

2.6.1 Selection of wild type *S. cerevisiae* strains with best properties for industrial bioethanol application.

Five potentially amylolytic *S. cerevisiae* strains (named as s1, s2, s3, s4, s5) with the highest growth rate on soluble starch (see Paragraph 2.4) were evaluated for their fermentative ability on MNS medium supplemented with different concentrations of glucose and/or xylose: 20% glucose, 15% glucose and 5% xylose, 10% glucose and 10% xylose. The following method was described by Delfini (1995). Every glass serum bottle was filled with 100 mL of MNS medium and then sealed with rubber stoppers. Pre-cultures of *S. cerevisiae* strains were inoculated with an average cell concentration of 7.5 x 10^6 cells per bottle and incubated in static condition at 25°C. *S. cerevisiae* H1 was used as benchmark strain.Two *S. cerevisiae* isolates, named as F6 and F9, were used as control strains from the collection of *S. cerevisiae* selected for their high fermentative vigour (Dipartimento di Biotecnologie Agrarie, University of Padova). The experiments were carried out in triplicate.

The fermentation vigour was daily monitored by measuring flask weight loss in relation to CO_2 production. Results were reported, as grams of glucose utilised per 100 mL of MSN medium, by a conversion factor of 2.118. Samples were drawn after 7 and 21 days, filtered through 0.22-μm filters and analyzed for detection of glucose, xylose, xylitol, glycerol and ethanol by HPLC as described in van Zyl et al. (1999).

2.6.2 Engineering *S. cerevisiae* yeasts by introducing the *sgaI* glucoamylase gene from *Aspergillus awamori* and/or *amyIII* amylase gene from *A. oryzae*.

Recombinant strains and plasmids.

The genotypes and sources of the plasmids, yeast and bacterial strains used in these experiments are summarised in Table 2.7.

Plasmid/Strains	Relevant genotype or phenotype	Source
ySYAG	*bla URA3 PGK1$_P$-XYNSEC-sgaI-PGK1$_T$*	Stellenbosch Univ.
yASAA	*bla URA3 ENO1$_P$-XYNSEC-amyIII-ENO1$_T$*	Stellenbosch Univ.
pBKD1	*amp* δ-sites-*TEF$_P$-KanMX-TEF$_T$*-δ-sites*	Stellenbosch Univ.
pBKD2	*amp* δ-sites-*TEF$_P$-KanMX-TEF$_T$*-δ-sites*	Stellenbosch Univ.
pBZD1	*amp* δ-sites-*TEF$_P$-ShBle-TEF$_T$*-δ-sites*	Stellenbosch Univ.
pBCF*sgaI*	*amp* δ-sites-*PGK1$_P$-XYNSEC-sgaI-PGK1$_T$ -Shble* -δ-sites	This work
pBCF*amyIII*	*amp* δ-sites- *ENO1$_P$-XYNSEC-amyIII-ENO1$_T$ - KanMX*-δ-sites	This work
pBGA	*amp* δ-sites-*PGK1$_P$-XYNSEC-sgaI-PGK1$_T$ -KanMX-ENO1$_P$-XYNSEC-amyIII-ENO1$_T$* -δ-sites	This work
E. coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F'proAB lacq ZΔM15 Tn10(tet)]*	Stratagene (USA)
S. cerevisiae LH3	*H1 recombinant strain with sgaI multiple copy integration*	This work
S. cerevisiae LH4	*H1 recombinant strain with sgaI multiple copy integration*	This work
S. cerevisiae LH18	*H1 recombinant strain with sgaI multiple copy integration*	This work
S. cerevisiae sBCF2	*s2 recombinant strain with sgaI multiple copy integration*	This work
S. cerevisiae sBCF6	*s2 recombinant strain with sgaI multiple copy integration*	This work

Table 2.7. Summary of plasmids and strains constructed for the development of an efficient amylolytic *S. cerevisiae* strain (**TEF1* promoter and terminator from *Ashbya gossypii*).

Recombinant plasmids were constructed and amplified in *E. coli* XL1-Blue. The bacterial strains were cultured at 37°C on a rotating wheel in Terrific Broth or on LB agar (Sambrook et al., 1989). Ampicillin was added to a final concentration of 100 μg mL^{-1} for the selection of resistant bacteria.

DNA manipulations.

Restriction enzyme digestion, electrophoresis, DNA ligation, transformation and DNA preparation from *E. coli* were performed using the standard methods according to Sambrook et al. (1989). DNA fragments were purified from agarose gels by using the GENE CLEAN kit (BIO 101, Inc., Vista, CA, USA) or phenol (Benson, 1984). Restriction enzymes, Klenow fragment, and T4 DNA ligase were supplied by either Roche or Fermentas.

Construction of integrative plasmids for secretion of glucoamylase and α-amylase.

A synthetic glucoamylase gene (*sgaI*) from *A. awamori* and the *amyIII* α-amylase gene from *A. oryzae* were selected for the construction of new integrative vectors targeted to the δ-sequences of the yeast retrotransposon Ty1. The fungal *sgaI* and *amyIII* sequences, encoding proficient raw starch degrading glucoamylase and α-amylase respectively, were recently described by de Villiers (2008). Both genes were subcloned into the pBKD1 and pBKD2 integrative plasmids.

These two vectors differ in that pBKD1 contains the *S. cerevisiae PGK1* (*Phosphoglycerate Kinase*) promoter and terminator sequences whereas pBKD2 contains the *S. cerevisiae ENO1* (*EnolaseI*) promoter and terminator sequences.

Dominant marker resistance tests.

To establish their dominant marker resistance, the wild type *S. cerevisiae* strains s1, s2, F6 and H1 were grown in YPD broth at 30°C for 24 h. Yeast cells were serially diluted in NaCl (0.9%) and plated onto YPD agar supplemented with increasing amounts of geneticin (0, 50, 100, 150, 200, 300 μg mL^{-1}) or zeocin (0, 50, 100, 150, 200 μg mL^{-1}). After 24 h incubation at 30°C, each strain was then evaluated for geneticin and zeocin sensibility.

Electrotransformation of yeast strains with delta vectors.

The wild type *S. cerevisiae* strains s1, s2, F6 and H1 were transformed with *Xho*I digested pBCF*sgaI*, pBCF*amyIII* and pBGA integrative plasmids for multi-copy chromosomal integration.

Host cells, grown overnight in YPD broth, were harvested in Eppendorf tubes by centrifugation at 4000 rpm for 1 min, washed twice with distilled deionized water and finally suspended in 1 mL of electroporation buffer containing 1 M sorbitol and 20 mM HEPES. After centrifugation at 4000 rpm for 1 min, the pellet was resuspended in 200 µL of electroporation buffer. The resuspended cells (50 µL) were transferred into electroporation cuvettes (0.2 cm electrode, Bio-Rad). After adding 10 µg of linearized plasmid, an electric pulse of 1.4 kV, 200 ohm was applied with various capacitances by using Gene-Pulser (Bio-Rad Lab., Hercules, CA, USA). In this pulsed cuvette, 1 mL of YPD supplemented with 1 M sorbitol was added. The cuvette was incubated for 3 h at 30°C.

The recombinant cells were plated onto YPD plates (containing 1M sorbitol) supplemented with zeocin (75-100 µg mL^{-1}) or geneticin (200-300 µg mL^{-1}) for selective pressure. The yeast transformants were transferred onto soluble starch agar plates at 30°C for 3 days. The plates were transferred to 4°C for 24 h to allow the starch to precipitate. Recombinant colonies expressing the amylase gene were surrounded by a clear halo due to starch hydrolysis.

Evaluation of mitotic stability of the transformants.

To study mitotic stability of the obtained mutants, the transformants with the largest starch hydrolysis halos were grown in sequential batch cultures. The integrants were cultivated in non-selective YPD broth (10 mL) on a rotating wheel and transferred (1% v/v) to fresh YPD after glucose depletion.

After 30, 60 and 120 generations, recombinant strains were plated onto YPD and incubated at 30°C for 24 h. Up to 250 colonies for each transformant were replicated onto soluble starch agar with and without zeocin (100 µg mL^{-1}) or geneticin (300 µg mL^{-1}) as

well as on YPD plates (with and without antibiotics). The stable transformants remained resistant to antibiotics and displayed hydrolytic activity on starch.

Enzymatic assays.

Stable mitotic transformants were studied for their ability to produce SgaI recombinant protein in the following broths: YPD, 2xSC (supplemented with gL^{-1}: glucose, 20 and yeast nitrogen base without amino acids, 13.4) and 2xSC supplemented also with 7.5 gL^{-1} yeast extract, then referred to as 2xSC-modified.

The enzymatic assays were conducted with wild type *S. cerevisiae* H1 and its mitotically stable recombinants LH3, LH4, LH18 as well as with wild type *S. cerevisiae* s2 and the recombinant strains sBCF2 and sBCF6.

Yeast cells were aerobically grown at 30°C up to 168 h. Five mL samples were withdrawn at 24 h intervals. After centrifugation (5000 rpm for 5 min), the supernatant was used for the assays and the dry biomass determined as described in Paragraph 2.3.3.

Samples of supernatant (50 μL) were mixed with 450 μL of the substrate (2% corn starch or 0.1% potato soluble starch in a 4.5 pH 0.05M citrate-phosphate buffer. The hydrolysing reaction was carried out at 30°C for 36 minutes and at 50°C for 12 minutes.

The optimal pH for glucoamylase hydrolysis at 50°C was determined by adding supernatant samples in citrate-phosphate buffers with the following pH values: 5.0-5.4-6.0 and 7.5. The enzymatic reactions were stopped by boiling in a waterbath for 5 minutes. Glucose concentration in a cooled sample was determined using the peroxidase-glucose oxidase method from a glucose assay kit (Boehringer Mannheim/R-Biopharm). Enzymatic activities were expressed as nanokatals per mL (nKat mL^{-1}) that is defined as the enzyme activity needed to produce 1 nmol of glucose per second per mL of culture. In addition, enzymatic activities were reported also as nanokatals per gram dry cell weight (nKat (g dw cells)$^{-1}$), which is defined as the enzyme activity needed to produce 1 nmol of glucose per second per gram dry cell weight. Two experiments were carried out in triplicate and each enzymatic assay was repeated three times.

Fermentation studies.

Recombinant amylolytic yeast strains were cultivated in: a) Raw Starch Fermentation Medium (RSFM) supplemented with (g L^{-1}) raw corn starch (Sigma) 20; yeast nitrogen base 6.7; peptone 20 and glucose 0.5, b) Starch Fermentation Medium (SFM) and c) Glucose Fermentation Medium (GFM) where the equivalent amount of raw starch was replaced with either soluble potato starch (Sigma) or glucose.

The raw starch was sterilised with ethanol and dried at 30°C overnight before adding to filter-sterilized medium. Streptomycin (Sigma) was added (0.5 g L^{-1}) to prevent bacterial contamination under non-sterile raw starch conditions.

Fermentation experiments were performed at 30°C in two different systems (Figure 2.3): fermentation flasks on orbital shaker and serum bottles sealed with rubber stoppers on multistirrer.

Precultures of *S. cerevisiae* s2 and recombinant strains sBCF2 and sBCF6 grown to stationary phase in YPD medium were used as inoculum. Cells were washed with a salt solution (0.9% NaCl) and used to inoculate 10% (v/v) 100 mL medium in triplicate experiments using 120 mL glass serum bottles or 120 mL Erlenmeyer flasks. Bottles and flasks were sealed with rubber stoppers, incubated at 30°C and mixed on a magnetic stirrer or on an orbital shaker, respectively.

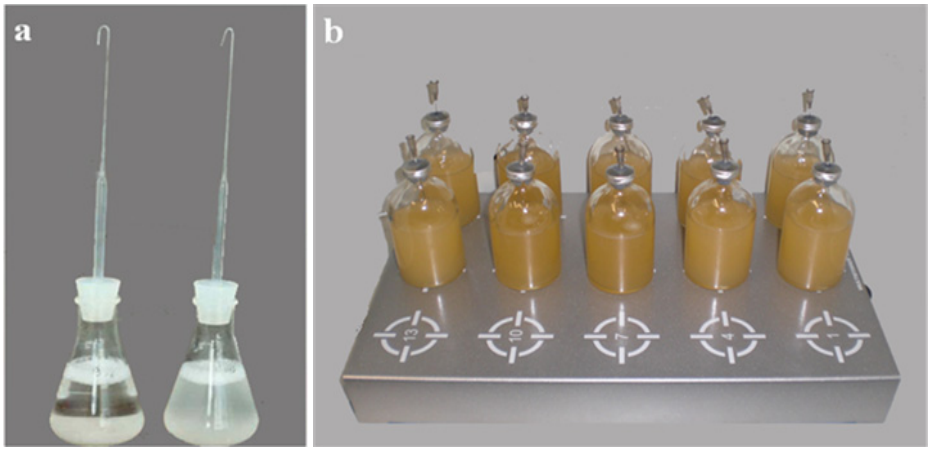

Figure 2.3. Fermentation systems used in this study: (a) Erlenmeyer flasks on orbital shaker; (b) serum bottles on magnetic multistirrer.

Samples were taken through a capped syringe needle pierced through the bottle stopper. Yeast cells were counted in triplicate using a Thoma chamber (depth, 0.02 mm) for raw starch fermentations. Anaerobic growth on glucose and starch fermentation medium was measured in triplicate as absorbance at 600nm.

Analytical methods.

A calibration chart was prepared to correlate dry weights (dw) with optical densities (OD_{600}) as well as cell counts determined using a Thoma chamber. Dry cell weights were determined from 5 mL culture samples. Cells were collected after centrifugation (5000 rpm, 5 min), washed several times with deionised sterile water, and dried in an oven (80°C) to constant weight.

Residual fermentable sugars present during anaerobic cultivations were determined in duplicate for each culture with the glucose and starch assay kit (Boehringer Mannheim/R-Biopharm) while raw starch concentration was determined with phenol-sulphuric acid method using glucose as standard (Dubois et al., 1956).

Ethanol concentrations were analysed by ethanol assay kit (Boehringer Mannheim/R-Biopharm) and by HPLC. Ethanol analysis was performed with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Ethanol was separated with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) as described in Paragraph 2.5.5..

Calculations.

The ethanol yield (g g^{-1} consumed sugar) from glucose or starch was calculated as the amount of ethanol produced per gram of consumed sugar. The volumetric productivity (Q) was based on grams of ethanol produced per litre of culture medium per hour: $(gL^{-1})h^{-1}$. The highest volumetric productivity value of each strain was defined as maximum volumetric productivity (Q_{max}).

The specific productivity (q), based on the respective volumetric productivity divided by the correspondent dry cell weight value, was also calculated. The highest specific productivity value (q_{max}) was defined as the maximum specific productivity of the strains.

3. RESULTS.

3.1 Isolation and characterization of proficient cellulolytic microbial species.

Isolation of cellulolytic microbial strains.

In order to obtain the largest possible collection of cellulose-degrading bacteria, three different biological matrices were selected from a forestry environment; carboxymethyl-cellulose (CMC) and Avicel were used as main carbon sources in Hankin-Anagnostakis medium (HA) and Tansey selective media, respectively.

After aerobic and anaerobic incubation at 30°C, culturable microbes were checked for their cellulolytic activity. On HA medium, many colonies showed a good cellulose-degrading activity since, after Congo Red staining, consistent cellulose degradation halos were detected. Among the bacterial strains grown on Tansey plates, several isolates were selected as efficient cellulase-producers.

Their activity on Avicel and CMC was used as criteria for the selection of 300 microbial colonies in order to obtain a collection of potentially high cellulolytic microbes. The selected isolates were then purified by streaking twice on fresh NA plates and stored as stock cultures at -80°C for further examinations.

Study on the enzymatic activity of the cellulose-degrading isolates.

The cellulolytic activity (CellA) of the isolates was compared to the cellulolytic ability of *C. persica* DSM 14784, used as reference strain since is well known for the production of proficient cellulase (Elberson et al., 2000).

New bacterial isolates and *C. persica* DSM 14784 were grown on HA medium plates and on LB agar supplemented with 0.25% CMC. Cellulolytic activity was evaluated according to the extent and intensity of hydrolytic clearing zones. All microbial strains

showed a remarkable cellulose degrading activity when grown on LB plates, suggesting that glucose and peptone as additional carbon and nitrogen sources could stimulate bacterial growth and hydrolytic activity. Five isolates selected on Tansey medium for their capability to hydrolyse Avicel showed high enzymatic activities also on CMC.

Moreover, twelve microbial strains showed cellulose degrading activity similar to that of the benchmark strain *C. persica* DSM 14784. In Figure 3.1, as an example, the hydrolytic ability of isolate s11 is compared to that of *C. persica* DSM 14784. The strain s11, isolated from a forest litter sample, produced large cellulose degradation halos after 72 h incubation on LB + 0.25% CMC.

Figure 3.1. CellA (Cellulolytic Activity) on LB + 0.25% CMC of isolate s11 compared to *C. persica* DSM 14784.

Genetic characterization of the most efficient cellulose-degrading isolates.

The twelve strains showing remarkable cellulolytic activity were firstly characterized by ARDRA technique (Amplified Ribosomal DNA Restriction Analysis). Prokaryotic small subunits rDNA were then amplified using universal bacterial primers 1389r and 63F (Hongoh et al., 2003) and subsequently digested with *Hinf*I and *Hin6*I. The resulting

fragments were separated in 1.8% agarose gel at 100 V (3 h) and the obtained restriction profiles were analyzed with the G*elCompar*II software (Applied Maths).

The numerical analysis of the combined patterns, reported as dendrogram in Figure 3.2, showed three distinct clusters with internal similarity levels ranging from 95 to 100%. The dendrogram, constructed by means of the UPGMA clustering algorithm, indicated a strict similarity between bacterial strain s2 and s8, s4 and s11, s5 and s7, respectively.

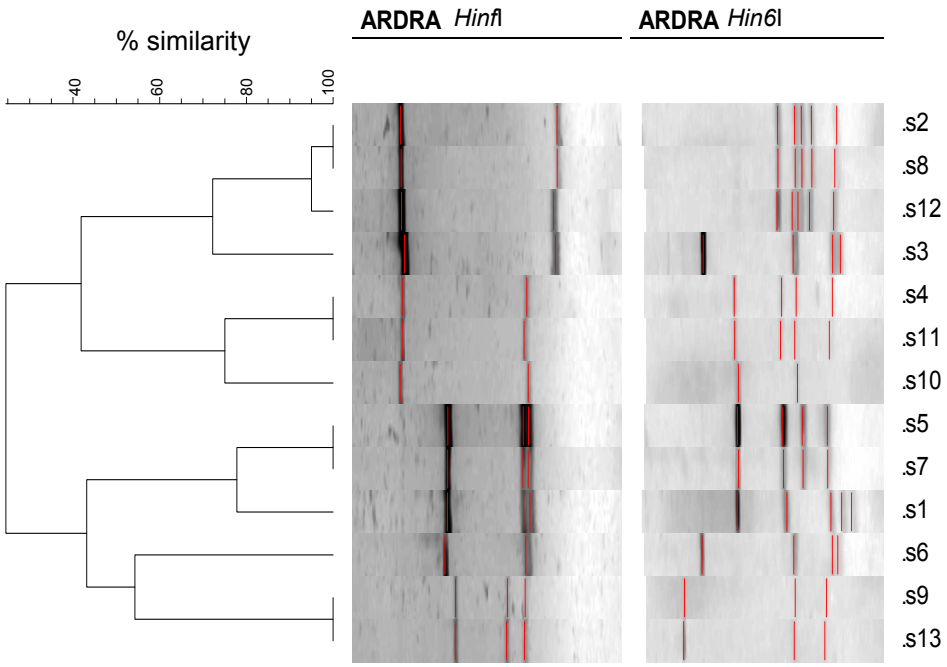

Figure 3.2. Dendrogram based on the UPGMA clustering with Dice correlation coefficients of ARDRA patterns, obtained with *Hinf*I and *Hin6*I.

In order to genetically identify the microbial isolates characterized with ARDRA method, amplification products, obtained with universal primers mentioned above, were subjected to sequencing. Species identification was completed after BLASTN alignment (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequences with those present in the GenBank database. The results of species identification, reported in Table 3.1, seem to confirm the ARDRA analysis: the high similarity showed by the three distinct clusters was validated by 16S rDNA sequencing.

.

Biological matrix	Number of isolates selected for CellA	Isolate	Activity on CMC	Activity on Avicel	Species identification	Omology (%)	Accession number
Forest litter	155	s12	+		*Pseudomonas brenneri*	100	EU169172
		s11	+	+	*Bacillus sp.*	100	EF693760
		s2	+		*Pseudomonas sp.*	100	EU057889
		s8	+	+	*Pseudomonas sp.*	100	AY166908
		s5	+		*Ewingella americana*	100	AB273745
		s7	+		*Ewingella americana*	100	AY581130
Decayed wood	97	s6	+		*Frateuria aurantia*	97	AB091197
		s9	+		*Bacillus pumilus*	100	EU231626
		s10	+		*Burkholderia sp.*	99	DQ419960
		s4	+		*Bacillus sp.*	100	AB366165
Brook bed	48	s1	+		*Rhanella sp.*	98	AM160791
		s3	+		*Stenotrophomonas sp.*	99	AJ534843

Table 3.1. Characterization of cellulolytic bacterial strains. The positive activity on CMC and Avicel is reported as (+).

Strains s2, s8 and s12 were identified as members of the genus *Pseudomonas* while isolates s4 and s11 were characterized as belonging to *Bacillus* genus. The microbial strains s5 and s7 were identified as *Ewingella americana*.

3.1.1 Discussion.

Over the years, culturable, cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry or agricultural residues, soil and organic matter, and extreme environments like hot-springs. The abundance of cellulolytic bacteria, including species of *Clostridium*, *Cellulomonas*, *Bacillus*, *Pseudomonas*, and species of *Cytophaga* and actinomycetes groups, has long been recognised due to the importance of cellulose decomposition in several ecosystems (Doi, 2008; Lynd et al., 2002; Wirth and Ulrich, 2002).

The cellulolytic activities of bacteria are mostly screened using agar-plate techniques with a variety of substrates, such as carboxymethyl-cellulose (CMC) or insoluble microcrystalline cellulose such as Avicel or filter paper (Li, 1997; Ruijssenaars and Hartmans, 2001).

Conventionally, endo-acting cellulase (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) is regarded as highly active towards CMC or amorphous cellulose with little or no activity towards microcrystalline cellulose, while exo-acting cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) hydrolyses micro-crystalline cellulose and hardly any CMC (Maki et al., 2009; Wood and Garcia-Campayo, 1990). Thus, cellulolytic bacteria can be divided into two groups based on their capability to degrade either soluble or insoluble celluloses, the latter being regarded as truly cellulolytic (Wirth and Ulrich, 2002). Activities of both endo- and exo-cellulases, however, are considered to act synergistically for efficient decomposition of native, crystalline cellulose (Coughlan and Ljungdahl, 1988; Davies and Henrissat, 1995; Weimer, 1991).

In this study, cellulose degrading microbial strains were isolated from a forestry ecosystem in which the environmental factors should be extremely selective for cellulolytic microbial populations as indicated by the high number of cellulolytic isolates obtained. The

majority of potential hydrolytic microbes was isolated from forest litter sample, as reported in Table 3.1.

A major proportion of strains degrading exclusively CMC could be distinguished, thus indicating only the activity of endocellulases. Few bacterial strains proved effective to degrade both CMC and Avicel suggesting that they should have also promising exocellulase enzymes.

Moreover, twelve strains were selected on the basis of their ability to hydrolyse cellulose strictly comparable with that of *C. persica* DSM 14784, isolated as efficient cellulose-degrading strain from forest humus soil (Malekzadeh et al., 1993).

Selected strains displayed a high phylogenetic diversity. The genetic differentiation of isolates was based on amplified ribosomal DNA restriction analysis (ARDRA) which is an approach, commonly used to discriminate among bacterial species from various habitats (Ross et al., 2000; Ulrich and Zaspel, 2000; Ventura et al., 2001). Using ARDRA with a set of two restriction enzymes provided sufficient discrimination power to both distinguish 16S rDNA sequences with high similarity and group isolates which are phylogenetically closely related.

The 16S rDNA sequencing showed that three strains belong to *Bacillus* genus and three isolates to *Pseudomonas* genus. Both genera are well known for their cellulolytic and xylanolytic aerobic species (Gordon et al., 1973; Kim, 1987; Kim et al., 2000; Li et al., 2008).

The other isolates were identified as *E. americana*, *F. aurantia* and as members of *Rhanella*, *Stenotrophomonas* and *Burkholderia* genera. These species and genera are not described to date in literature for their interesting cellulose-degrading activities and could be considered as novel sources of hydrolytic enzymes with specific applications in the bioprocessing industry.

Furthermore, the selected strains of *Rhanella* sp. and *Stenotrophomonas* sp., isolated in the extreme environment of a brook bed soil, could be adapted to grow under anaerobic conditions during the winter time. The strains, facing this environmental selective pressure in the brook bed, should have developed different fermentative pathways in order to yield energy from the abundant decaying cellulose even at very low oxygen concentrations.

These strains may contribute to a decrease in the current cost of bioconversion of lignocellulose to ethanol by their efficient cellulase and their potential ability to ferment resulting sugars into ethanol. However, further analyses using molecular and physiological approaches are required to study their fermentative vigour and other technologically related characteristics.

3.2 Screening for the production of extracellular hydrolytic enzymes by *S. cerevisiae* and non-*Saccharomyces* strains.

Two hundred and twenty *S. cerevisiae* strains and 180 non-*Saccharomyces* yeasts were screened for the production of saccharolytic enzymes. The strains, were grown on selective media in order to verify the presence of amilolytic, cellulolytic, hemicellulolytic, lipolytic, pectinolytic and proteolytic activities. The results, derived from three experiments, each with two replicates, are reported in Table 3.2.

		n. positive strains					
Strains	**n. of strains**	**CellA**	**LipA**	**PectA**	**PrA**	**StA**	**XylA**
S. cerevisiae	220	18	6	12	9	13	-
Non-*Saccharomyces*	180	1	14	-	-	1	-

Table 3.2. Extracellular enzymatic activity profile of 400 yeast strains (CellA: cellulolytic activity; LipA: lipolytic activity; PectA: pectinolytic activity; PrA: proteolytic activity; StA: starch-degrading activity; XylA: xylan-degrading activity).

Cellulolytic activity (CellA).

Eighteen *S. cerevisiae* strains were selected for their potential capability of hydrolysing cellulose, although their growth on rich medium containing CMC (carboxymethyl-

cellulose) was slow. All strains, indeed, produced small hydrolytic halos after a prolonged incubation under both aerobic (Figure 3.3a) and anaerobic conditions (Figure 3.3b).

The minimal medium MMY supplemented with CMC as sole carbon source was used to further evaluate the cellulose degrading activity of the selected strains. As shown in Figure 3.2c, the strains were unable to use CMC since no cellulose degradation halos were observed. This evidence might be due to MMY medium lacking any of component (micronutrient or co-factor) essential for growth as well as for production of cellulose degrading enzymes.

However, at this stage it is unclear if the cellulose-degrading activity detected in the strains depends on non-specific hydrolytic mechanisms or on a truly cellulolytic enzyme(s). Further studies are then required to better understand their weak capability of hydrolysing cellulose.

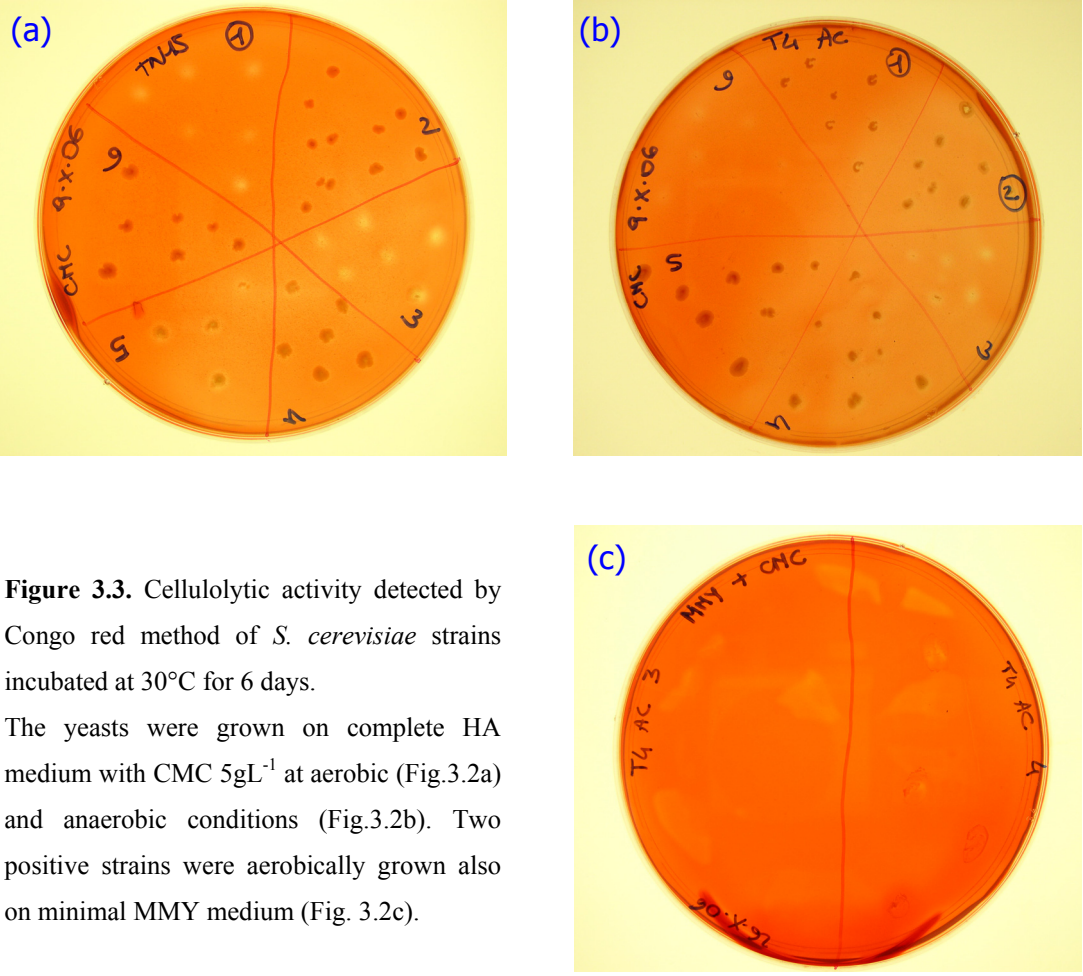

Figure 3.3. Cellulolytic activity detected by Congo red method of *S. cerevisiae* strains incubated at 30°C for 6 days.
The yeasts were grown on complete HA medium with CMC 5gL^{-1} at aerobic (Fig.3.2a) and anaerobic conditions (Fig.3.2b). Two positive strains were aerobically grown also on minimal MMY medium (Fig. 3.2c).

Among the non-*Saccharomyces* strains tested, one isolate was selected as interesting cellulase producer. After 4 days of incubation on HA medium, the yeast showed consistent cellulose degradation halos (Figure 3.4).

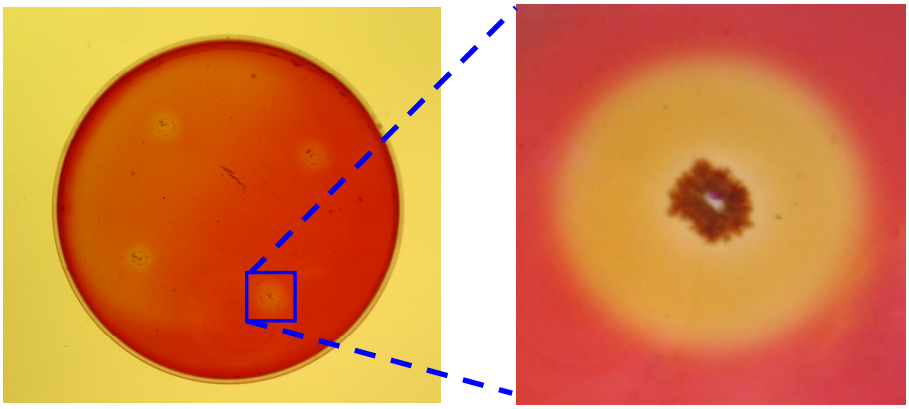

Figura 3.4. Cellulolytic activity of a non-*Saccharomyces* isolate grown at 30°C for 4 days on HA plate supplemented with CMC (5 gL^{-1}).

On the basis of such hydrolytic activity, much more efficient than *S. cerevisiae* yeasts described above, it seemed interesting to investigate the extracellular cellulolytic activity of this non-*Saccharomyces* yeast through several *in vitro* enzymatic assays as described in the Paragraph 3.2.1.

Lipolytic activity (LipA).

Six *S. cerevisiae* isolates and fourteen non-*Saccharomyces* strains were selected for their potential activity on tributyrin. All strains were able to hydrolyse tributyrin although very weakly: they produced in fact only tiny degradation halos around the colonies (data not shown).

Pectinolytic activity (PectA).

Twelve *S. cerevisiae* strains showed pectinolytic activity on plates (Table 3.2). All strains grew well on plates containing polygalacturonic acid. Their enzymatic activity revealed good performance since the degradation halos, after staining the plates with HCl (6N), were consistent (Figure 3.5).

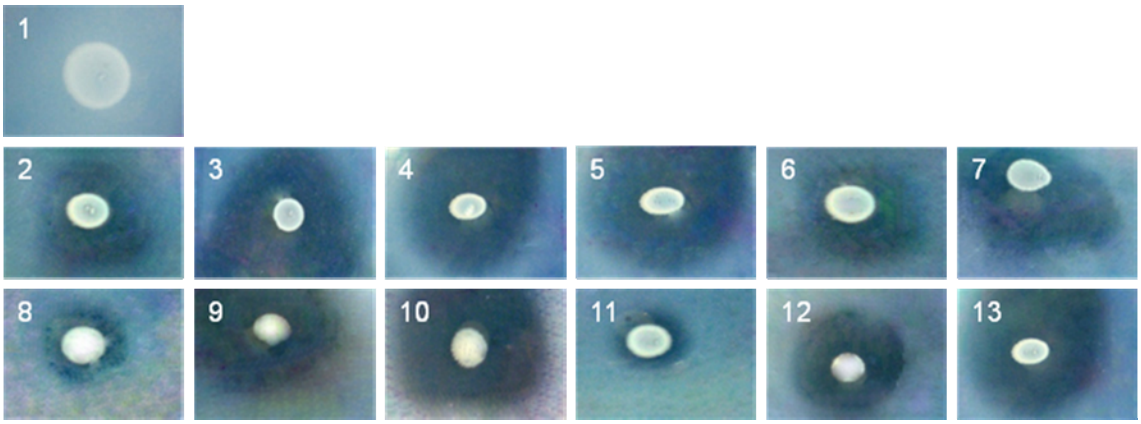

Figure 3.5. Growth of *S. cerevisiae* strains on polygalacturonic acid (7.5 gL^{-1}). Negative strain (1) and potential pectinolytic strains (2-13) were grown for 6 days at 30°C and then stained with HCl solution (6N).

McKay (1990) reported that the secretion of polygalacturonases by some strains of *S. cerevisiae* was constitutive and the medium must be supplemented with 1% glucose for enzyme production. For this reason, a small amount of glucose (1 gL^{-1}) was included in the composition of the PGM medium used in the present study. However, it is possible that the presence of glucose in the medium could have inhibited the production of these enzymes in some of the tested isolates.

Proteolytic activity (PrA).

Table 3.2 indicates that nine isolates of *S. cerevisiae* gave positive results for protease activity on skim milk agar plates. As described in the literature, a clear zone around their colonies was considered as a proof of their extracellular protease production. However, their protease ability should be further confirmed through several biochemical and

physiological approaches since the selected strains produced very small hydrolysis halos (data not shown).

Starch-degrading activity (StA).

Yeast strains were screened for their starch-degrading activity on two different soluble starch media (Wollum and NA+0.2% starch). From the collection of non-*Saccharomyces* strains tested, no isolate was found effective for the production of starch-degrading enzymes.

As reported in Table 3.2, thirteen strains of *S. cerevisiae* were selected as potential amylolytic yeasts on the basis of their starch degradation halos. All isolates produced cell biomass and hydrolysing activity on both tested selective media. Figure 3.6 shows the amylolytic ability of two *S. cerevisiae* strains grown for 5 days on NA medium supplemented with soluble potato starch (0.2%).

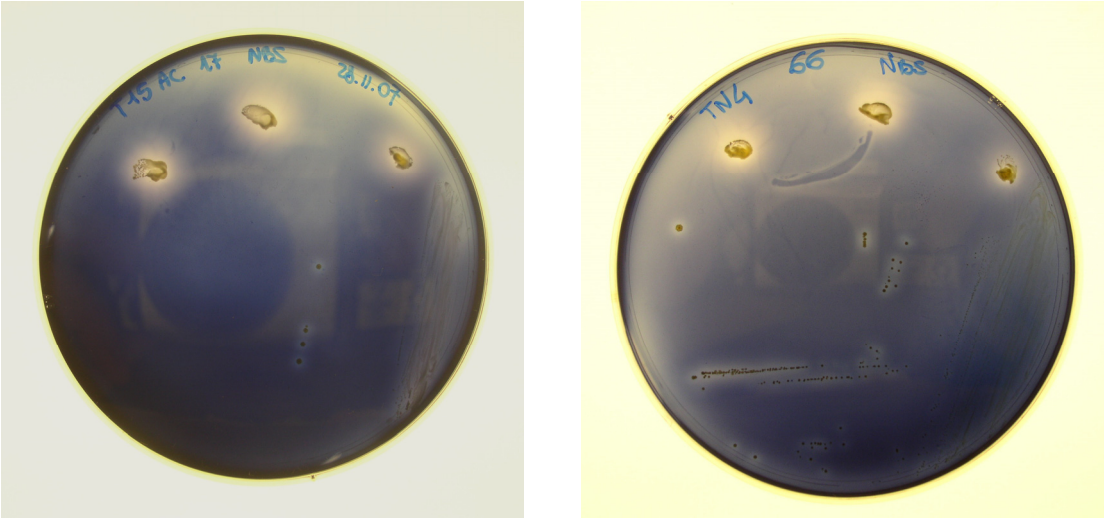

Figura 3.6. Starch-degrading activity of two *S. cerevisiae* strains grown for 5 days at 30°C on NA + 0.2% soluble potato starch (Sigma) plates.

The selection of high fermentative *S. cerevisiae* strains with potential starch-degrading abilities was unexpected since the species is considered in literature unable to use and ferment polysaccharides, such as cellulose, xylan and starch (Lynd et al., 2002).

In order to confirm this unpredicted finding, the selected strains were evaluated for their amylolytic activities on different agar media. They were grown on YEPS complete medium and on media with progressively decreased additives (yeast extract and peptone) which could supply to yeasts other carbon sources. All strains grew well on complete medium (data not shown) and slowly on Wollum, supplemented with only 1 gL^{-1} yeast extract (Figure 3.7A). Moreover, the isolates grew also on the minimal medium EMM that lacks any biological nitrogen source (Figure 3.7B).

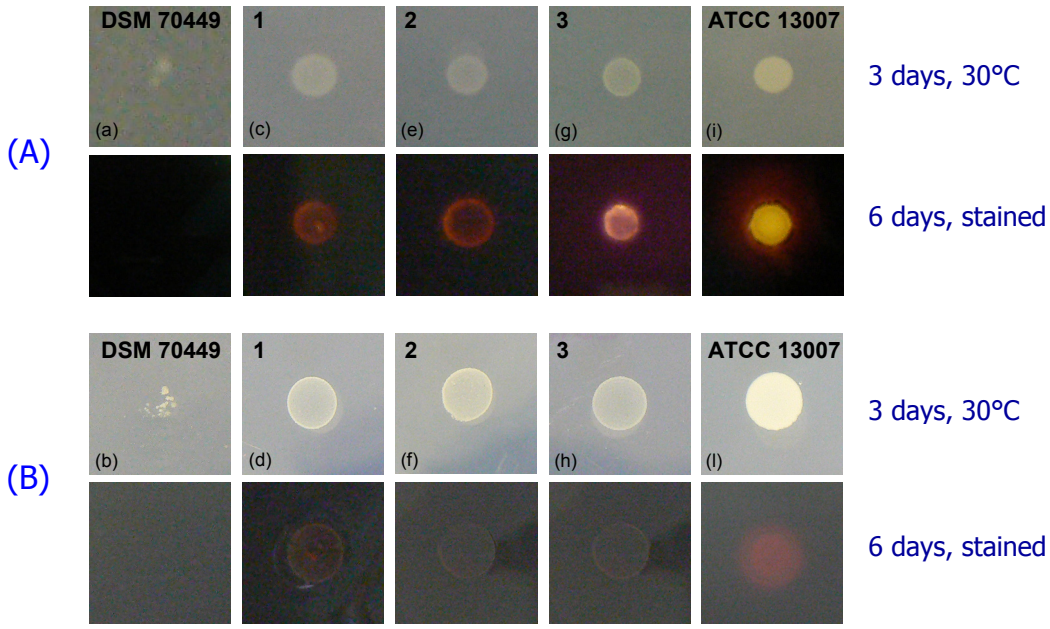

Figure 3.7. Growth of *Saccharomyces* sp. strains on complete Wollum medium (A) and on minimal EMM plates (B). Reference strains (negative: *S. cerevisiae* DMS 70449; positive: *S. diastaticus* ATCC 13007) and potentially amylolytic strains (1-3) were grown at 30°C for 6 days and then stained with iodine solution.

The Figure 3.7 shows the growth, after 3 days incubation at 30°C, of the potentially amylolytic *S. cerevisiae* strains (c-d; e-f, g-h) on complete Wollum and minimal EMM plates. The type strain *S. cerevisiae* DSM 70449, used in the experiments as negative control, showed a very feeble growth pattern (Figure 3.7 a-b). *S. diastaticus* ATCC 13007, having glucoamylolytic activity, grew very well on both media (Figure 3.7 i-l).

After 6 days incubation, Petri dishes were flooded with iodine solution. In Figure 3.7, the halos of the strains were compared. Complete Wollum medium (Figure 3.7A) seems to support amylolytic activity better than EMM agar: the halos around the colonies grown on Wollum medium were larger in diameter than those on the minimal medium (Figure 3.7B). However, the presence of a weak starch-degrading activity on EMM plates is clear evidence of the ability of the *S. cerevisiae* strains to use starch as the only carbon source.

Xylan degrading activity (XylA).

Yeasts strains were screened also for xylan degrading activity by growth onto agar plates of modified HA medium containing 0.5% xylan. No xylanolytic yeast was selected: all strains were able to grow slowly on HA plates without producing any xylan degradation halo.

3.2.1 Non-*Saccharomyces* strain identification and determination of optimal pH and temperature for extracellular enzymatic activity.

One non-*Saccharomyces* strain was selected for the production of cellulose degrading enzymes (Table 3.2). In order to identify the isolate, a subunit of ITS regions was amplified with primers ITS1 and ITS4 (Guillamon et al., 1998) and the resulting amplification products were sequenced by BMR Genomics (University of Padova). Species identification was completed after BLASTN alignment (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequence with those present in the GenBank database. ITS sequencing identified the cellulolytic strain as *Arthroascus schoenii*.

The cellulolytic activity of *A. schoenii*, was studied using CMC as substrate at 50°C with three different pH values of the citrate phosphate buffer (0.05 M, 1% CMC). *A. schoenii* was grown in YPD medium (5 gL^{-1} glucose) for 168 h: the highest enzymatic values, reported in Table 3.3, were obtained after 72 hour incubation. Cellulase activity was determined by measuring the reducing sugar groups enzymatically released from cellulose with the DNS method (Miller, 1959).

The cellulolytic yeast strain expressed its maximum enzymatic activity at pH 6.0 while at The enzymatic assay conducted in a neutral buffer resulted in a strong reduction of cellulolytic activity: at pH 7.5, the enzymatic efficiency was not detectable.

This result was in accordance with the literature that reported an optimal pH value for fungal extracellular cellulases ranging from 4.0 to 6.5 (Johnson et al., 1982; Lynd et al., 2002; Mourino et al., 2001; Nidetzky et al., 1994; Yu et al., 1995).

	Cellulase activity at 50°C (nKat mL^{-1})		
	pH 4.5	**pH 6.0**	**pH 7.5**
Arthroascus schoenii	30,48 ± 3,14	37,13 ± 2,24	ND

Table 3.3. Extracellular cellulolytic activity (nKat mL^{-1}) of *A. schoenii* cultured in YPD broth (5 gL^{1} glucose) for 72 hours. The enzymatic activity was detected at 50°C in citrate-phosphate buffers (1% CMC) at pH 4.5-6.0-7.5. ND: not detectable.

In order to determine the optimal pH and temperature values for the yeast cellulolytic activity, enzymatic assays were performed at three incubation temperatures (40-50-60 °C) and with four buffer pH values (4.5-5.0-5.5-6.0). The data, obtained with the cell-free supernatant of a 72 hours culture, are shown in Figure 3.8. The enzymatic activity is expressed as nKat(g dw cells)$^{-1}$.

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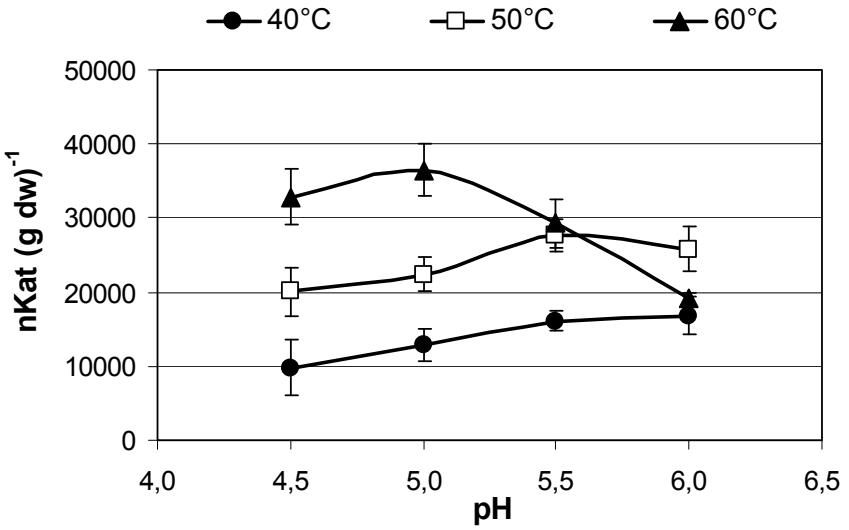

Figure 3.8. Extracellular cellulolytic activity in cell-free culture supernatant of *A. shoenii* grown in YPD broth for 72 hours. The activity was detected at the incubation temperature of 40-50-60 °C in buffers at 4.5-5.0-5.5-6.0 pH values. The enzymatic activity is expressed as nKat(g dw cells)$^{-1}$ that is the enzyme activity needed to produce 1\ nmol of glucose per second per gram dry cell weight.

The optimal pH value was in the range 5.0 to 5.5. As expected, the enzymatic activity was influenced by temperature incubation. The cell-free supernatant increased the cellulolytic activity as the enzymatic assay temperature increased: the maximum activity was 16864 and 27603 nKat(g dw cells)$^{-1}$ at 40° and 50°C, respectively, while the yeast cellulase produced the highest enzymatic value, 36502 nKat(g dw cells)$^{-1}$, when incubated at 60°C.

3.2.2 Evaluation of technologically related properties for the *Arthroascus schoenii* isolate.

The non-*Saccharomyces* yeast, identified by ITS sequencing as *A. schoenii*, showed a consistent cellulase activity both on agar plates and in enzymatic assays. Since the strain was isolated in oenological environments, the yeast could possess the combination of technological traits required for the Consolidated BioProcessing of lignocellulose into

ethanol: polysaccharides utilisation (e.g., high-level production of hydrolytic enzymes and efficient consumption of the resulting sugars) and fermentative performance (high selectivity and ethanol tolerance).

In order to confirm this hypothesis, several experiments were designed for the determination of *A. schoenii* fermentative vigour, ethanol resistance and yeast-killer activity.

Fermentative performance and ethanol tolerance in *A. schoenii*.

A. shoenii strain did not show any fermentative vigour since, after an extended incubation period, the yeast was unable to consume glucose in the MNS medium (data not shown). This result was consistent with the taxonomic keys recently proposed for the species by Naumov et al. (2006).

However, the yeast ethanol tolerance on agar plates and in liquid broth was interesting. When cultured on YPD Petri dishes with increasing concentrations of ethanol (Figure 3.9), the yeast exhibited a good alcohol tolerance showing the capacity to grow on a medium supplemented with ethanol up to 5% v/v.

This ability was comparable with that of the *S. cerevisiae* laboratory strains (s288C and Y294) used as benchmarks (Figure 3.9). As expected, the wild type *S. cerevisiae* strains, s2, H1, ECC1118 displayed remarkable growth pattern: they grew well on YPD supplemented with ethanol up to 10% v/v (Figure 3.9).

To further evaluate the alcohol tolerance, the ability of *A. schoenii* to grow in presence of increasing ethanol concentrations was tested in YPD broth. The cell growth of yeast strains was monitored measuring optical density at 600nm as well as cells counting using the Thoma chamber. Figure 3.10a shows that *A. schoenii* was able to grow in concentration of alcohol up to 2.5% v/v. However growth was slowed as the concentrations of ethanol increased. No growth was observed in media with concentrations of 7.5 or 10 %.

The *A. schoenii* pattern was very similar to *S. cerevisiae* Y294 with the exception that the latter strain showed some adaptation to the 5% ethanol after prolonged incubation (Figure 3.10b).

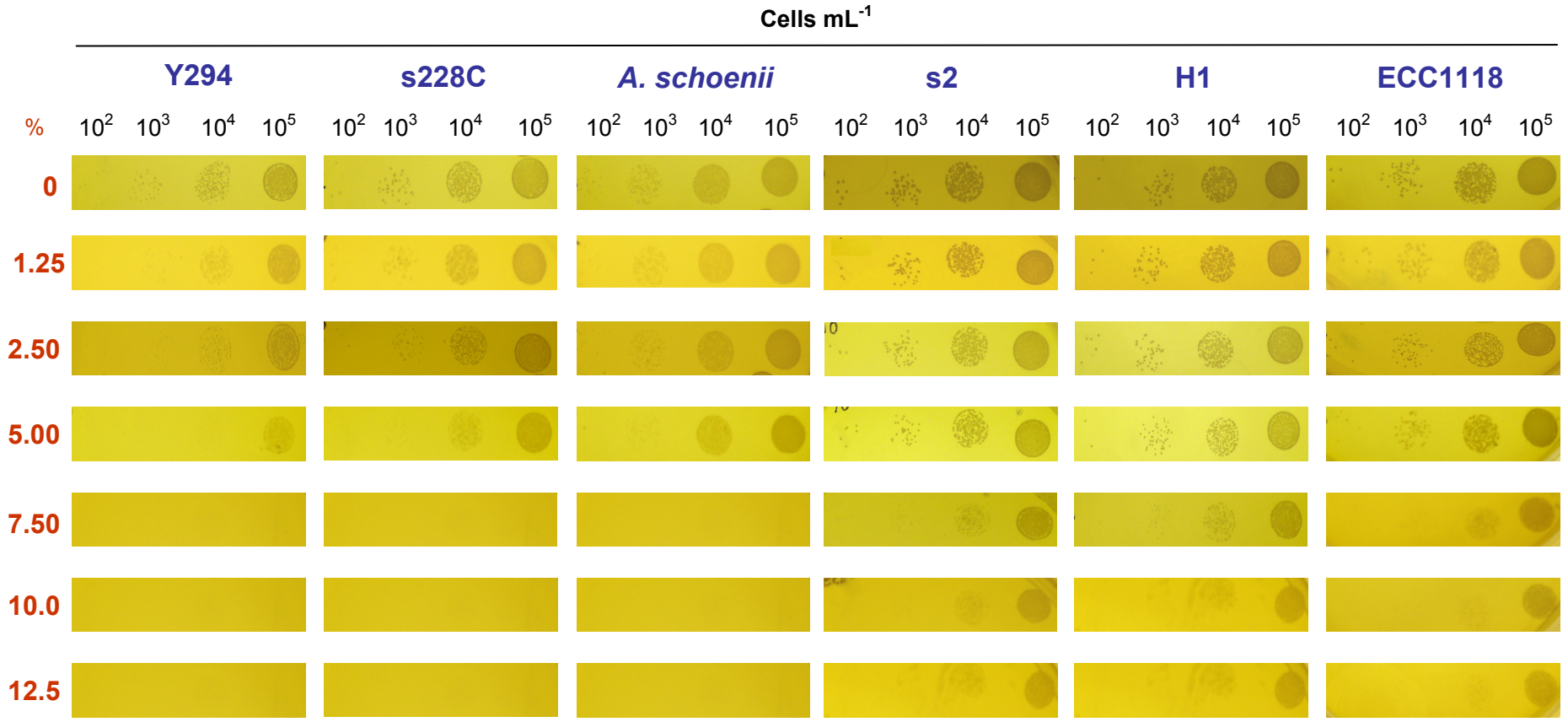

Figure 3.9. Ethanol tolerance of yeast strains (*A. schoenii* and *S cerevisiae* Y294, s288C, s2, H1, ECC1118) grown for 24 h on YPD in the presence of different ethanol concentrations (% ,v/v). Samples (10 µL) of cell suspensions at increasing densities (3 x 10^2, 10^3, 10^4, 10^5 cells mL^{-1}) were spotted on agar plates and incubated at 30°C.

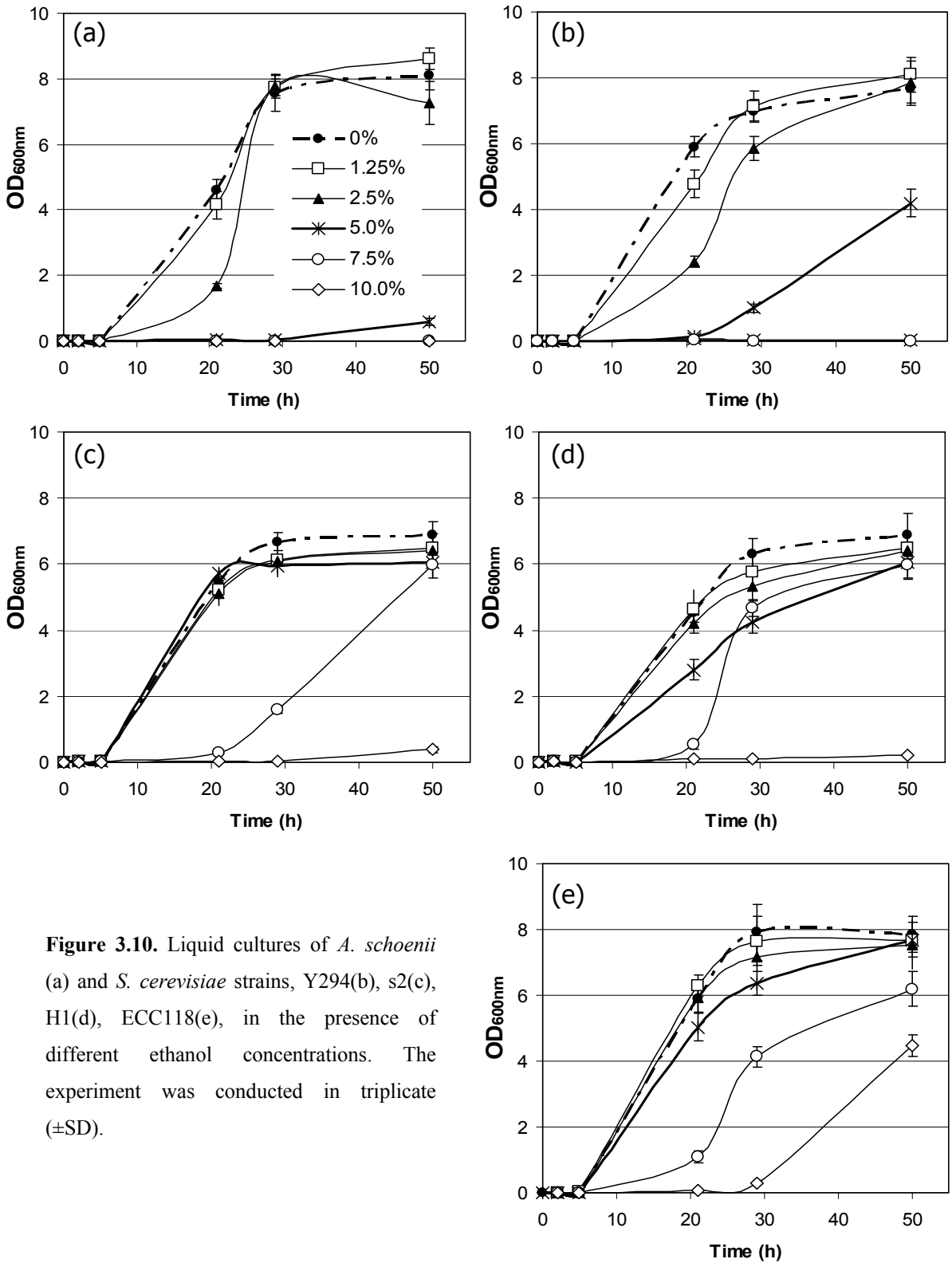

Figure 3.10. Liquid cultures of *A. schoenii* (a) and *S. cerevisiae* strains, Y294(b), s2(c), H1(d), ECC118(e), in the presence of different ethanol concentrations. The experiment was conducted in triplicate (±SD).

This finding seems very interesting because *S. cerevisiae* has traditionally been used in the fermented food and beverage industries. Moreover, *S. cerevisiae* is well known for its fast growth and fermentation rates, ethanol tolerance and resistance to virus infection. Therefore *A. schoenii*, although without a significant fermentative vigour, is able to tolerate ethanol as a laboratory strain of high fermentative yeast species.

A. schoenii alcohol resistance was compared also with wild type *S. cerevisiae* isolates. The *S. cerevisiae* yeasts confirmed their robustness growing at higher ethanol concentrations. *S. cerevisiae* s2, reference strain of the high fermentative yeast collection belonging to the Dipartimento di Biotecnologie Agrarie, grew well at 7.5% ethanol but did not show any significant growth at 10% (Figure 3.10c).

The strain H1, isolated from a distillery, showed a comparable alcohol tolerance (Figure 3.10d) while the commercial strain ECC1118 could be considered as the best ethanol tolerant strain tested: the isolate grew even at 10% ethanol concentration although the growth was slower than that in 7.5% (Figure 3.10e).

Study on the putative inhibitory activity of *A. schoenii.*

On the basis of its promising ethanol tolerance and cellulase activity, the *A. schoenii* isolate could be successfully used in a CBP microbial consortium toghether with a high fermentative yeast strain. Moreover, *A. schoenii*, grown in several media, demonstrated slow glucose consumption and therefore minimal glucose requirement (data not shown). Theoretically, the strain could proficiently hydrolyse cellulose, using only a little amount of the enzymatically released glucose for growth purpose. As a result, the non utilised sugar could be efficiently converted into ethanol by a properly selected *S. cerevisiae* strain.

In order to apply such a process, the strains of the mixed culture should not have the ability to inhibit each other leading to reduced growth and therefore ethanol conversion rate. This perspective is of great importance especially in the case of *Arthroascus* since this genus has been reported in literature to include killer strains, able to inhibit growth of other yeasts and fungi (Kreger-van Rij and Veenhuis, 1973; Suh et al., 2006). Few *A. schoenii*

and *A. javanensis* strains were recently described for their antimicrobial activity mainly due to the penetration of prey cells by means of infection pegs (Lachance et al., 2000).

In order to detect any potential yeast killer activity of the *A. schoenii* strain, well diffusion assays and spot tests were performed with either culture samples or cell-free supernatant of the yeast grown overnight at 30°C.

In the experimental conditions tested, the *A. schoenii* isolate did not show any antimicrobial activity against *S. cerevisiae* s2. The non-*Saccharomyces* yeast was unable to grow at 37°C both on spot and well diffusion assays (Figure 3.11 and 3.12). At lower temperatures (20-30 °C), it grew well on YPD soft agar inoculated with *S. cerevisiae* s2 without producing any killer activity.

Figure 3.11 shows the results of spot tests carried out on soft agar with two pH values (4.5 and 6.4): even at pH 4.5, previously reported as optimal value for killer toxins activity (Barre, 1992; Wingfield et al., 1990), no inhibitory halos were detected. The target *S. cerevisiae* s2 strain grew well into the soft agar medium at both cell densities ($1x10^5$ and $1x10^6$ cells per mL) used as inoculum.

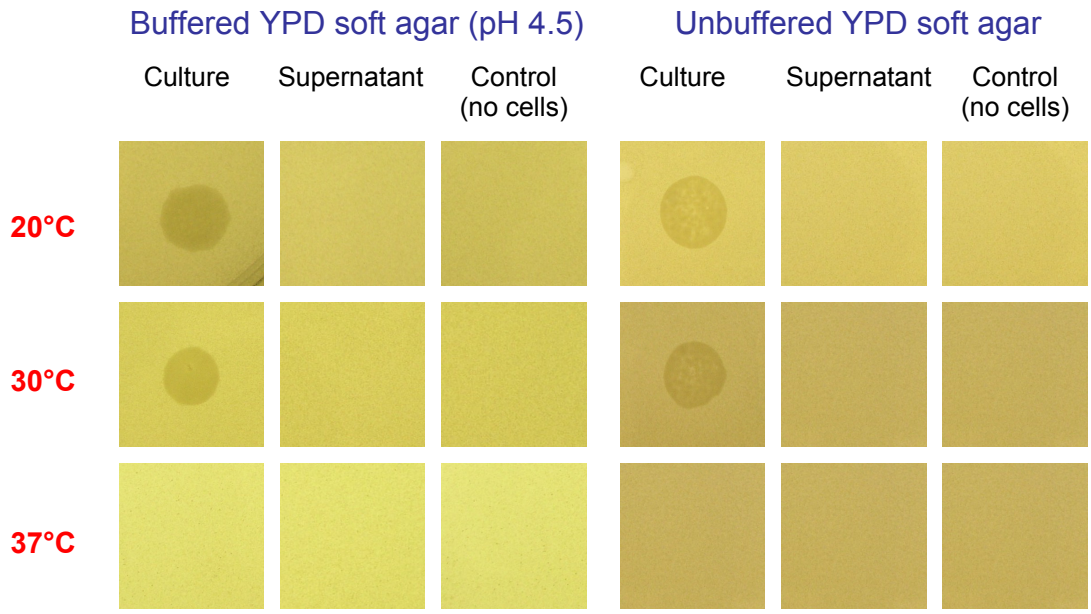

Figure 3.11. Antimicrobial activity (spot test assay) of culture and supernatant samples of *A. schoenii* against *S. cerevisae* s2 inoculated ($1x10^5$ cells mL^{-1}) in buffered (pH 4.5) and unbuffered YPD soft agar after 24 h incubation at three temperatures. YPD broth was used as control.

The results of the well diffusion assays, reported in Figure 3.12, confirm the inability of *A. schoenii* of inhibiting the growth of the target yeast: the potential yeast killer activity was not detectable both in cell-free culture supernatant and in cultured broth.

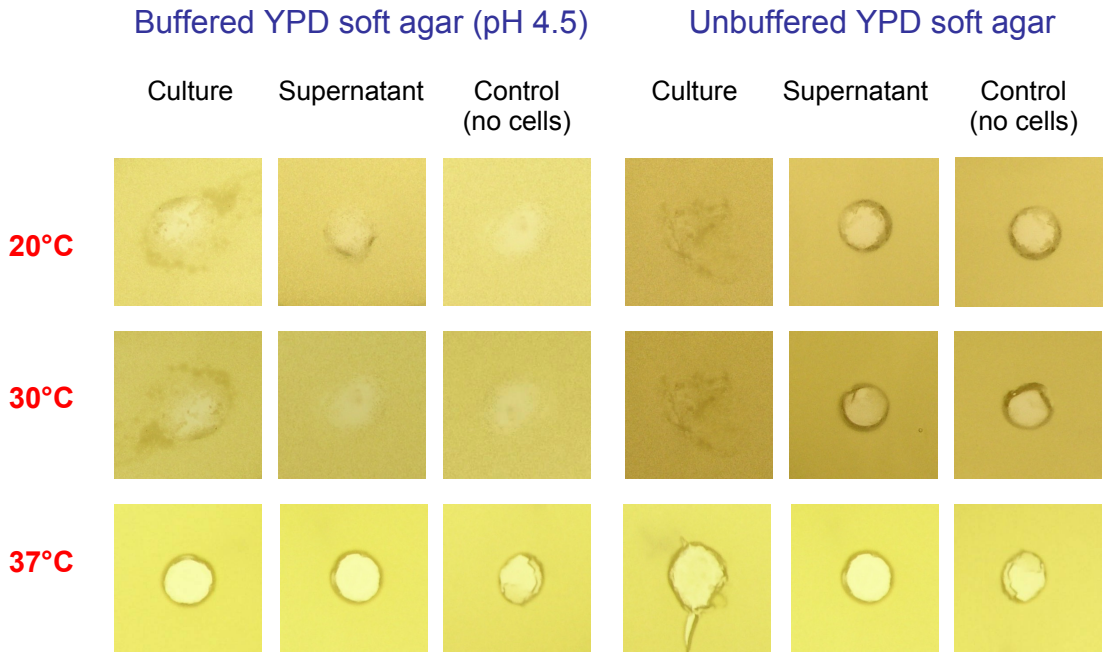

Figure 3.12. Antimicrobial activity (well diffusion assay) of culture and supernatant samples of *A. schoenii* against *S. cerevisae* s2 inoculated ($1x10^5$ cells mL^{-1}) in buffered (pH 4.5) and unbuffered YPD soft agar after 24 h incubation at three temperatures. YPD broth was used as control.

3.2.3 Discussion.

Several reviewers still stress the fact that, although advances in genetics and microbial physiology had a strong impact on enzyme production, screening programmes for the selection of microbes able to produce bioactive molecules continue to be an important biotechnological aspect (Steele and Stowers 1991; Bull et al., 1992). New commercial opportunities, indeed, could be revealed by systematic programmes of screening microbial strains aimed at well-defined industrial targets.

The above presented screening clearly revealed the potential of yeasts isolated from oenological environments to produce a wide range of extracellular enzymatic activities. The

selection of such yeast possessing interesting enzymatic profiles could be very promising for future applications in bioethanol production.

For example, this study indicated that oenological yeasts are able of producing pectolytic activity: twelve *S. cerevisiae* strains have the potential to hydrolyse polygalacturonic acid. This finding could be explained considering the high selective pressure of the environment the strains originated from. The isolates were selected from grape marcs, a complex material quite rich in pectin, that may have selected the strains with the ability to use this polysaccharide as carbon source. Renouf et al. (2005) argued a similar ecological significance of the pectolytic and lypolytic activities produced by the microbial population developed on the grape berry surface.

Pectic enzymes are mainly produced by moulds and bacteria. However yeast as a group have long been known to be capable of producing pectin-degrading enzymes and utilising pectin as a carbon source (Biely and Slavikova, 1994). Pectic enzymes from yeasts are mainly endo-polygalacturonases, enzymes which randomly degrade the main chain of the pectic backbone, mainly via hydrolysis of α-1,4-glycosidic linkages. Yeast pectinase production is mainly a constitutive capacity because pectin, polygacturonic acid and galacturonic acid are not required to induce the synthesis of these enzymes (Blanco et al., 1998).

Earlier screening surveys of yeast for pectolytic activity have shown that the property is not generally distributed and is limited to several genera only. Luh and Phaff (1951) were the first to report the ability of *Kluyveromyces fragilis* and *Candida tropicalis* to clarify liquid media containing citrus pectin. Roelofsen (1953) observed the production of pectin-degrading enzymes by strains of the genera *Candida*, *Pichia* and *Zygosaccharomyces*. Later screenings have established that yeasts depolymerising pectin-substances include mainly the genera *Rhodotorula*, *Cryptococcus* and *Saccharomyces* (Frederici et al., 1988; Vaughn et al., 1969; Wimborne and Rickard, 1978).

Several *Saccharomyces* species were reported to have polygalacturonase activity: *S. carlsbergensis*, *S. chevalieri*, *S. cerevisiae*, *S. oviformis*, *S. uvarum* and *S. vini* (Kotomina and Pisarnitskii 1974). It was later claimed that certain strains of *S. cerevisiae* have the ability to degrade polygalacturonic acid in the presence of glucose (McKay, 1990). Recently, a single culture of *S. cerevisiae*, that potentially produces pectinesterase,

polygalacturonase and pectin lyase, was isolated (Gainvors et al., 1994). Blanco et al. (1994) reported that at least 75% of oenological strains tested showed limited pectolytic activity. Blanco et al. (1998) speculated that all *S. cerevisiae* strains contain a promoter-less polygalacturonase gene or else a nonfunctional one. The structural polygalacturonase-encoding gene (*pgu1*) from *S. cerevisiae* IM1-8b was cloned and sequenced. This polygalacturonase gene showed 54% homology with the fungal polygalacturonases and only 24% homology with its plant and bacterial counterparts. *pgu1* sequence was present in a single gene copy per haploid genome and it was detected in all strains, regardless of their phenotype (Gognies et al., 1999).

This screening has also confirmed that oenological yeasts could produce limited proteolytic activity. The weak proteolytic activity showed by nine *S. cerevisiae* strains selected in this study should be further investigated through several physiological and biochemical approaches. However, the production of proteolytic halos on selective media could be considered as an evidence in *S. cerevisiae* of the ability of hydrolysing skim milk protein.

So far, the production of yeast proteases has been studied in relation to protein haze reduction in beer and wine industry. The vacuolar protease A plays an important role during the autolysis process, which occurs in wines kept on yeast lees during ageing. However, because of the particular conditions found in wine, only a few proteases are active. Eight yeast strains were identified which consistently exhibited proteolytic activity in model wine solutions. These included *Candida olea*, *C. flavus*, *Metschnikowia pulcherrima*, *Pichia pinus*, *Torulopsis magnolia*, *T. monosa* and *Yarrowia lipolytica*. The extracellular proteolytic activities produced by *C. olea*, *C. lipolytica* and *Cryptococcus flavus* could be correlated with their ability to reduce wine haze. In a recent study Dizy and Bisson (2000) reported that strains of *Kloeckera* and *Hanseniaspora* produced the most proteolytic activity in grape juice, and affected the protein profile of the finished wines.

Lipases from yeasts are gaining industrial interest with applications in laundry detergents and in dairy industries (Burden and Eveleight, 1990; Ratledge and Tan, 1990), while little attention has so far been paid to esterases from yeasts (Basaran and Hang, 2000; Buzzini and Martini, 2002; Lloyd et al., 1971). Apparently, in the present study, the occurrence of lipase activity was essentially associated with non-*Saccharomyces* strains

(Table 3.2). Fourteen strains, indeed, have the potential to produce extracellular lipolytic activity. These enzymes could be interesting in wine making process for the degradation of lipids originating from the grape or any autolytic reactions of yeasts. Therefore their activity may potentially impact on wine quality.

However, the ability to hydrolyse tributyrin does not necessarily mean that these yeasts would degrade grape juice lipids and further research is needed to determine this attractive possibility. The lipase capability of the positive strains selected in this study should be improved in order to enhance their hydrolytic activity as a source of enzymes potentially exploitable for biotechnological purposes.

Hemicellulose is a group of polysaccharides associated with cellulose in plant cell walls. These complex carbohydrate polymers contain xylan as their main component and β-1,4-xylans are mainly found in the secondary cell walls of plants, where it acts as one of the major components of woody tissue (Thomson, 1993). A high fermentative yeast capable of hydrolysing xilan could be a promising microbial strain for the conversion of lignocellulose into ethanol. Unfortunately, no *S. cerevisiae* and non-*Saccharomyces* strains were selected for their xylan-degrading activity. This study clearly confirmed that to date the xylanolytic activity is a feature rarely detected in oenological yeast strains (Strauss et al., 2001).

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls. This complex polysaccharide is hydrolysed into glucose by a wide range of multiple enzymes. Microorganisms able to completely degrade cellulose have been described but to date no one showed the ability to convert cellulose into ethanol with high conversion rate and high yield. The non-*Saccharomyces* yeast strain, identified in this study as *A. schoenii*, proved effective for the production of cellulose-degrading enzymes both on plates and enzymatic assays. This finding revealed to be novel since in literature no oenological yeast strain has been described for such high cellulolytic activity on CMC. In addition, *A. schoenii* showed a good and improvable ethanol tolerance when incubated in the presence of increasing alcohol concentrations. Moreover, the non-*Saccharomyces* yeast could be efficiently used in a microbial CBP consortium since did not produce any antimicrobial activity against the selected high fermentative *S. cerevisiae* strain. Killer phenotype is present in *S. cerevisiae* and in several other yeast genera (Magliani et al., 1997). The natural distribution of yeast producing killer toxins and

sensitivity to those toxins were demonstrated to be related to phylogeny as well as to ecological habitats of the strains (Ganter and Starmer, 1992). Killer phenotype has also been reported as a tool to estimate yeast diversity (Buzzini and Martini, 2000). However, further studies are required for a deeper evaluation of antimicrobial activity in the *A. schoeenii* isolate. If the future results will confirm that the yeast has no killer or predacious activity against selected *S. cerevisiae* strains, the perspective of a CBP microbial consortium could be efficiently applied using *A. schoeenii* as proficient cellulolytic yeast.

The degradation of starch is not important from an oenological perspective. This ability may be more interesting for whisky fermentations but is considered essential for the CBP conversion of starchy materials by a single microbe with proper fermentative abilities. Considerable cost savings, indeed, could be realised by the use of a yeast that both produces its own α-amylase and glucoamylase and ferments into ethanol the yielding glucose.

From this point of view, the results about amylolytic activities screened in this study should be considered as significant. Thirteen strains of *S. cerevisiae* were selected for their starch degrading activity on selective media.

There have been several reports about yeasts that could produce extracellular α-amylase and glucoamylase. These include *Candida tsukubaensis* CBS 6389, *Filobasisium capsuligenum* (de Mot and Verachtert, 1985), *Lipomyces starkeyi* (Kelly et al., 1985), *Saccharomycopsis capsularis*, *Saccharomycopsis fibuligera* (Ebertova, 1966; Gasperik et al., 1985), *Schwanniomyces alluvius* and *Schwanniomyces castelli* (Sills et al., 1984; Simoes-Mendes, 1984). Amylase secretion by yeast is highly dependent on medium composition, with soluble starch and dextrin being the best carbon sources for inducing this activity. Some amylases are produced constitutively and require the glucose as well as starch substrate (De Mot and Verachtert, 1987).

However, no *S. cerevisiae* strain have been described to date for starch-hydrolytic activity. Thus the novel side of this screening is the selection of *S. cerevisiae* strains able to grow on soluble starch and to produce starch-degrading halos both on complete and minimal media. Their weak growth on starch minimal agar plates (Figure 3.7) was unexpected since the common dogma (Pardo et al., 1986; Pretorius, 1997) is that wild type *S. cerevisiae* cannot grow on starch as carbon source. Although their starch degrading

activity was still low in comparison with *S. diastaticus*, producer of extracellular glucoamylases, this observation provided the basis for a multi-disciplinary study with biochemical, physiological and genetic approaches in order to give advance in knowledge about their potentially amylolytic enzyme(s).

3.3 Extensive biochemical, physiological and genetic study on the starch-hydrolytic mechanism showed by *S. cerevisiae* strains.

Thirteen strains showed the potential of starch-hydrolysis when incubated on complete and minimal media supplemented with soluble starch as the only carbon source. Their capability was further evaluated with multi-disciplinary approaches in order to look into this possible new starch-hydrolytic mechanism. The research was carried out by means of a) studies on their amylolytic activity in liquid cultures, b) definition of a reliable method for *in vitro* enzymatic assays c) genetic identification of putative glucoamylolytic sequence(s) in *S. cerevisiae* strains.

3.3.1 Determination of amylolytic activity in liquid media.

All potentially amylolytic yeasts were checked for their ability to grow in different broths using starch. To evaluate if starch-hydrolysing ability in *S. cerevisiae* strains is dependent on medium composition, both complete (YPD) and minimal media (MMY and EMM) were used.

Firstly, their starch degrading activity was checked in complete YPS broth supplemented with soluble starch (20 gL^{-1}). The selected strains grew at good levels reaching OD_{600} values up to 1.6. However, the isolates showed variable ability to use starch as carbon source. In Figure 3.13 the growth in liquid cultures of five strains, selected for their highest growth rate, is reported.

The growth of *S. cerevisiae* type strain DSM 70449 was much slower: the yeast, used as negative control, reached an OD_{600} of only about 0.75 after 48 h incubation at 30°C (Figure 3.13).

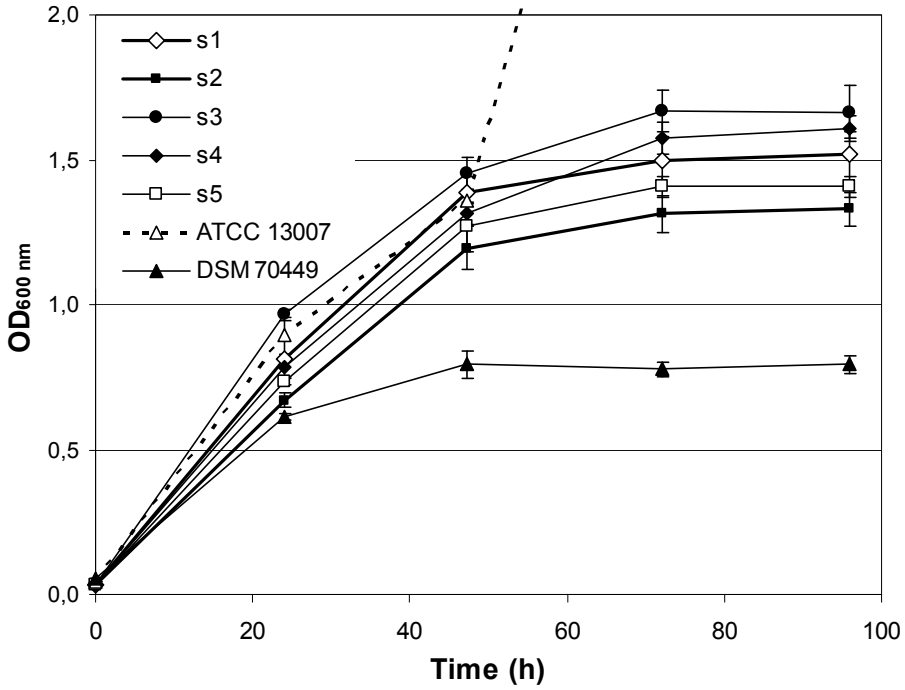

Figure 3.13. Liquid cultures in YPS broth (supplemented with gL^{-1}: yeast extract, 10; peptone, 20 and soluble starch, 20) of potentially starch-degrading *S. cerevisiae* strains (s1-s5), *S. diastaticus* (ATCC 13007) and *S. cerevisiae* type strain (DSM 70449). Data are the means of three replicates (± SD).

As expected, *S. diastaticus* ATCC 13007 performed the best growth rate and after 48 h incubation it resulted induced by soluble starch for the production of extracellular glucoamylases. The strain reached a final OD_{600} of 8.5 after 4 days incubation.

To test whether the growth of *S. cerevisiae* strains was depending closely on soluble starch and not on peptone or yeast extract added to the complete YPS medium, the strains were also grown in modified YP broth without starch (Figure 3.14). Yeasts did not exhibit growth comparable to that shown in the same medium supplemented with starch.

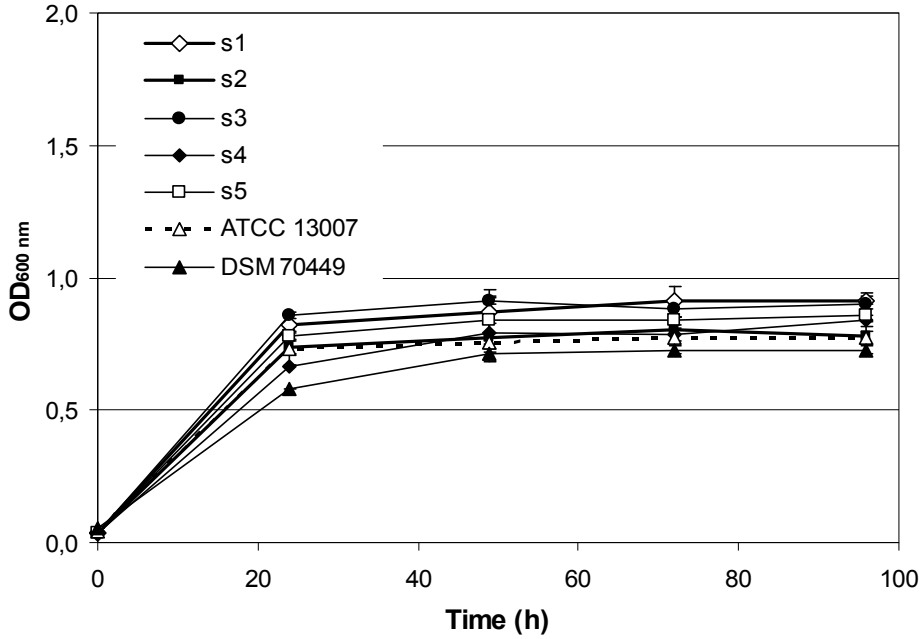

Figure 3.14. Liquid cultures in YP broth (supplemented with gL^{-1}: yeast extract, 10 and peptone, 20) of potentially starch-degrading *S. cerevisiae* strains (s1-s5), *S. diastaticus* (ATCC 13007) and *S. cerevisiae* type strain (DSM 70449). Data are the means of three replicates (± SD).

The thirteen strains were also cultured in MMY medium supplemented with starch (5 gL^{-1}). As indicated in Figure 3.15a, the five strains with the highest growth rate in YPS broth, confirmed their ability to use soluble starch as carbon source. The negative strain DSM 70449 gave no significant growth in terms of measurable absorbance: the yeast grew only up to 0.3 (OD_{600}) after a prolonged incubation at 30°C.

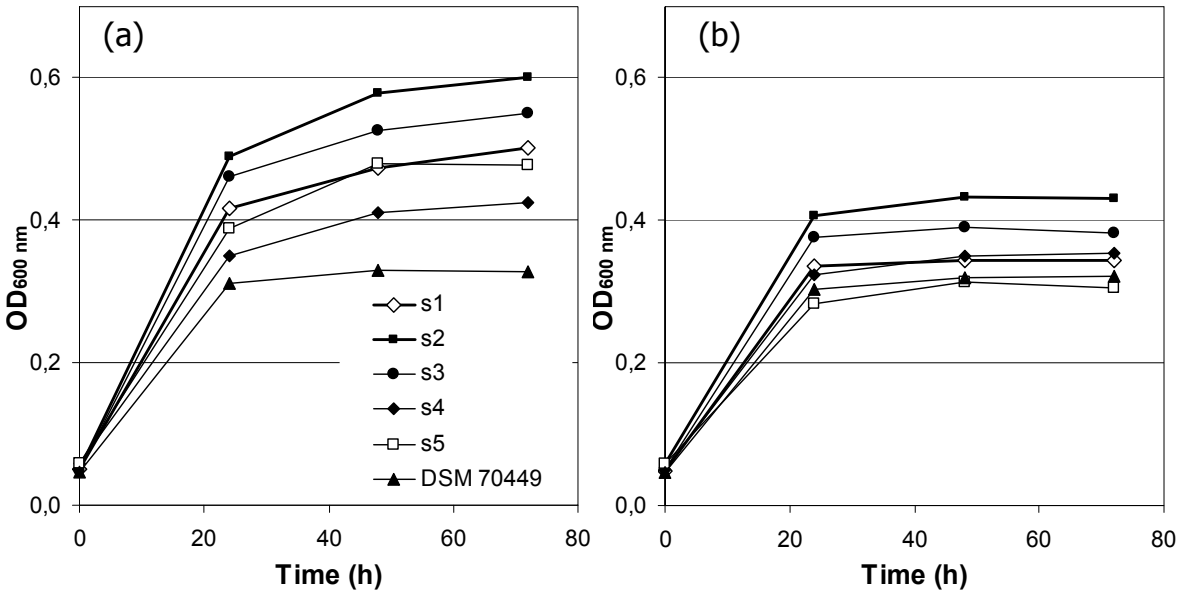

Figure 3.15. Liquid cultures of potentially starch-degrading *S. cerevisiae* strains (s1-s5) in Minimal Medium Yeast with (a) or without (b) added soluble starch (5 gL^{-1}). *S. cerevisiae* DSM 70449 was used as negative control strain. Data represent the means of two replicates.

However, on the basis of the growth exhibited by amylolytic yeasts, MMY formulation could be a limiting factor for their enzymatic activity on soluble starch. Once incubated in the same medium without starch, the strains grew at levels quite similar to those showed in MMY supplemented with soluble starch (Figure 3.15b). For example, *S. cerevisiae* s2 was able to grow up to 0.43 (OD_{600}) after 48 h incubation while, in the presence of starch as available carbon source, it grew only up to 0.57. This evidence may be due to the MMY restricted amount of any component essential for amylase production by *S. cerevisiae* strains. This suggestion is consistent with that of De Mot and Verachtert (1987) who reported that amylase secretion by yeast is highly dependent on medium composition. Therefore, MMY broth could be not effective for supporting amylolytic enzymes production or activity by the selected yeasts.

The five strains with the highest growth rate in YPS broth (Figure 3.13) were grown also in Edimburgh Minimal Medium (EMM). Data reported in Figure 3.16 indicate that amylolytic yeasts grew well on soluble starch in a liquid minimal medium, too. All wild type strains showed starch degrading activity within 72 h incubation at 30°C.

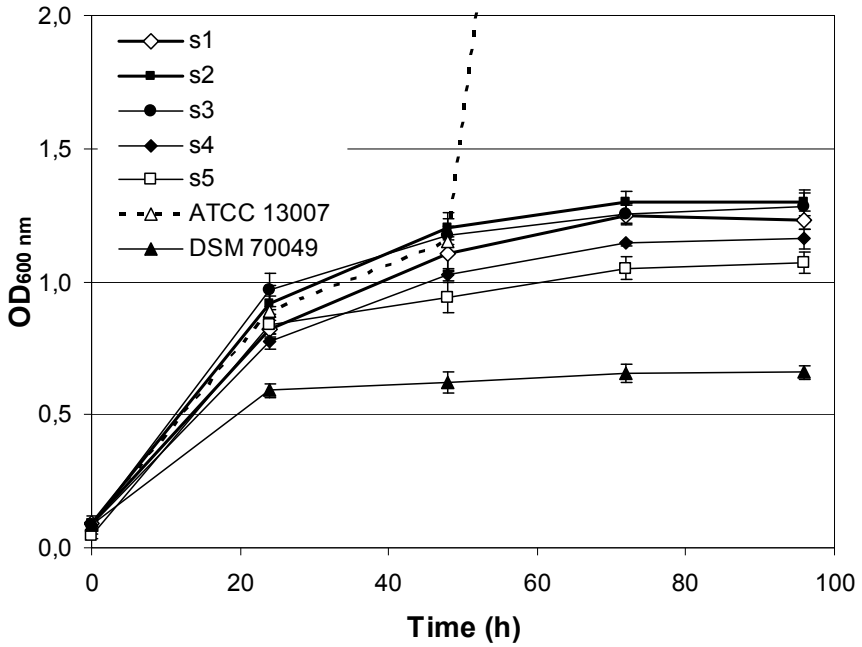

Figure 3.16. Liquid cultures in EMM broth (20 gL^{-1} soluble starch) of potentially starch-degrading *S. cerevisiae* strains (s1-s5), *S. diastaticus* (ATCC 13007) and *S. cerevisiae* type strain (DSM 70449). Data are the means of three replicates (± SD).

The presence of starch in the broth was essential for the yeast growth: in EMM medium formulated without the polysaccharide, the yeasts showed only limited growth within 24 h incubation. Moreover, the addition of glucose at the concentration equivalent to that present as impurities of starch into fresh EMM (0.18 gL^{-1}) did not sustain the extra growth of potentially amylolytic strains (data not shown). Thus, the greater number of generations accomplished by the cells in the minimal broth was ascribed to result from starch utilisation.

S. cerevisiae DSM 70449 grew up to 0.65 OD_{600} within 72 h incubation. This growth was probably due to the presence in the broth of a small amount of potassium hydrogen phthalate, utilised by yeast for growth. On the other hand, *S. diastaticus* resulted more proficient in growth rate. After 48 h incubation, the yeast showed consistent growth on soluble starch. Therefore, its glucoamylases should support the cell growth much better than the putative starch-degrading enzymes of the selected *S. cerevisiae* strains.

Analyses of the residual starch in exhausted EMM broth seem to confirm this hypothesis: as reported in Table 3.4, while *S. diastaticus* hydrolysed 75% of the soluble starch, *S. cerevisiae* strains were able to use no more than 6%.

Strain	Starch in exhausted medium (gL^{-1})	Starch utilised by strain (gL^{-1})
ATCC 13007	4.25 (±0.24)	15.75
s1	18.72 (±0.19)	1.28
s2	18.94 (±0.19)	1.06
s3	19.15 (±0.18)	0.85
s4	19.09 (±0.23)	0.91
s5	19.12 (±0.16)	0.88
DSM 70449	19.77 (±0.13)	0.23

Table 3.4. Starch utilisation by *Saccharomyces* sp. strains grown in EMM (20 gL^{-1} soluble starch) for 6 days at 30°C. Data are the means of three independent experiments (± SD).

The deficiency observed in *S. cerevisiae* strains could be probably due to the activity of different enzymes involved in starch degradation. Alternatively, the yeasts could have glucoamylase(s) with lower enzymatic efficiency than that secreted by *S. diastaticus* ATCC 13007.

3.3.2 Glucoamylase production and enzymatic assays.

In order to better understand the nature of the hydrolytic mechanism exhibited by *S. cerevisiae* yeasts, it was necessary to assess a reliable method for amylolytic enzymatic assay. On agar plates, the selected *S. cerevisiae* strains showed starch hydrolysis halos very similar to that detected in *S. diastaticus* (Figure 3.7). On the basis of this evidence, glucoamylolytic activity of the *S. cerevisiae* isolates was estimated according to Verma method, recently described for *S. diastaticus* (Verma et al., 2000). Cell-free culture

supernatant of the yeasts grown in YPS broth was used in the enzymatic assays measuring the glucose units enzymatically released from soluble starch.

S. diastaticus had glucoamylolytic activity values comparable to those previously reported in literature (Laluce and Mattoon, 1984; Verma et al., 2000). As shown in Figure 3.17, *S. diastaticus*, aerobically grown in YPS supplemented with starch 20 gL^{-1}, gave the highest enzymatic activity (261.38 UmL^{-1}) after 96 h incubation. On the other hand, *S. cerevisiae* strains did not show any glucoamylolytic activity.

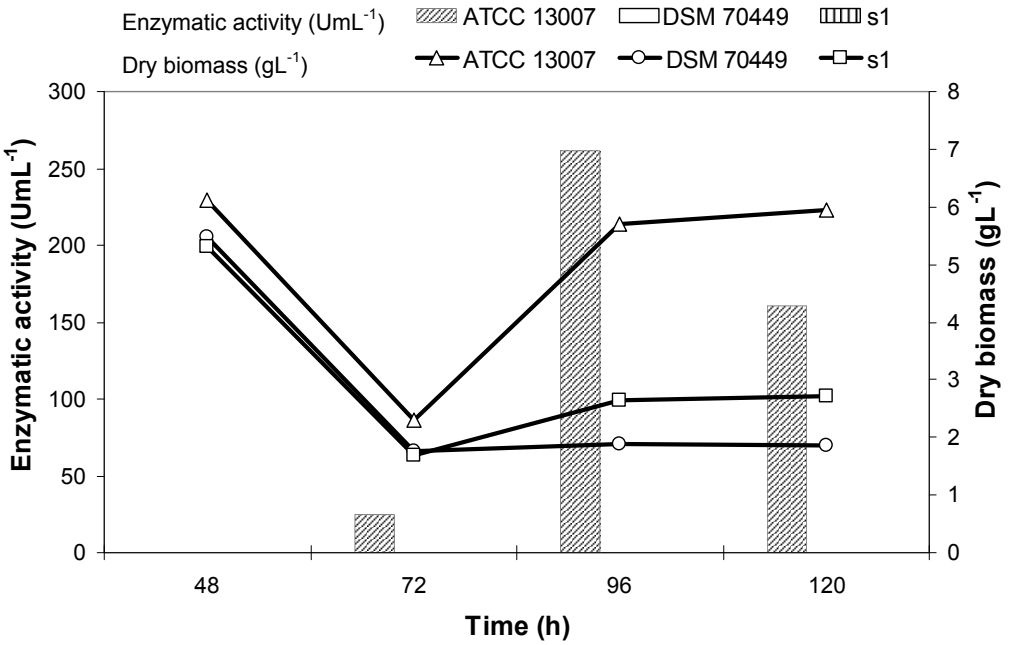

Figure 3.17. Dynamic of glucoamylolytic activity (bars) and dry biomass (open symbols) over 120 h in YEPS broth (20 gL^{-1} starch) of *Saccharomyces* sp. strains: *S. cerevisiae* type strain (DSM 70449), *S. diastaticus* (ATCC 13007) and the starch-degrading *S. cerevisiae* s1. The experiment was conduted in triplicate.

S. cerevisiae s1, reported in Figure 3.17 as representative for the selected *S. cerevisiae* yeasts, did not exhibit any detectable glucoamylolytic activity but produced dry biomass 35% higher than *S. cerevisiae* DSM 70449.

In order to detect even low enzymatic activities performed by *S. cerevisiae* strains, Verma method was modified. Several assays were performed changing either the ratio of starch solution/culture supernatant and the incubation time. In the tested experimental conditions, the wild type *S. cerevisiae* yeasts did not produce any glucoamylolytic activity.

This result indicates that their starch-degrading potential could be due to a different enzymatic mechanisms. Moreover, the method used in this study proved effective for the detection of secreted glucoamylase. The activity of the *S. cerevisiae* isolates may be related to cell-bound enzyme(s). In this case, culture samples and not cell-free supernatant should be used for enzymatic assays.

3.3.3 Genetic study on putative glucoamylolytic sequence(s) of *S. cerevisiae* strains.

The hydrolysis halos on starch agar dishes performed by the selected *S. cerevisiae* strains were very similar to *S. diastaticus* (Figure 3.7). The yeast *S. diastaticus* is clearly related to *S. cerevisiae*, except for ethanol performance and extracellular glucoamylase production. Starch utilisation in *S. diastaticus* depends on the expression of the three unlinked genes, *sta1* (chr. IV), *sta2* (chr. II) and *sta3* (chr. XIV), each encoding one of the extracellular glycosylated glucoamylases isoenzymes GaI, GaII, or GaIII, respectively (Pretorius et al., 1991).

Moreover, glucoamylase has already been identified in *S. cerevisiae* (Pugh et al., 1989). The expression of glucoamylase activity in *S. cerevisiae* is confined only to the sporulation phase of the life cycle. The sporulation glucoamylase, encoded by the *sga* gene, is intracellularly produced to breakdown glycogen stores in the cell at the time of spore formation (James and Lee, 1997).

The *S. diastaticus sta1-2* and *S. cerevisiae sga* gene sequences were aligned using CLUSTALw program. Nucleotide sequences were obtained from GenBank and the corresponding accession numbers are reported in Table 2.3. The accurate evaluation of various alignments revealed that *sga* is homologous to the middle and 3′ regions of the *sta* genes but lacks a 5′ sequence that encodes the secretion domain of the extracellular glucoamylases. The primer pairs (STA1F-STA1R; STA2F-STA2R designed on *sta1* and *sta2* sequences, respectively) derived from that 5’ region, highly conserved in other extracellular glucoamylase genes. Genomic DNA amplifications of both primer pairs are presented in Figure 3.18 and 3.19.

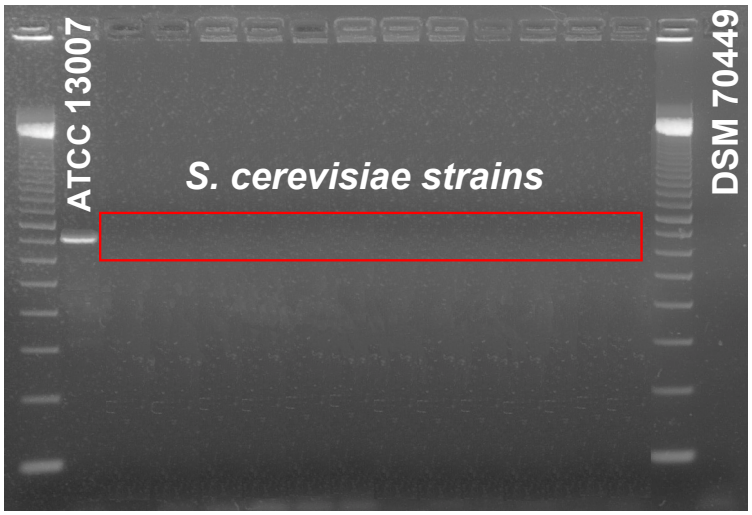

Figure 3.18. Gel electrophoresis of STA1F-STA1R primers PCR amplification product of *Saccharomyces* sp. strains: *S. cerevisiae* type strain (DSM 70449), *S. diastaticus* (ATCC 13007) and the starch-degrading *S. cerevisiae* strains. M: Molecular weight marker 'GeneRulerTM 100-bp DNA ladder', Fermentas.

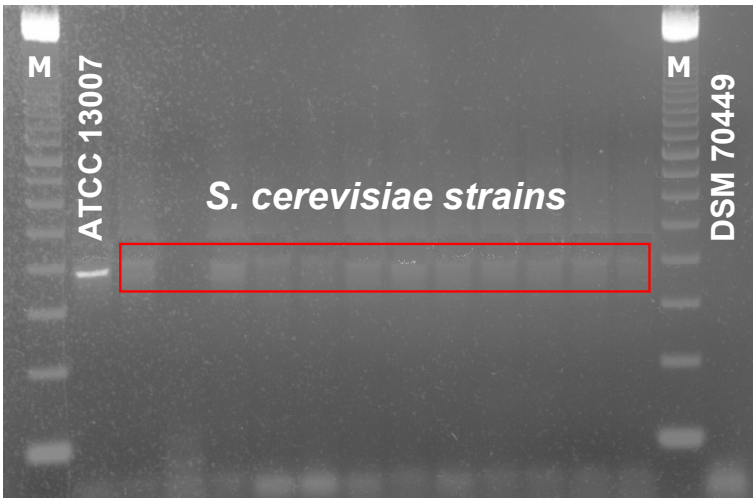

Figure 3.19. Gel electrophoresis of STA2F-STA2R primers PCR amplification product of *Saccharomyces* sp. strains: *S. cerevisiae* type strain (DSM 70449), *S. diastaticus* (ATCC 13007) and the starch-degrading *S. cerevisiae* strains. M: Molecular weight marker 'GeneRulerTM 100-bp DNA ladder', Fermentas.

As expected, *S. diastaticus* produced single clear bands of 700 bp and of 388 bp with STA1F-STA1R and STA2F-STA2R primers, respectively. No signal was detected with the thirteen potentially amylolytic *S. cerevisiae* strains nor with *S. cerevisiae* DSM 70449.

This evidence may confirm that their starch hydrolysing activity could be ascribed to different enzymatic mechanisms.

3.3.4 Discussion.

In contrast to the accepted view that *S. cerevisiae* cannot use starch as a sole carbon source for growth, thirteen wild type strains of this species were found able of utilise starch in both complete and minimal broths. Heterogeneity in growth rate was also observed, suggesting that there is some genetic variability in the starch growth phenotype.

Their growth and starch-utilising capability were strongly dependent on the media composition. Complete medium supported amylolytic activity better than minimal broths. These results were consistent with the literature on yeast amylase (De Mot and Verachtert, 1987; Fogarty and Kelly, 1979; Pandey et al., 2000; Sun et al., 2009; Vihinen and Mantsiila, 1989). Gupta et al. (2003), in a recent review, indicated pH, nitrogen and phosphate sources as the main physico-chemical parameters affecting microbial amylase production.

The highest growth rates were indeed detected in YPS complete medium. However, the complex additives (yeast extract and peptone) included in YPS formulation supplies other carbon sources that yeast can metabolise. In EMM broth, which was supplemented only with NH_4Cl as nitrogen source, the strains proved effective for their growth on soluble starch.

In addition, extensive biochemical and genetic study on their potentially amylolytic enzyme(s) was applied. In the experimental conditions tested, the *S. cerevisiae* strains did not show any extracellular glucoamylolytic activity. Considering that their starch hydrolysis halos on agar plates were similar to those produced by *S. diastaticus* (Paragraph 3.2), the absence of an extracellular amylolytic activity was unexpected.

To verify whether these potential amylolytic enzymes have cell-bound localization, a new enzymatic assay was defined. In this way, further studies on their potentially cell-bound glucoamylolytic enzymes will be performed. Alternatively, the absence in *S. cerevisiae* strains of detectable glucoamylolytic activity may indicate that their starch-

degrading potential could be due to different enzyme(s). The yeasts could utilise starch by the action of endo-hydrolysing enzymes. It is the case of α-amylase that cleaves α-1,4 linkages yielding reducing groups with the α-configuration and/or pullulanase that hydrolyse α-1,6 linkages.

Therefore, the *S. cerevisiae* strains could be able to use starch for growth by a cell bound glucoamylolytic mechanism and/or endo-hydrolysing enzymes. However, their enzymes should be different from the glucoamylase secreted by *S. cerevisiae.*

This suggestion seems to be confirmed by genetic investigations on the putative glucoamylolytic sequence(s) in *S. cerevisiae* strains. All yeasts did not possess sequences similar to *sta* genes encoding extracellular glucoamylases in *S. diastaticus*. Nevertheless, the search of the *sta* genes or genes with the same function is still in progress. Further study on the gene-regulation and enzymatic efficiency of these DNA-sequences started in order to enhance and improve the starch-degrading activity of the selected *S. cerevisiae* strains.

On the basis of the preliminary results presented above, the starch-hydrolytic mechanism showed by *S. cerevisiae* yeasts seems related to amylolytic enzyme(s) with cell-bound localization and low activity. This hypothesis may explain both the slow starch degradation detected in all tested media and the absence of amylolytic activity in cell-free supernatants of *S. cerevisiae* strains.

The observations that wild type yeasts do in fact grow, though slowly, using starch as a sole carbon source, and that different strains vary in this phenotype, provide the basis for a new approach using breeding and natural selection to derive non-GM yeasts with the ability to grow rapidly on starch. A similar work was successfully achieved for the development of non recombinant xylose-utilising strains of *S. cerevisiae* (Attfield and Bell, 2006). The authors improved the native ability of few yeasts to grow slowly on xylose as a sole carbon source. Therefore, breeding and natural selection are interesting tools that may open an alternative route to the development of efficient starch-converting yeasts. Furthermore, population-genetics approach could be coupled with the genetic-engineering strategies to obtain optimally performing strains for bioethanol production.

3.4 Study of a Separated Hydrolysis and fermentation (SHF) process for the conversion of wheat bran into ethanol.

The study presented aimed to optimise few features of the Separate Hydrolysis and Fermentation (SHF) process recently proposed for wheat bran conversion into ethanol (Favaro et al., 2009). In particular, the previous work described various physical and chemical methods of hydrolysing the wheat bran polysaccharides. Firstly, acid hydrolysis, heat pre-treatment followed by enzymatic hydrolysis and direct enzymatic hydrolysis were compared in terms of total sugar yield. The maximum total sugar level was achieved when small amounts of acid (H_2SO_4 1-2% w/w) were added at the pre-treatment step prior to enzymatic hydrolysis. The hydrolysates were then fermented by two *S. cerevisiae* strains with good ethanol performances.

This study was designed to enhance the efficiency of that SHF method through the 1) definition of pre-treatments with low costs and easy industrial applicability 2) dosage optimisation of commercial enzymes 3) minimization of inhibitory compounds released during hydrolysis phase 4) optimisation of the process ethanol yield relying on *Saccharomyces* sp. strains previously selected for their high fermentative and amylolytic properties.

3.4.1 Wheat bran as feedstock.

The analysis of the wheat bran (WB) used in this study was conducted by LAZ laboratories (Department of Animal Science, University of Padova). As reported in Table 3.5, the major polysaccharide components are hemicellulose (29.73 g/100 g), starch (23.29 g/100 g), and cellulose (10.64 g/100 g).

While hemicellulose and cellulose are present as generally indicated in literature, this bran is quite rich in starch. This implies an unusually consistent starch amount that is not extracted during milling processes. Starch content is quite high if compared to the maximum 20 g starch/100 g previously reported by other authors (Bergmans et al., 2006;

Maes and Delcour, 2001) and it is nearly 75% of the value (34 g starch/100 g bran) described in Palmarola-Adrados et al. (2005).

Component	g/100 g WB
DM	88.71
Ash	5.85
Cellulose	10.64
Hemicellulose	29.73
Protein	15.12
Lignin	2.42
Starch	23.29

Table 3.5. Composition of the wheat bran (WB) used in this study.

Protein in bran accounted for 15%. The value agree well with recently published results (Palmarola-Adrados et al., 2005; Theander et al., 1995) while lignin is present in low value (2.42 g/100 g), approximately half of that reported by Palmarola-Adrados et al. (2005).

On the basis of its composition, wheat bran has the great potential to serve as low-cost feedstock for ethanol production. In many ethanol production plants from cereals, bran is not yet utilised for ethanol and could considerably increase the alcohol yield and productivity of the process.

3.4.2 Pre-treatment.

Since pure enzymatic treatments were not enough to give high yields of pentoses and hexoses (Favaro et al., 2009), wheat bran was heat treated at 121°C with increasing concentrations of sulphuric acid. Results, reported as released monosaccharides, are presented in Table 3.6 and 3.7 for raw and milled wheat bran, respectively.

The heat and acid treatments gave no significant releases of pentoses and hexoses. The highest yield was 0.26 and 0.09 gL^{-1} for glucose and xilose, respectively. Arabinose was detected at levels of about 0.35 gL^{-1} in both raw and milled bran. However, pre-treatment

step should have rendered the feedstock more susceptible to the subsequent enzymatic hydrolysis.

PRE-TREATMENT YIELD ON RAW WHEAT BRAN

(gL^{-1})

% H_2SO_4 w/w	Glucose	Xylose	Galattose	Arabinose	Mannose
0%	0.14 ± 0.03	0.09 ± 0.02	ND	0.33 ± 0.02	0.14 ± 0.03
0.1%	0.03 ± 0,01	0.03 ± 0.01	ND	0.33 ± 0.04	0.15 ± 0.03
0.3%	0.07 ± 0.01	0.04 ± 0.02	ND	0.38 ± 0.05	0.18 ± 0.04

Table 3.6. Pre-treatment yield on raw bran: monosaccharides (gL^{-1}) released from raw wheat bran treated at 121°C (30 min) with increasing concentrations of H_2SO_4. Data report the means of 5 replicates (±SD). ND: not detectable.

PRE-TREATMENT YIELD ON MILLED WHEAT BRAN

(gL^{-1})

% H_2SO_4 w/w	Glucose	Xylose	Galattose	Arabinose	Mannose
0%	0.09 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.31 ± 0.05	0.21 ± 0.08
0.1%	0.03 ± 0.01	0.02 ± 0.02	0.05 ± 0.01	0.40 ± 0.04	0.22 ± 0.05
0.3%	0.26 ± 0.02	0.08 ± 0.01	0.03 ± 0.01	0.42 ± 0.04	0.33 ± 0.08

Table 3.7. Pre-treatment yield on milled bran: monosaccharides (gL^{-1}) released from milled wheat bran treated at 121°C (30 min) with increasing concentrations of H_2SO_4. Data are the means of 5 replicates (±SD).

3.4.3 Enzymatic hydrolysis.

After pre-treatment, wheat bran was enzymatically hydrolysed in two sequential steps. Firstly, the material was treated adding enzyme solutions containing cellulase and xylanase. The enzymatic hydrolysis yields are presented in Table 3.8 for raw bran and in Table 3.9 for milled wheat bran.

The physico-chemical pre-treatment synergistically acted with the commercial enzymes ($p \leq 0.01$) giving consistent sugar releases. The highest glucose yield, 20.74 gL^{-1}, was reached in milled bran treated with 0.3% H_2SO_4 (w/w). The feedstock mechanical milling influenced the most important sugar yields in all experimental theses. Enzymatic hydrolysis of raw wheat bran gave only 87 and 82% of the glucose and xylose respectively measured in the milled material.

SUGAR YIELD AFTER PRE-TREATMENT AND FIRST ENZYMATIC HYDROLYSIS ON RAW WHEAT BRAN

(gL^{-1})

% H_2SO_4 w/w	**Glucose**	**Xylose**	**Galattose**	**Arabinose**	**Mannose**
0%	17.97 ± 2.14	4.99 ± 0.64	0.46 ± 0.05	0.95± 0.33	0.20 ± 0.02
0.1%	18.09 ± 1.55	5.34 ± 0.67	0.51 ± 0.11	1.00 ± 0.19	0.22 ± 0.08
0.3%	18.89 ± 2.13	6.26 ± 0.88	0.61 ± 0.12	1.23 ± 0.12	0.26 ± 0.07

Table 3.8. Sugar yield on raw bran after pre-treatment and first enzymatic hydrolysis (cellulase and xylanase). Data are the means of 5 replicates (±SD).

SUGAR YIELD AFTER PRE-TREATMENT AND FIRST ENZYMATIC HYDROLYSIS ON MILLED WHEAT BRAN

(gL^{-1})

% H_2SO_4 w/w	**Glucose**	**Xylose**	**Galattose**	**Arabinose**	**Mannose**
0%	19.27 ± 1.62	6.07 ± 0.62	0.76 ± 0.28	1.13 ± 0.21	0.30 ± 0.12
0.1%	20.95 ± 1.80	6.78 ± 0.47	0.78 ± 0.26	1.36 ± 0.20	0.35 ± 0.08
0.3%	22.35 ± 2.23	8.19 ± 0.89	0.82 ± 0.38	1.65 ± 0.21	0.44 ± 0.11

Table 3.9. Sugar yield on milled bran after pre-treatment and first enzymatic hydrolysis (cellulase and xylanase). Data are the means of 5 replicates (±SD).

Moreover, acid pre-treatment resulted in significant difference on pentoses yield ($p \leq 0.01$). For instance, rising amounts of xylose were detected as sulphuric acid concentrations increased. Also arabinose concentrations obtained in acid-treated raw and milled bran were statistically higher ($p \leq 0.01$) than those resulting from wheat bran pre-treated without sulphuric acid.

In Figure 3.20, the efficiency after the first enzymatic treatment is reported as sugar yield per 100 grams of dry bran. The highest value of sugar release, 37.6 g/100 g dry bran, was reached in the milled bran treated with 0.3% H_2SO_4 (w/w). However, physical-chemical and enzymatic treatments applied to the feedstock resulted in high hydrolysis yield both in raw and milled bran.

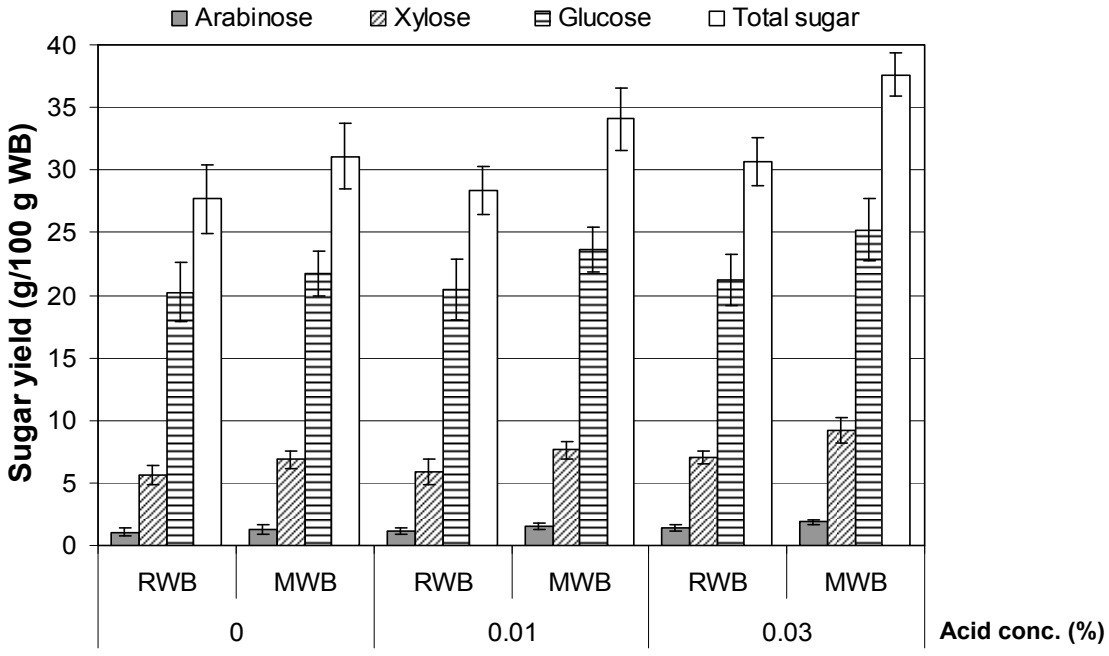

Figure 3.20. Sugar yield after pre-treatment and first enzymatic hydrolysis (cellulase and xylanase): g/100 g dry Wheat Bran (WB). Raw and milled wheat bran are reported as RWB and MWB, respectively. Data are the means of 5 replicates (±SD).

The sugar levels indicated in Figure 3.20 were obtained from the cellulose and hemicellulose fractions of the bran. On the basis of the bran composition (Table 3.6) the yield was interesting, corresponding to more than 87% of the theoretical maximum value (40.4 g/g 100 g dry bran).

After the enzymatic treatment of cellulose and hemicelllulose, the starchy component of the material was partially hydrolysed adding α-amylase. This second enzymatic hydrolysis, as reported in Table 3.10 and 3.11, resulted in additional glucose releases. However, the glucose amounts obtained were lower than that expected as wheat bran is rich in starch (Table 3.6). This finding may be explained considering that α-amylase randomly liberates reducing groups with the α-configuration (Solomon, 1978).

SUGAR YIELD AFTER PRE-TREATMENT AND SECOND ENZYMATIC HYDROLYSIS ON RAW WHEAT BRAN

(gL^{-1})

% H_2SO_4 w/w	**Glucose**	**Xylose**	**Galattose**	**Arabinose**	**Mannose**
0%	20.84 ± 1.81	5.19 ± 0.60	0.70 ± 0.20	1.11 ± 0.23	0.88 ± 0.38
0.1%	21.33 ± 1.91	5.50 ± 1.01	0.75 ± 0.19	1.03 ± 0.14	0.94 ± 0.29
0.3%	21.01 ± 1.48	6.85 ± 0.86	0.73 ± 0.21	1.29 ± 0.27	1.11 ± 0.25

Table 3.10. Sugar yield on raw bran after pre-treatment and second enzymatic hydrolysis (α-amylase). Data are the means of 5 replicates (±SD).

SUGAR YIELD AFTER PRE-TREATMENT AND SECOND ENZYMATIC HYDROLYSIS ON MILLED WHEAT BRAN

(gL^{-1})

% H_2SO_4 w/w	**Glucose**	**Xylose**	**Galattose**	**Arabinose**	**Mannose**
0%	21.96 ± 2.22	6.23 ± 0.69	0.82 ± 0.18	1.33 ± 0.29	0.94 ± 0.28
0.1%	22.18 ± 2.42	6.62 ± 0.85	0.97 ± 0.19	1.38 ± 0.21	1.10 ± 0.34
0.3%	23.28 ± 1.80	8.44 ± 0.64	1.05 ± 0.11	1.61 ± 0.38	1.54 ± 0.34

Table 3.11. Sugar yield on milled bran after pre-treatment and second enzymatic hydrolysis (α-amylase). Data are the means of 5 replicates (±SD).

In raw bran, the amylolytic enzyme released higher glucose amounts (2.87, 3.24, 2.12 gL^{-1} for bran treated with 0, 0.1, 0.3 % H_2SO_4, respectively) that those detected in milled bran hydrolysates. However, α-amylase addition had the effect to obtain hydrolysates with similar glucose concentrations in each kind of bran. Glucose levels reached an average level of about 21 and 22.5 gL^{-1} in raw and milled wheat bran, respectively.

The final sugar yield of pre-treatment and enzymatic hydrolysis were consistent (Figure 3.21). The highest monosaccharide value, 40.1 g/100 g dry WB, corresponding to 63% of the theorical yield, was obtained in the hydrolysis of the H_2SO_4 0.3% treated milled wheat bran. The same material gave the highest yield of xylose and arabinose while glucose content was higher in milled bran heat-treated without acid.

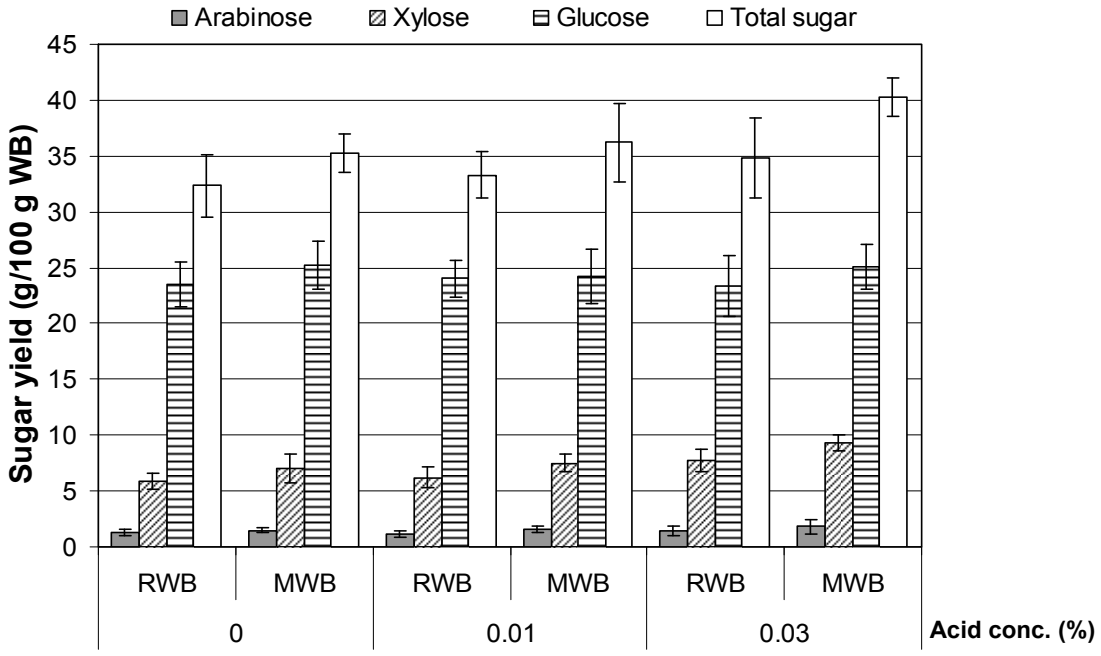

Figure 3.21. Sugar yield after pre-treatment and enzymatic hydrolysis (α-amylase): g/100 g dry Wheat Bran (WB). Raw and milled wheat bran are reported as RWB and MWB, respectively. Data are the means of 5 replicates (±SD).

3.4.4 By-product formation.

In addition to the sugars, several by-products are formed or released after the pre-treatment step (Larsson et al., 1999; Klinke et al., 2004). Among them, the most important are weak acids and mainly furfural and 5-hydroxymethyl-2-furaldehyde (HMF), formed by decomposition of pentoses and hexoses, respectively.

The yields of the fermentation-inhibiting compounds were similar in the monitored experimental theses (Table 3.12). Aliphatic acids were produced at low levels in both raw and milled bran. Acid acetic formation was influenced by sulphuric acid hydrolysis and milling treatment. Furaldehydes, furfural and HMF, were not detectable in all bran hydrolysates.

YIELDS OF INHIBITORY BY-PRODUCTS AFTER PRE-TREATMENT AND ENZYMATIC HYDROLYSIS

g/100 g dry WB

	Acetic acid	Lactic Acid	Furfural	HMF
RWB H_2SO_4 0%	0.94 ± 0.04	0.26 ± 0.10	ND	ND
RWB H_2SO_4 0.1%	1.12 ± 0.05	0.26 ± 0.09	ND	ND
RWB H_2SO_4 0.3%	1.21 ± 0.15	0.29 ± 0.02	ND	ND
MWB H_2SO_4 0%	1.04 ± 0.08	0.27 ± 0.04	ND	ND
MWB H_2SO_4 0.1%	1.13 ± 0.11	0.29 ± 0.06	ND	ND
MWB H_2SO_4 0.3%	1.24 ± 0.15	0.31 ± 0.09	ND	ND

Table 3.12. Inhibitory compounds production after pre-treatment and enzymatic hydrolysis of wheat bran: g/100 g dry Wheat Bran (WB). Raw and milled wheat bran are reported as RWB and MWB, respectively. Data are the means of 4 replicates (±SD). ND: not detectable.

3.3.5 Fermentation studies on wheat-bran hydrolysates.

In order to determine if the produced hydrolysates had inhibitory effects on the yeast, fermentation tests were performed. The unfiltered hydrolysates were fermented by *S. cerevisiae* s1, selected as the most efficient amylolytic *S. cerevisiae* isolate, and

S. diastaticus, producer of extracellular glucoamylase. The hydrolysates fermentation was also conducted with *S. diastaticus* once induced for the production of glucoamylase as described in the Paragraph 2.5.4.

Both yeasts should ferment the free glucose available even in unfiltered hydrolysates since they have been efficiently used in wine and beer factories. Moreover, their amylolytic activities on the starchy oligosaccharides liquefied by α-amylase treatment should support additional ethanol production.

The average sugar and inhibitor compositions of wheat bran hydrolysates are reported in Table 3.13. Glucose was the main monosaccharide with concentration ranging from 18.59 to 20.49 gL^{-1}. Because pentoses are not fermented by wild type *Saccharomyces* sp strains, this study focused mainly on glucose conversion to ethanol.

The highest concentration of sugar degradation by-products, 1.10 gL^{-1} acetic acid and 0.27 gL^{-1} lactic acid, were obtained in milled wheat bran. It has been reported that acetic acid could be inhibitory to yeast metabolism at a level of about 2-5 gL^{-1} (Martin and Jönsson, 2003; Roberto et al., 1991) while lactic acid at concentration higher than 3-6 gL^{-1} (Dorta et al., 2006; Olsson and Hahn-Hägerdal, 1996). Therefore, since no detectable level of furaldehydes was measured and low amounts of aliphatic acids were present in the hydrolysates, subsequent fermentation should not be compromised.

The data of fermentative kinetics on wheat bran hydrolysates derived from the means of two replicates conducted for each experimental thesis: the difference between the values was about 5%.

The fermentative performance of *S. diastaticus* on wheat bran hydrolysates is reported in Figure 3.22 and Figure 3.23. The yeast proved effective in ethanol production. On the basis of HPLC analysis monitoring both pentose and hexose sugars, the strain was not able to metabolise xylose, arabinose, galactose and mannose. Once free glucose was depleted, the total sugar amount remained constant.

On raw bran hydrolysates, *S. diastaticus* produced the highest ethanol value, 11.4 gL^{-1}, after a long incubation on H_2SO_4 0.1% treated bran (Figure 3.22b). Similar fermentative performance was detected in the H_2SO_4 0.3% treated bran (Figure 3.22c) while the ethanol production (8.9 gL^{-1}) from raw wheat bran without sulphuric acid was much lower (Figure 3.22a).

Products (gL^{-1})	Inhibitory yeast level (gL^{-1})	RWB H_2SO_4 0%	RWB H_2SO_4 0.1%	RWB H_2SO_4 0.3%	MWB H_2SO_4 0%	MWB H_2SO_4 0.1%	MWB H_2SO_4 0.3%
Lactic Acid	3.0-6.0[a]	0.23 ± 0.08	0.23 ± 0.08	0.26 ± 0.02	0.24 ± 0.03	0.25 ± 0.05	0.27 ± 0.11
Acetic acid	2.5-5.0[b]	0.83 ± 0.04	0.99 ± 0.09	1.08 ± 0.13	0.92 ± 0.07	1.00 ± 0.09	1.10 ± 0.13
HMF	1.5-2.0[c]	ND	ND	ND	ND	ND	ND
Furfural	1.0-2.0[d]	ND	ND	ND	ND	ND	ND
Glucose		18.59 ± 1.61	18.88 ± 1.69	18.63 ± 1.64	19.43 ± 1.96	19.54 ± 2.14	20.49 ± 1.41
Xylose		4.60 ± 0.53	4.88 ± 0.96	6.06 ± 0.76	5.51 ± 0.61	5.86 ± 0.75	7.29 ± 0.56
Galactose		0.62 ± 0.27	0.66 ± 0.17	0.64 ± 0.18	0.72 ± 0.20	0.86 ± 0.17	0.92 ± 0.10
Arabinose		0.98 ± 0.21	0.91 ± 0.12	1.14 ± 0.24	1.18 ± 0.26	1.22 ± 0.36	1.43 ± 0.24
Mannose		0.78 ± 0.31	0.84 ± 0.43	0.98 ± 0.40	0.83 ± 0.27	0.98 ± 0.30	1.37 ± 0.30

Table 3.13. Sugar and inhibitory by-product composition (gL^{-1}) of wheat bran hydrolysates. Data are the means of 4 replicates (±SD). ND: not detectable. Inhibitory yeast levels of toxic by-products are derived from [a] Essia Ngang et al., 1989; Olsson and Hahn-Hägerdal, 1996; Sjöström, 1991; van Maris et al., 2006 [b] Almeida et al., 2007; Klinke et al., 2004; Larsson et al., 2001; Martin and Jönsson, 2003; Nigam, 2001 [c] Klinke et al., 2004; Larsson et al., 2000; Martin and Jönsson, 2003 [d]Almeida et al., 2007; Martin and Jönsson, 2003; Palmqvist et al., 1996; Taherzadeh et al., 1997.

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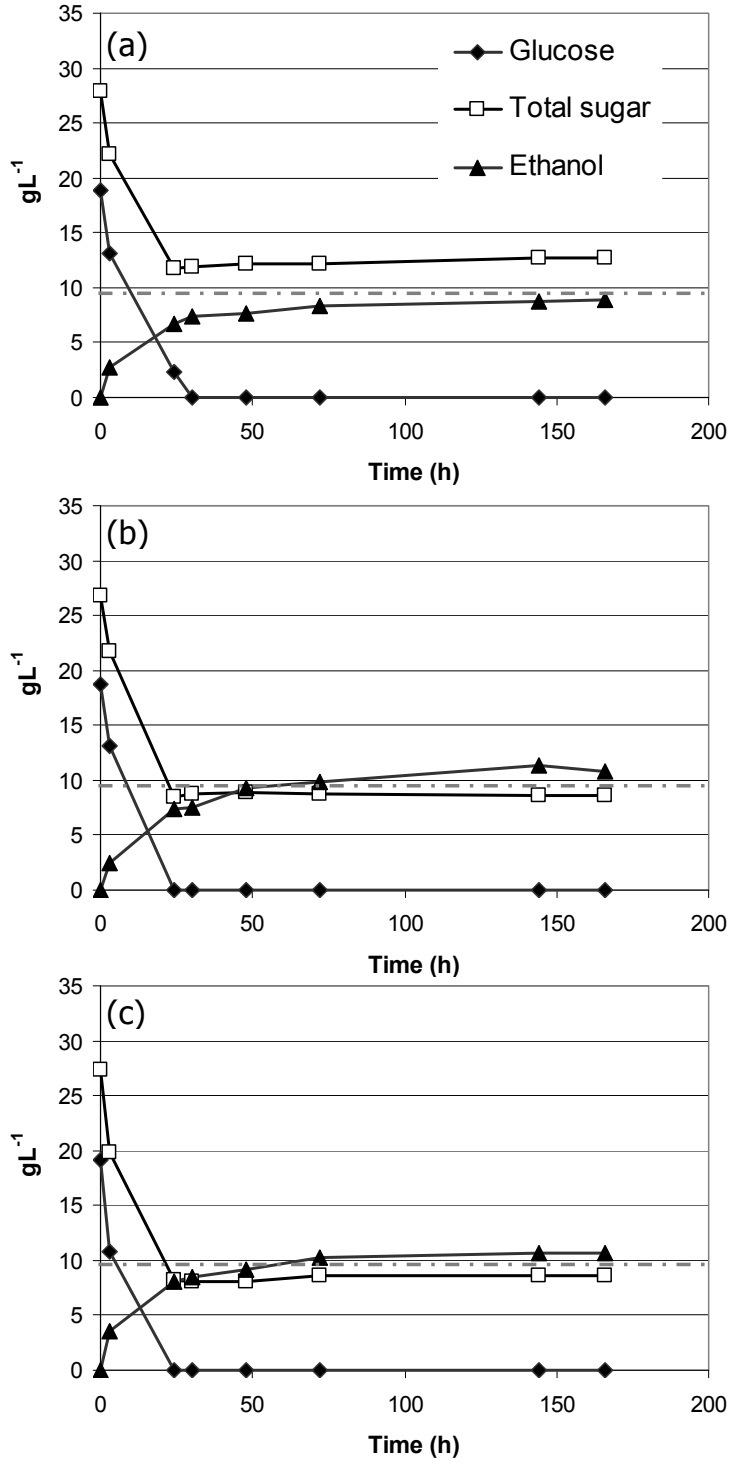

Figure 3.22. Ethanol production of *S. diastaticus* ATCC 13007 from hydrolysates of raw wheat bran pre-treated without H_2SO_4 (a), with H_2SO_4 0.1% (b) and with H_2SO_4 0.3% (c). Dash line (--) indicates maximum ethanol level obtainable from glucose present at the beginning of fermentation according to the theoretical yield of 0.51g ethanol per gram consumed glucose. Data reported are means of two replicates.

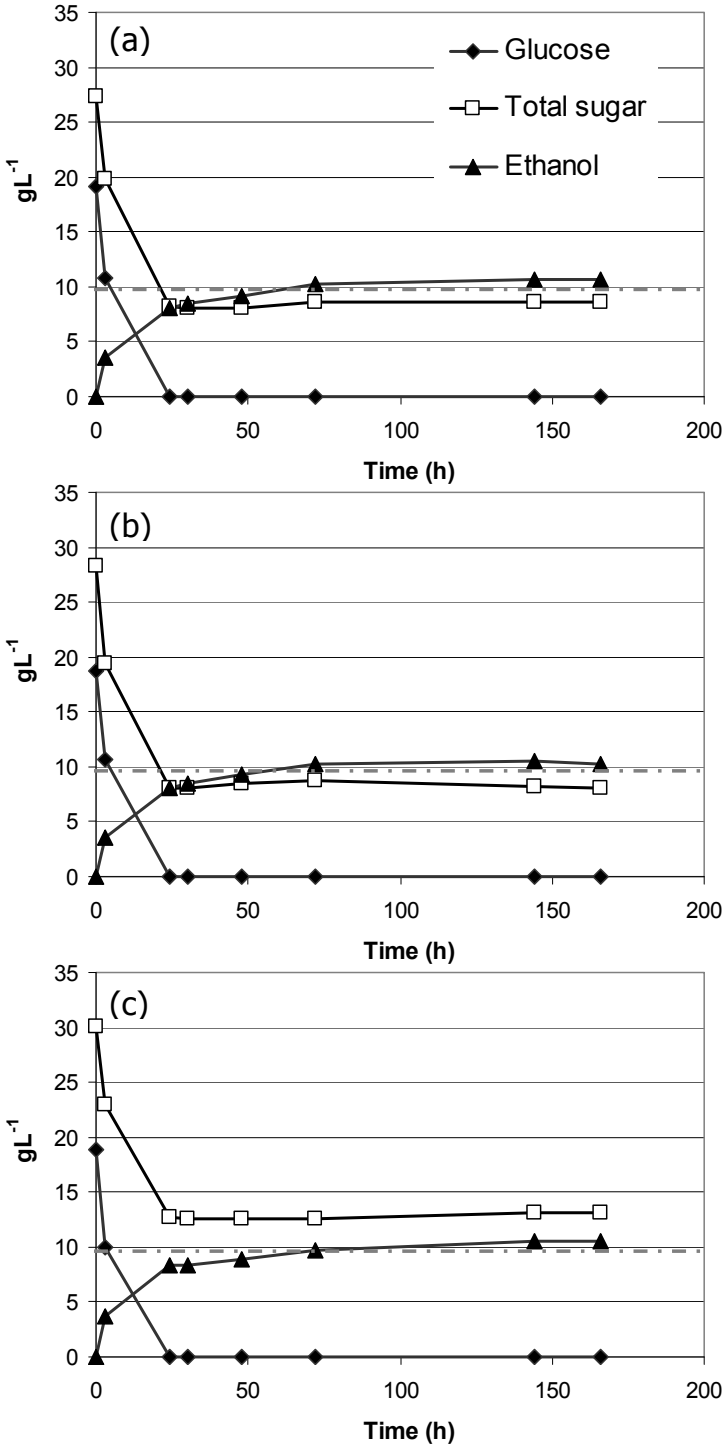

Figure 3.23. Ethanol production of *S. diastaticus* ATCC 13007 from hydrolysates of milled wheat bran pre-treated without H_2SO_4 (a), with H_2SO_4 0.1% (b) and with H_2SO_4 0.3% (c). Dash line (--) indicates maximum ethanol level obtainable from glucose present at the beginning of fermentation according to the theoretical yield of 0.51g ethanol per gram consumed glucose. Data reported are means of two replicates.

The yeast showed interesting performance also on milled bran hydrolysates (Figure 3.23). The maximum ethanol value (10.8 gL^{-1}) was produced after 144 h incubation in milled bran hydrolysed without sulphuric acid addition (Figure 3.23a). However, comparable amounts were detected in both acid-pre-treated milled brans (Figure 3.23b,c).

S. diastaticus seemed to be not affected by the low amount of inhibitory compounds of the hydrolysates and exhibited the highest ethanol productions after long incubation at 25°C.

According to the maximum theoretical yield of 0.51 g ethanol per gram of consumed glucose, the fermentative performance of *S. diastaticus* was interesting. In raw wheat bran 0.1% H_2SO_4-treated (Figure 3.22b), the yeast produced 11.4 gL^{-1} that is 1.2-fold the maximum amount of 9.5 gL^{-1} theoretically obtainable from the glucose present at the beginning of fermentation.

In addition, similar ethanol production were achieved on milled wheat bran where ethanol reached level of 10.8 gL^{-1} that is 1.18-fold that the maximum theoretical amount of 9.2 gL^{-1} (Figure 3.23b). This result may be explained considering that the yeast, producing extracellular glucoamylases, could have hydrolysed the starchy oligomeric chains of the hydrolysates into glucose and then fermented it into ethanol.

To confirm this hypothesis, the hydrolysates were fermented by *S. diastaticus* once induced as inoculum for the production of glucoamylase. The fermentative kinetics of induced *S. diastaticus* on raw and milled wheat bran are reported in Figure 3.24 and 3.25, respectively.

On raw bran, the strain produced the highest ethanol value (10.3 gL^{-1}) after 48 h incubation (Figure 3.24a). Similar concentrations were measured also on bran acid-pre-treated (Figure 3.24b,c). The yeast showed consistent ethanol production also from milled bran: in all monitored fermentations, the ethanol concentration reached values of about 10.7 gL^{-1} within 48 h incubation (Figure 3.25a,b,c).

The data confirmed that *S. diastaticus* was able to utilise glucose in the hydrolysates as starchy oligosaccharides: ethanol reached levels higher than those expected from the glucose present at the beginning of fermentation according to the maximum theoretical yield (0.51 g ethanol per gram of consumed glucose).

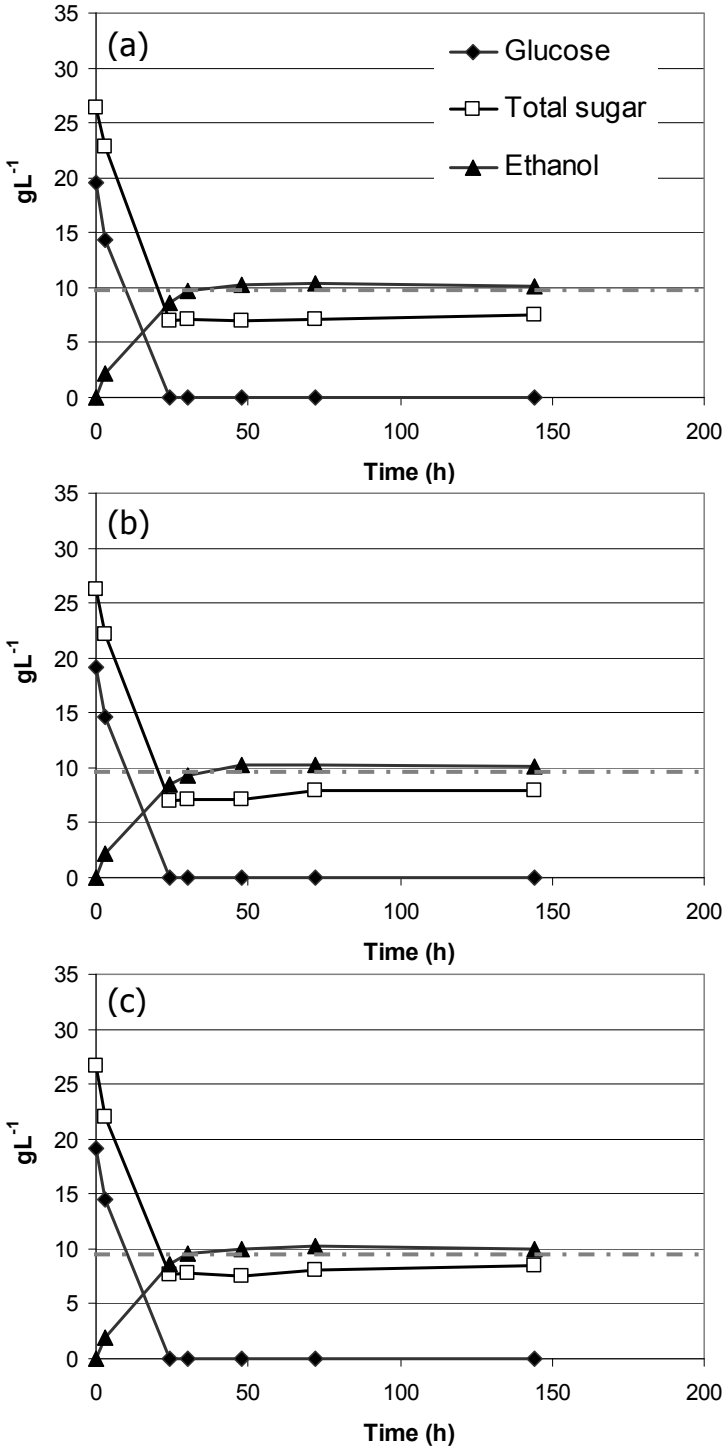

Figure 3.24. Ethanol production of *S. diastaticus* ATCC 13007, induced for glucoamylase secretion, from hydrolysates of raw wheat bran pre-treated without H_2SO_4 (a), with H_2SO_4 0.1% (b) and with H_2SO_4 0.3% (c). Dash line (--) indicates maximum ethanol level obtainable from glucose present at the beginning of fermentation according to the theoretical yield of 0.51g ethanol per gram consumed glucose. Data reported are means of two replicates.

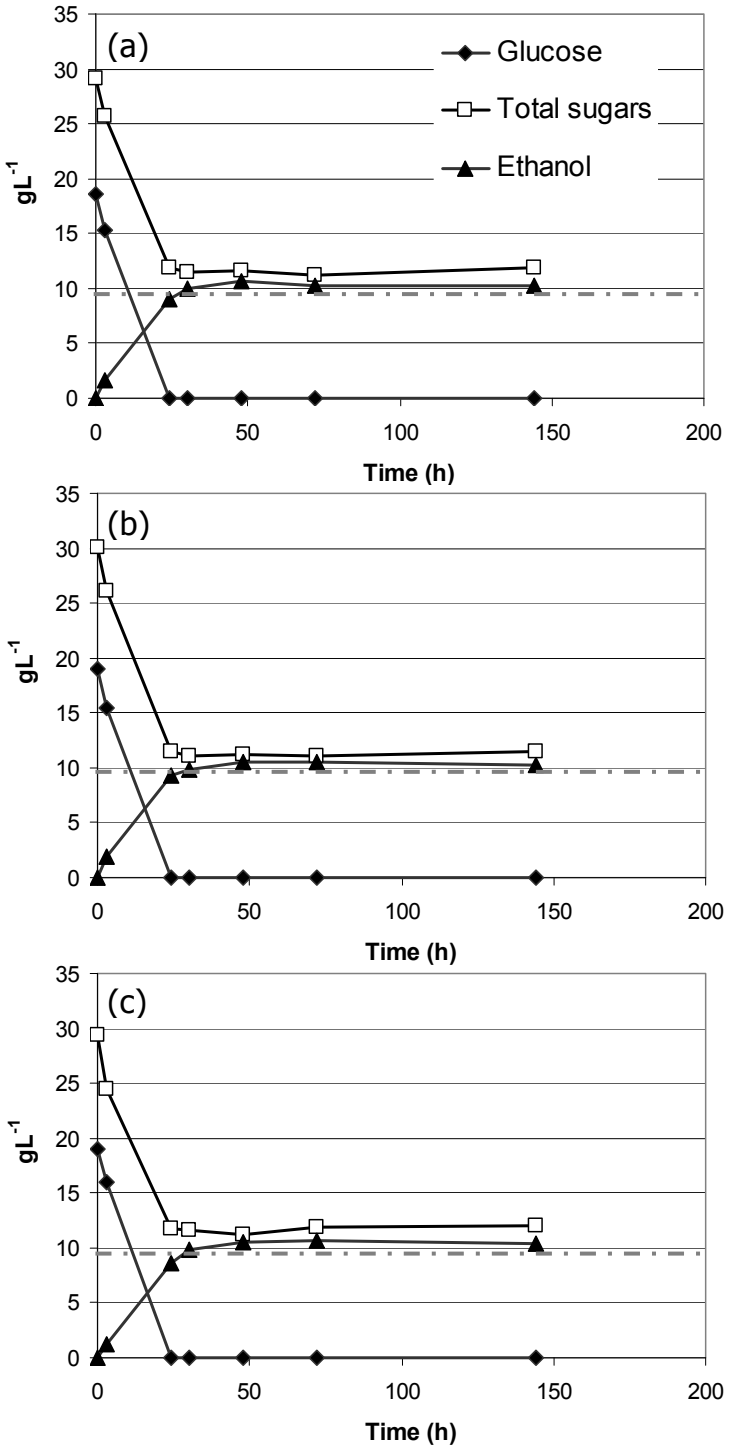

Figure 3.25. Ethanol production of *S. diastaticus* ATCC 13007, induced for glucoamylase secretion, from hydrolysates of milled wheat bran pre-treated without H_2SO_4 (a), with H_2SO_4 0.1% (b) and with H_2SO_4 0.3% (c). Dash line (--) indicates maximum ethanol level obtainable from glucose present at the beginning of fermentation according to the theoretical yield of 0.51g ethanol per gram consumed glucose. Data reported are means of two replicates.

For instance, from milled bran un-treated with H_2SO_4, the yeast produced 10.7 gL^{-1} ethanol that is 1.14-fold that the maximum amount of 9.4 gL^{-1} theoretically obtainable (Figure 3.25a).

S. cerevisiae s1, selected as the most efficient amylolytic *S. cerevisiae* isolate (Paragraph 3.3), exhibited good fermentative vigour from both raw and milled bran as shown in Figure 3.26 and 3.27, respectively.

As expected, *S. cerevisiae* s1 was unable to use for growth other sugars except glucose. Once free glucose was depleted, the total sugar level remained constant.

Regarding raw wheat bran hydrolysates, the yeast produced the maximum ethanol level (10.4 gL^{-1}) in the material treated with 0.3% H_2SO_4 (Figure 3.26c). On bran treated without acid (Figure 3.26a), *S. cerevisiae* s1 fermented well up to 9.3 gL^{-1} and in the hydrolysate with 0.1% H_2SO_4 the ethanol level was about 9.7 gL^{-1} (3.26b).

From milled bran, the highest ethanol values (9.8 gL^{-1}) were detected in the fermentation of the material treated with 0.1% H_2SO_4 (Figure 3.27b). However, the strain displayed comparable amounts of ethanol in the other milled bran hydrolysates (Figure 3.27a,c). Considering the maximum theoretical ethanol yield per gram of consumed glucose (0.51 g g^{-1}), the strain produced fermentative performances ability conformed to yeasts with high ethanol vigour.

Nevertheless, on both raw and milled bran hydrolysates with H_2SO_4 0.3%, *S. cerevisiae* s1 produced alcohol levels slightly higher than those expected from the glucose available at the beginning of fermentation (Figure 3.26c and Figure 3.27c). This finding could be ascribed to the potential amylolytic activity exhibited by *S. cerevisiae* s1 both in liquid and on solid media (Paragraph 3.3).

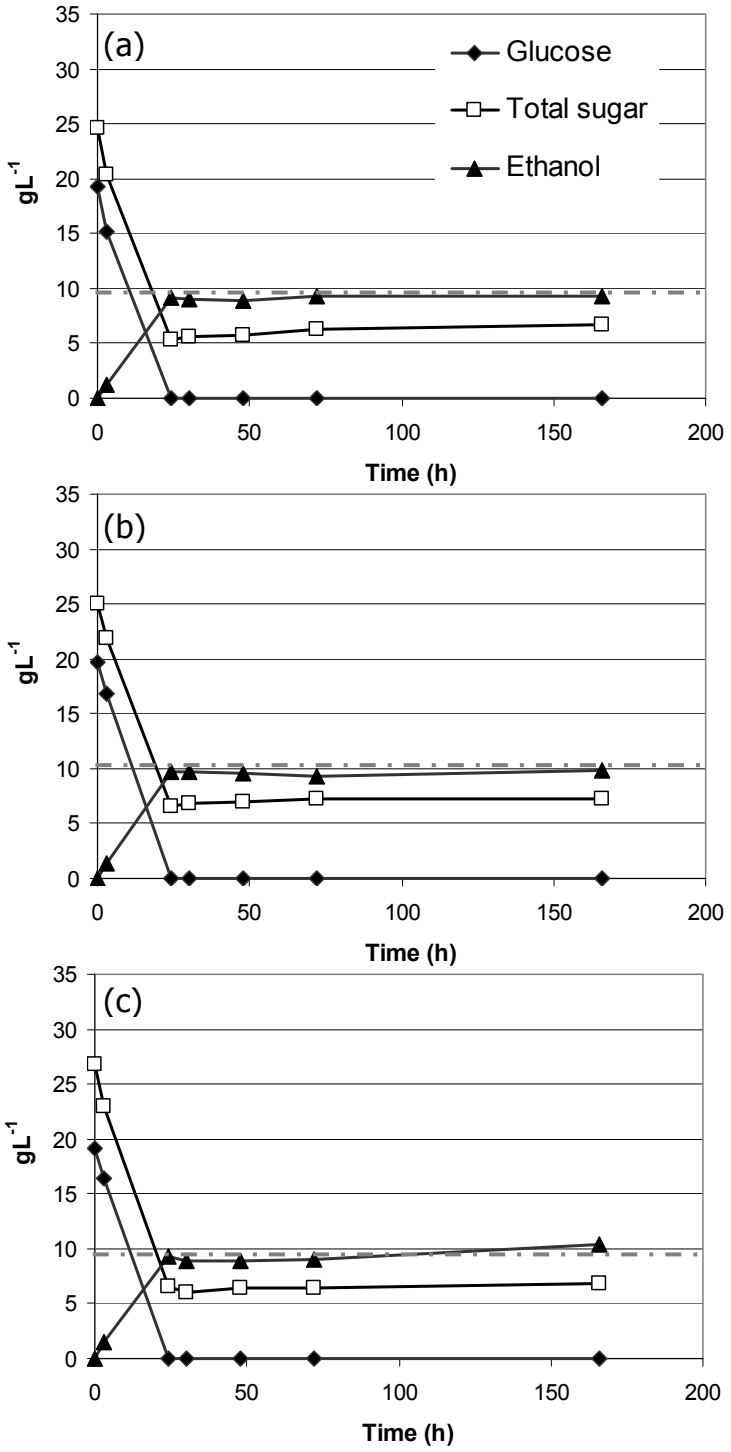

Figure 3.26. Ethanol production of *S. cerevisiae* s1 from hydrolysates of raw wheat bran pre-treated without H_2SO_4 (a), with H_2SO_4 0.1% (b) and with H_2SO_4 0.3% (c). Dash line (--) indicates maximum ethanol level obtainable from glucose present at the beginning of fermentation according to the theoretical yield of 0.51g ethanol per gram consumed glucose. Data reported are means of two replicates.

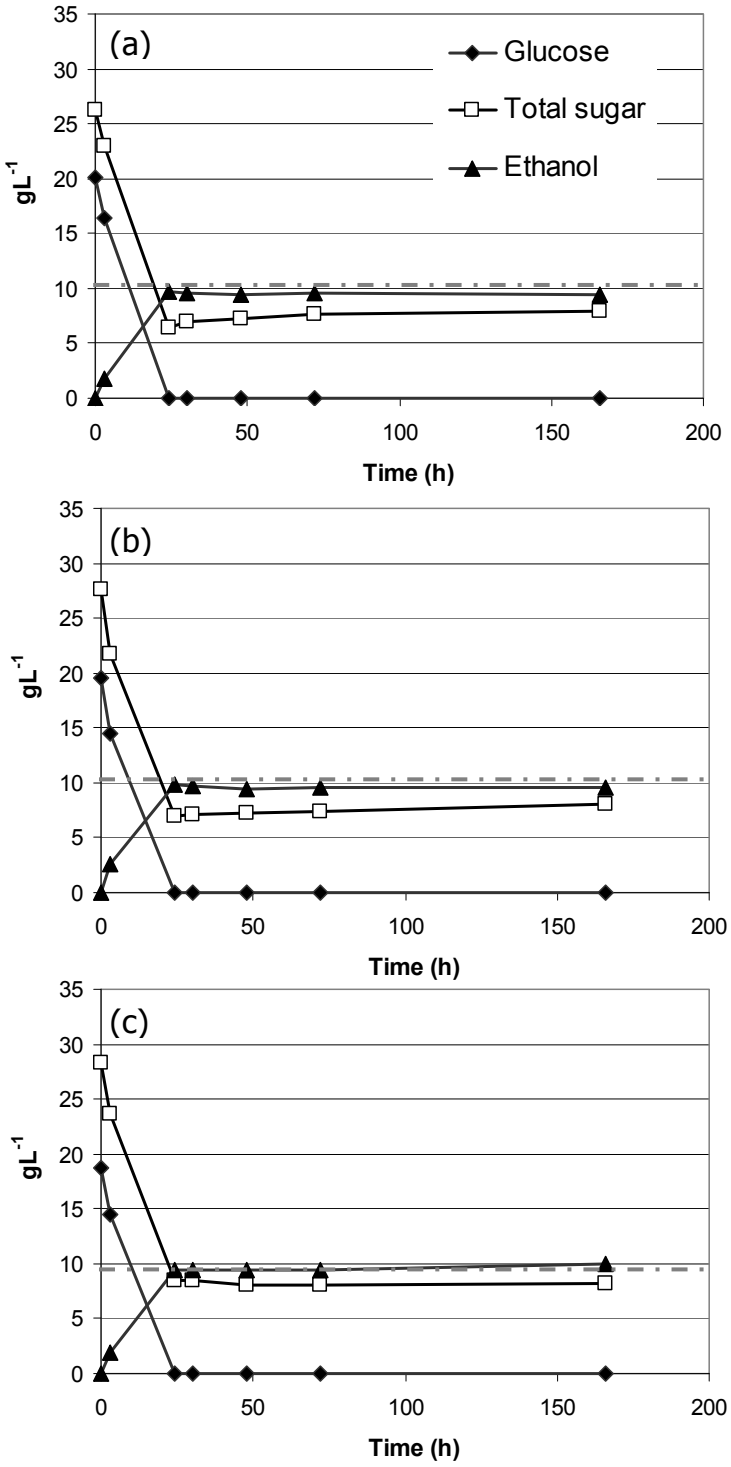

Figure 3.27. Ethanol production of *S. cerevisiae* s1 from hydrolysates of milled wheat bran pre-treated without H_2SO_4 (a), with H_2SO_4 0.1% (b) and with H_2SO_4 0.3% (c). Dash line (--) indicates maximum ethanol level obtainable from glucose present at the beginning of fermentation according to the theoretical yield of 0.51g ethanol per gram consumed glucose. Data reported are means of two replicates.

3.3.5 Discussion.

Pre-treatment and enzymatic hydrolysis.

Different approaches to perform pre-treatment of wheat bran were compared with respect to yield of sugars, formation of inhibitors and fermentability of the resulting hydrolysates. The acid catalyst and milling treatment were shown to have the major impact on all these parameters.

The yields of glucose and xylose under different pre-treatment conditions provided an indication of the efficiency of the conversion of cellulose and hemicellulose to the corresponding monosaccharides. The breakdown of hemicellulose was also reflected by the yield of acetic acid, which is formed mainly by the hydrolysis of acetylated β-D-xylopyranose residues in lignocellulose.

The low glucose yield obtained after the thermal treatment indicated that most of the cellulose was still intact at the pre-treatment stage. Limited hydrolysis of hemicellulose also took place since low concentrations of xylose, arabinose and mannose were detected (Table 3.6 and 3.7).

Pre-treatment alone resulted in low total sugar releases but, when combined with enzymatic hydrolysis, the sugar yield notably increased (Figure 3.20). This indicated that cellulose was not significantly hydrolysed before the hemicellulose was converted to a mixture of oligomeric and monomeric sugars. Therefore, after the first enzymatic hydrolysis, the consistent sugar yields pointed at an almost complete conversion of cellulose and partial de-polymerisation of hemicellulose. The high glucose release suggested that a significant fraction of cellulose, β-glucan or unstable starch was solubilized during enzymatic hydrolysis.

However, the yield of pentose sugars was lower than glucose. Hemicellulose does not have the crystallinity of cellulose and consequently is more susceptible to hydrolysis. Therefore, in this case, it appears that the activity of endo-xylanases and arabinoxylan-degrading enzymes could be affected. Thus, pentose release did not achieve optimal levels.

The second enzymatic treatment with α-amylase was applied to improve the potential amylolytic activity of the *Saccharomyces* sp. strains used in the following fermentation phase. The commercial enzyme cleaving α-1,4-glycosidic bonds of amylose and

amylopectin has released mixtures of starchy oligosaccharides in the hydrolysates. The α-amylolytic treatment proved effective since no significant amount of starch was detectable in the solid fraction of the hydrolysates (data not shown).

The final sugar yields of the SHF process were consistent with Palmarola-Adrados et al. (2005) who described glucose values even higher than those obtained in this study. However, the severe conditions of the Palmarola-Adrados pre-treatment consisted of much higher temperatures (up to 160°C) and residence times. Furthermore, massive enzyme dosages and up to ten-fold H_2SO_4 concentrations (0.5-2% w/w) have been used. Therefore, the concentrations of inhibitory compounds were higher, reaching about 4 and 0.3 gL^{-1} of furfural and HMF, respectively (Palmarola-Adrados et al., 2005).

Interestingly the mild pre-treatment conditions of the present study did not cause consistent production of inhibitory by-products. This is indicated by the quite high amounts of xylose and glucose (Figure 3.21) and the low concentrations of aliphatic acids (Table 3.12). This finding suggested that the SHF system proposed was efficient in the minimization of toxic compounds otherwise released in large quantities in many other lignocellulosic hydrolysates (Almeida et al., 2007).

Hydrolysis method which involves treatment of lignocellulose at high temperature leads to the formation of a wide range of compounds. Acetic acid is ubiquitous in hemicellulose hydrolysates from all lignocellulosics, where the hemicellulose and to some extent lignin is acetylated (Sarkanen and Ludwig 1971; Torssell, 1997). Hydroxycarboxylic acids such as glycolic acid and lactic acid are common degradation products from alkaline carbohydrate degradation (Alén et al. 1990; Sjöström, 1991).

The low levels of weak acids measured in all bran hydrolysates confirmed that xylose and arabinose hydrolysis took place during enzymatic treatment. The arabinose release (0.35 gL^{-1}) in both raw and milled bran during thermal pre-treatment (Table 3.6 and 3.7) may have caused the acetic acid production.

Statistical evaluation of the pre-treatment and hydrolysis phase conditions.

ANOVA analysis revealed significant effects of the hydrolysis process parameters on six monitored products (Table 3.14). The interactions between the factors were also tested.

Milling and treatment were the most important parameters followed by acid. The milling of wheat bran significantly influenced the releases of monosaccharides and the formation of acetic acid. This acid was produced in higher amounts ($p \leq 0.01$) in wheat bran heat-treated with sulphuric acid. This finding is confirmed by the fact that acid significantly influenced arabinose and xylose releases ($p \leq 0.01$) and mannose yield ($p \leq 0.05$). These sugars together with galactose derived from bran hemicellulose. Thus, partial hemicellulose hydrolysis occurred during heat-treatment with the liberation in the hydrolysates of acetic acid.

Products	Milling	Acid	Treatment	Milling x Treatment	Acid x Treatment
Glucose	**	ns	**	*	ns
Xylose	**	**	**	**	**
Galactose	*	ns	**	ns	ns
Arabinose	**	**	**	ns	ns
Mannose	*	*	**	ns	ns
Acetic acid	*	**	ns	ns	ns

Table 3.14 Effect of milling, acid hydrolysis, treatment and their interactions on the products released from wheat bran evaluated by ANOVA (ANalysis Of VAriance). Products not showing any significant effect are not included (furfural, HMF and lactic acid). The interaction 'milling x acid' is not reported because not significant for all the products. (ns: not significant; * $p \leq 0.05$; ** $p \leq 0.01$)

The interaction between acid and treatment influenced glucose and xylose levels. In particular, the milling significantly acted with thermal treatment and the first enzymatic hydrolysis. Milling, indeed, is a common method of reducing particle size (Hendriks and Zeeman, 2009; Mosier et al., 2005; Wyman et al., 2005). The enhancement in specific surface area and reduction of the degree of polymerization (DP) are factors that improve the total enzymatic hydrolysis yield of the lignocellulose in most cases by 5-25%. In addition, the technical digestion time is reduced by 23-59% (Delgenés et al., 2002). In this study, the milling increased hydrolysis yield of about 13% and 22% for glucose and xylose, respectively.

Acid hydrolysis interacted with treatment giving significant difference on xylose release from the feedstock. The hemicellulolytic enzyme mixture hydrolysed increasing levels of xylose as the acid catalyst concentration in the heat-treatment increased.

No significant interaction occurred between sulphuric acid and milling treatment. The result may be explained considering the limited amounts of acid used (0.1-0.3 % w/w wheat bran) and the DP of the raw wheat bran. Moreover, this evidence was in accordance with Chang and Holtzapple (2000) that reported small effect of particle size reduction below 40 mesh on hydrolysis yield as well as hydrolysis rate of biomass.

Fermentation studies on wheat-bran hydrolysates.

Under the condition used, all hydrolysates were found to be fermentable and the low amounts of aliphatic acids seemed not to have negative influence on the ethanol yield process. The fermentation phase of SHF system was efficient with alcohol production higher than 11 gL^{-1}.

S. diastaticus produced ethanol converting both raw and milled bran. On the basis of its fermentation kinetics reported in Figure 3.22 and 3.23, the yeast generated the maximum ethanol values when the hydrolysates were totally depleted of glucose. Indeed, the hexose was not detectable in all fermented hydrolysates within 30 h incubation. Therefore, the yeast should have utilised other sugars to produce energy via the Embden-Meyerhof pathway of glycolysis. Since no references are available for *S. diastaticus* on the use of pentoses and hexoses for growth, with the exception of glucose, the strain must have obtained glucose from the mixture of oligosaccharides released from starch by the α-amylase treatment.

S. diastaticus, indeed, was able to ferment bran sugars at levels exceeding the maximum theoretical yield of ethanol from glucose available at the beginning of fermentation. *S. diastaticus* is described for good but not excellent glucose-to-ethanol performance having yield of 0.44 g g^{-1} (Verma et al., 2000). Therefore, the strain had necessarily utilised the oligomeric chains released by α-amylase in the hydrolysates yielding glucose and fermenting it into ethanol.

Moreover, the glucoamylase production in *S. diastaticus* is inducible (James and Lee, 1997; Pretorius et al., 1991) and could explain that the highest levels of alcohol were reached after a long incubation time (144 h). The starch oligosaccharide chains should have induced the yeast to secrete glucoamylase. Thus the higher alcohol production exhibited by *S. diastaticus* seemed related to its amylolytic enzymes that cleaved α 1-4 linkages in the hydrolysed bran starch.

This hypothesis is corroborated by the fermentative performance of the yeast inoculated once induced for glucoamylase production. *S. diastaticus* consumed all glucose within 24 h and produced ethanol values exceeding the maximum theoretical yield. The ethanol production kinetics were more rapid (Figure 3.24 and 3.25) showing that the inoculum induction for glucoamylase influenced the yeast productivity: *S. diastaticus* displayed the highest ethanol values within 48-72 h. The results confirmed that glucoamylase production was essential for the high conversion yields of the yeast. Moreover, ethanol performance of this strain was noteworthy since *S. diastaticus* has been described to date only for ethanol production from simple substrates such as dextrin or soluble starch (Laluce and Mattoon, 1984; Verma et al., 2000). In this study, the yeast proved effective for ethanol conversion from complex hydrolysates of wheat bran, used as model of other starchy agricultural residues.

The high fermentative performance of *S. cerevisiae* s1 showed that the strain is suitable for industrial ethanol production. The yeast produced only in two cases ethanol concentration higher than those expected according to the maximum theoretical yield from glucose. This finding could confirm its weak ability of yielding glucose from starch. However, this activity was still lower than *S. diastaticus* able of producing more consistent ethanol amounts from the same hydrolysates.

All kinetics fermentations conducted in this study were also evaluated considering the difference between the observed ethanol yields, displayed by the yeasts at the end of the fermentations, and the expected ones according to the maximum alcohol yield described for *S. diastaticus*, 0.44 g g^{-1} (Verma et al., 2000) and *S. cerevisiae*, 0.49 g g^{-1} (this work). The values were subjected to statistical analysis with chi-square test. As a result, *S. diastaticus* had ethanol conversion rate significantly more consistent than *S. cerevisiae* s1. Moreover,

S. cerevisiae s1 did not exhibit ethanol conversion efficiency considerably different from the maximum rate reported above (0.49 g g^{-1}). On the other hand, *S. diastaticus* presented ethanol yields statistically higher than the maximum value expected (0.44 g g^{-1}). This finding confirmed that *S. diastaticus* must have utilised bran starch as glucose substrate for the production of ethanol.

Nevertheless, the *S. cerevisiae* s1 showed the highest ethanol yield from glucose available at the beginning of fermentation in all hydrolysates. The strain consumed glucose within 24 h, with a conversion yield much higher than *S. diastaticus*. The yeast produced about 0.49 g ethanol(g glucose)$^{-1}$ corresponding to 95% of the theoretical yield. This efficiency was significantly different from those exhibited by *S. diastaticus* and induced *S. diastaticus*, 0.43 and 0.46 g g^{-1}, respectively. The latter yield may be due to the enzymatic activity of induced glucoamylase resulting in additional glucose consequently fermented by the yeast.

The alcohol levels of the SHF process was interesting. The ethanol yield were consistent with the results of similar works performed on wheat bran (das Neves et al., 2006; Palmarola-Adrados et al., 2005). In addition, the ethanol conversion efficiency obtained in this study was much higher than the value reported by das Neves et al. (2006). The choice of utilising the hydrolysates without filtration proved remarkable: the yeast strains were able to ferment well in static incubation with significant amounts of solid particles. The high dry matter content (8.7%) could have negatively influenced the productivity of the process. Nevertheless, the non-filtration of hydrolysates may be an interesting process variable to be carefully evaluated in techno-economical analyses in order to determine the actual feasibility of the SHF system proposed.

Furthermore, such yeasts able to ferment in presence of high dry matter content could be efficiently used for the production of ethanol from other unfiltered lignocellulosic hydrolysates.

3.5 Development of an efficient amylolytic yeast strain for industrial ethanol production.

3.5.1 Selection of wild type *S. cerevisiae* strains with proper traits for industrial bioethanol application.

Production of bioethanol from agricultural residues requires a fermenting organism converting all sugars of the raw material to ethanol in high yield and with high rate. The main properties for an industrial yeast strain are: (1) process water economy, (2) inhibitor tolerance, (3) ethanol yield, and (4) specific ethanol productivity (Hahn-Hägerdal et al., 2007). Moreover, the complete substrate utilisation is one of the prerequisites to render lignocellulosic ethanol processes economically competitive (Galbe and Zacchi, 2002). This means that all types of sugars in cellulose, starch and hemicellulose must be converted to ethanol, and that microorganisms must efficiently perform this conversion under industrial conditions.

In addition to easily metabolised sugars, industrial substrates may also contain a mixture of unusual sugars. The simultaneous presence of multiple monosaccharides may pose limitations such as incomplete substrate utilisation and inhibition of sugar uptake pathways.

In this study, the five *S. cerevisiae* strains with the most interesting amylolytic capability (Paragraph 3.3) were tested for their robustness and fermentative vigour in defined medium with low amounts of additives and high sugar levels. MNS broth, chosen for fermentation trials, has been used in several works for the comparison of yeast fermentative abilities (Agnolucci et al., 2007; Delfini and Formica, 2001). Glucose and xylose were selected as representative of hexose and pentose sugars since they are abundantly present as substrates in the industrial scale ethanol processes. It is known that wild type *S. cerevisiae* does not utilise xylose for growth, but the presence of the sugar in the medium could interfere with the ethanol performance of the tested yeast strains.

Delfini method was followed using MNS medium with different concentrations of glucose and/or xylose as described in Paragraph 2.6.1.

Figure 3.28 shows the yeast fermentative performance on MNS supplemented with glucose (20%) reported both as cumulative sugar utilisation (grams of consumed glucose per 100 mL of MNS medium) and daily glucose consumption rate.

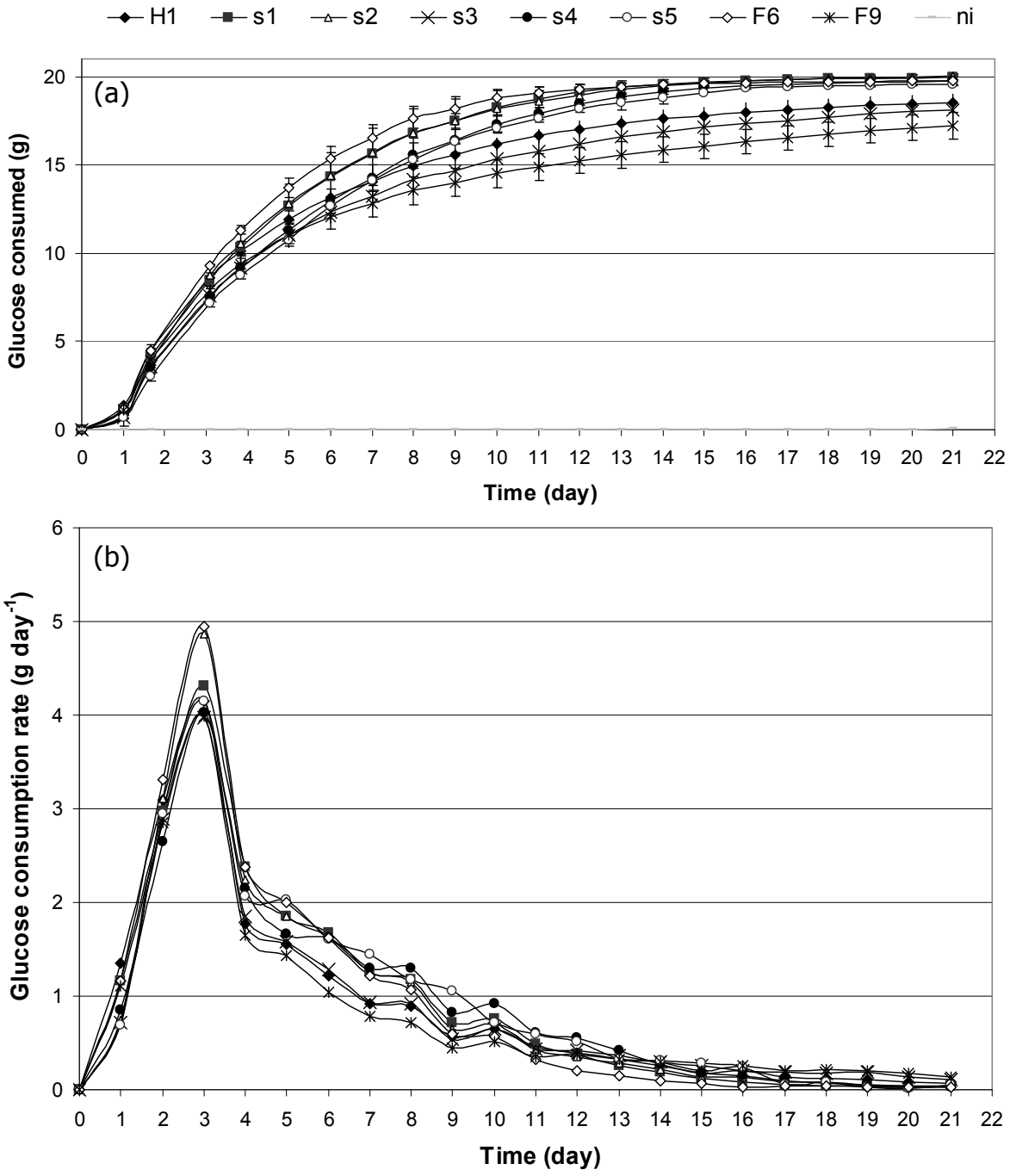

Figure 3.28. Fermentative performance of *S. cerevisiae* strains in MNS medium with glucose (200 gL^{-1}) reported as (a) cumulative sugar utilisation (grams of glucose consumed per 100 mL of MNS) and (b) daily glucose consumption rate. The experiment was conducted in triplicate (±SD).

The strains displayed variable sugar utilisation kinetics. Four to five yeasts were able to utilise all sugar available in the medium while strain s3 exhibited lower fermentative ability. The potentially amylolytic strains performed remarkable fermentative vigour since they consumed glucose with a rate much higher than *S. cerevisiae* H1, used as reference strain. Interestingly, the ability of the five yeasts was comparable also with that showed by F6 and F9 strains, selected among the high fermentative yeasts of the collection belonging to the Department of Agricultural Biotechnology (University of Padova).

The yeasts produced significant daily sugar consumption and two strains showed the highest rate of about 5 grams at the third incubation day (Figure 3.28b). The data obtained following Delfini method were confirmed by HPLC analysis of samples taken at the end of fermentation (Table 3.15).

Product (gL^{-1})	**H1**	**s1**	**s2**	**s3**	**s4**	**s5**	**F6**	**F9**
Substrate remaining								
Glucose	15.2	3.1	-	21.2	-	2.5	-	28.0
Products formed								
Glycerol	7.3	5.3	5.9	6.3	5.5	7.2	6.5	5.9
Ethanol	84.8	93.3	95.6	85.7	87.9	87.1	93.9	79.6
Ethanol yield [a]	0.46 (90%)	0.47 (91%)	0.48 (94%)	0.48 (94%)	0.44 (86%)	0.44 (86%)	0.47 (92%)	0.46 (91%)

Table 3.15. Product formation by *S. cerevisiae* strains after 21 days fermentation at 25°C in MNS with glucose (200 gL^{-1}) as substrate. [a] Ethanol yield as g g^{-1} and % of theoretical maximum (0.51 g g^{-1} from glucose) indicated in brackets.

The best fermentative activities were performed by the strains s1, s2 and F6 that produced the highest ethanol levels. Their ethanol yield was interesting: 0.47, 0.48 and 0.46 g ethanol per gram of consumed glucose, respectively. Glycerol was produced by all yeasts in small amount.

The fermentative ability of the *S. cerevisiae* strains were tested also on MNS supplemented with glucose (15%) and xylose (5%) as reported in Figure 3.29. The presence of xylose seemed to affect glucose consumption. After 21 days of incubation, indeed, the medium still contained 1% glucose.

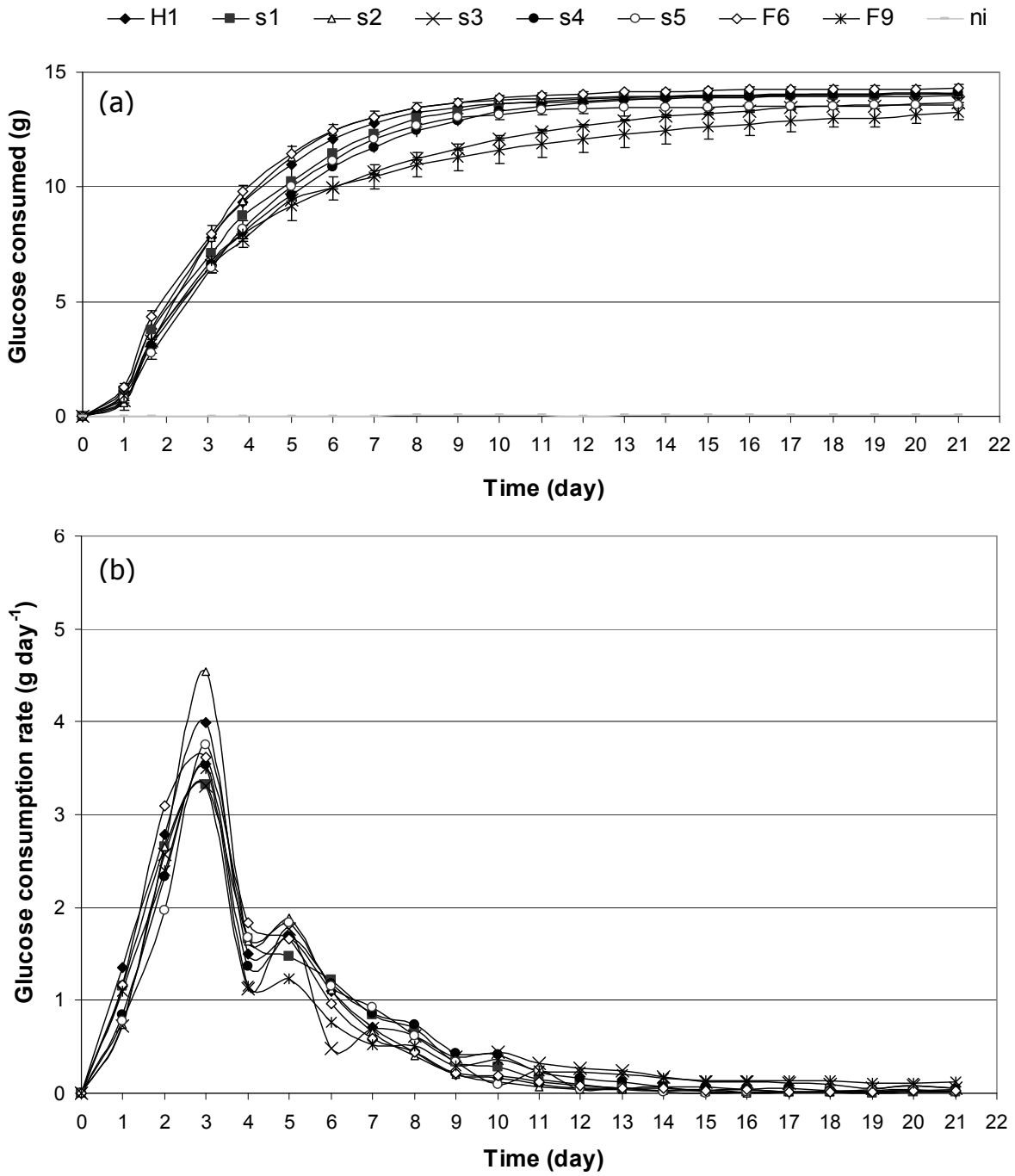

Figure 3.29. Fermentative performance of *S. cerevisiae* strains in MNS medium with glucose (150 gL^{-1}) and xylose (50 gL^{-1}) reported as (a) cumulative sugar utilisation (grams of glucose consumed per 100 mL of MNS) and (b). daily glucose consumption rate.The experiment was conducted in triplicate (±SD).

However, the five strains displayed interesting fermentative performance also in the presence of high xylose concentration. In particular the isolates s1, s2 and s4 showed the

highest glucose utilisation. Strain H1 consumed the glucose available at rate similar to *S. cerevisiae* F6 that exhibited the best cumulative sugar consumption.

In Figure 3.29b, the yeasts were compared for daily glucose uptake. The rate of utilisation was influenced by the presence of xylose. The strain s2 confirmed high fermentative vigour with values slightly lower than those exhibited in MNS 20% glucose (Figure 3.28b). The isolate F6, even if at a consumption rate significantly slower than in MNS 20% glucose, showed high vigour at the beginning of fermentation. As a result, the strain showed one of the best fermentative performances.

H1 isolate seemed to be slightly affected by xylose: in both MNS media, *S. cerevisiae* H1 displayed the maximum sugar consumption rate of about 4 grams glucose per day.

The HPLC analysis showed that all strains were able to convert little amounts of xylose into xylitol (Table 3.16). However, the majority of the pentose was still present at the end of fermentation. The alcohol conversion efficiency was remarkable in the strains s2, s3, s4 with ethanol yields of about 0.47 g g^{-1} which corresponds to 92% of the theoretical maximum yield of 0.51 g ethanol per g glucose.

Product (gL^{-1})	**H1**	**s1**	**s2**	**s3**	**S4**	**s5**	**F6**	**F9**
Substrate remaining								
Glucose	10.9	8.6	3.7	3.2	-	15.0	5.5	20.0
Xylose	46.0	45.3	44.8	45.0	44.5	44.2	44.7	45.4
Products formed								
Xylitol	3.6	3.5	4.8	3.8	4.1	4.8	4.0	4.0
Glycerol	5.7	5.1	5.3	4.9	5.5	6.0	6.2	5.9
Ethanol	60.6	59.6	68.6	64.7	65.5	58.0	62.4	56.6
Ethanol yield [a]	0.46 (85%)	0.42 (83%)	0.47 (92%)	0.44 (86%)	0.44 (86%)	0.43 (84%)	0.43 (85%)	0.44 (85%)

Table 3.16. Product formation by *S. cerevisiae* strains after 21 days fermentation at 25°C in MNS with glucose (150 gL^{-1}) and xylose (50 gL^{-1}) as substrates. [a] Ethanol yield as g g^{-1} and % of theoretical maximum (0.51 g g^{-1} from glucose) indicated in brackets.

The yeasts were also incubated in MNS supplemented with glucose and xylose at 10% each (Figure 3.30).

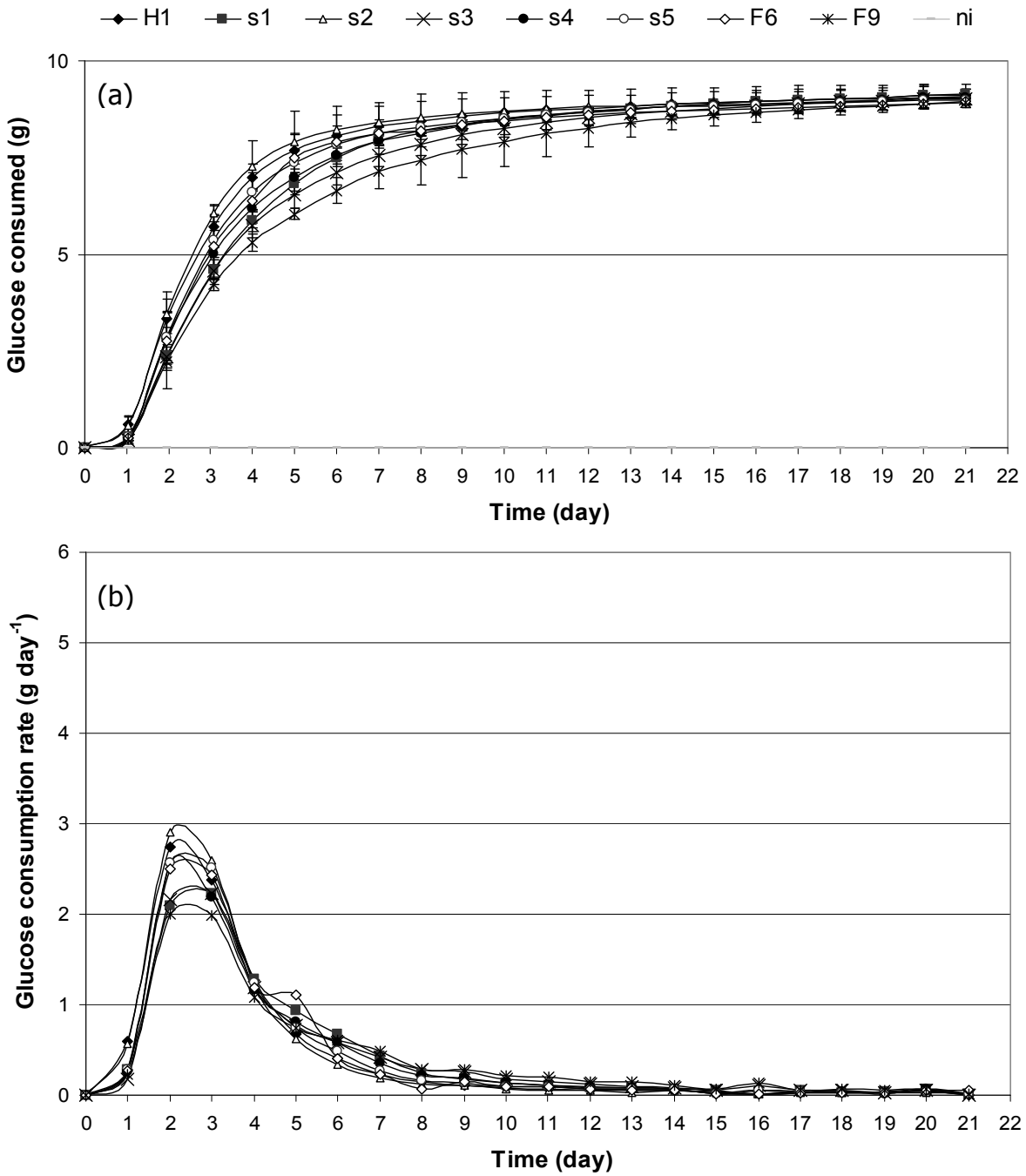

Figure 3.30. Fermentative performance of *S. cerevisiae* strains in MNS medium with glucose (100 gL^{-1}) and xylose (100 gL^{-1}) reported as reported as (a) cumulative sugar utilisation (grams of glucose consumed per 100 mL of MNS) and (b). daily glucose consumption rate.The experiment was conducted in triplicate (±SD).

Sugar consumption was slower than those reported in the other two MNS broths. As a result, the glucose utilisation rate was lower and the highest values were obtained earlier, after two days of fermentation. Strains s2 and H1 presented the most consistent

fermentative vigour (about 3.0 grams) which was 1.5-fold that shown by isolate F9 (Figure 3.30b). As reported in Table 3.17, xylitol conversion was limited although the medium was supplemented with high xylose concentration. The alcohol productions were significantly influenced by the presence of the xylose: the yeasts produced less consistent ethanol levels. *S. cerevisiae* s2 showed the most important yield of about 0.43 g ethanol per gram of glucose consumed, corresponding to nearly 84% of the maximum theoretical yield.result, the glucose utilisation rate was lower and the highest values were obtained earlier, after two days of fermentation. Strains s2 and H1 presented the most consistent

Product (gL^{-1})	**H1**	**s1**	**s2**	**s3**	**S4**	**s5**	**F6**	**F9**
Substrate remaining								
Glucose	-	-	-	-	-	-	-	-
Xylose	97.4	93.3	95.8	94.5	95.5	96.0	95.2	95.4
Products formed								
Xylitol	2.9	3.7	3.1	3.5	3.3	3.1	3.5	3.3
Glycerol	5.2	4.9	5.3	7.9	4.8	4.6	5.3	4.5
Ethanol	37.0	40.5	43.0	33.9	36.5	32.0	39.4	30.9
Ethanol yield [a]	0.37 (73%)	0.40 (79%)	0.43 (84%)	0.34 (66%)	0.37 (72%)	0.32 (63%)	0.39 (77%)	0.31 (61%)

Table 3.17. Product formation by *S. cerevisiae* strains after 21 days fermentation at 25°C in MNS with glucose (100 gL^{-1}) and xylose (100 gL^{-1}) as substrates. [a] Ethanol yield as g g^{-1} and % of theoretical maximum (0.51 g g^{-1} from glucose) indicated in brackets.

On the basis of the fermentative kinetics reported in Figure 3.28, 3.29 and 3.30, the strains s1, s2 and F6 were selected in order to start a molecular biology programme for the development of an efficient amylolytic yeast. The isolate H1 was also included as reference strain.

3.5.2 Integrative plasmids construction.

The glucoamylase *sgaI* codon-optimised gene of *Aspergillus awamori* and the *amyIII* α-amylase gene from *A. oryzae* were inserted in frame with the *XYNSEC* secretion signal

(Den Haan et al., 2007) for constitutive expression under the transcriptional control of the *S. cerevisiae PGK1* and *ENO1* promoters and terminators, respectively.

The yeast integrative expression plasmids were constructed as follows. The *XYNSEC-amyIII* sequence was retrieved from yASAA by digesting with *Eco*RI, treated with Klenow enzyme and then digested with *Bgl*II. The resulting fragment was sub-cloned into pBKD2 (vector digested with *Pac*I, treated with T4 DNA polymerase and digested with *BamH*I). The constructed plasmid was named pBCF*amyIII* (Figure 3.31).

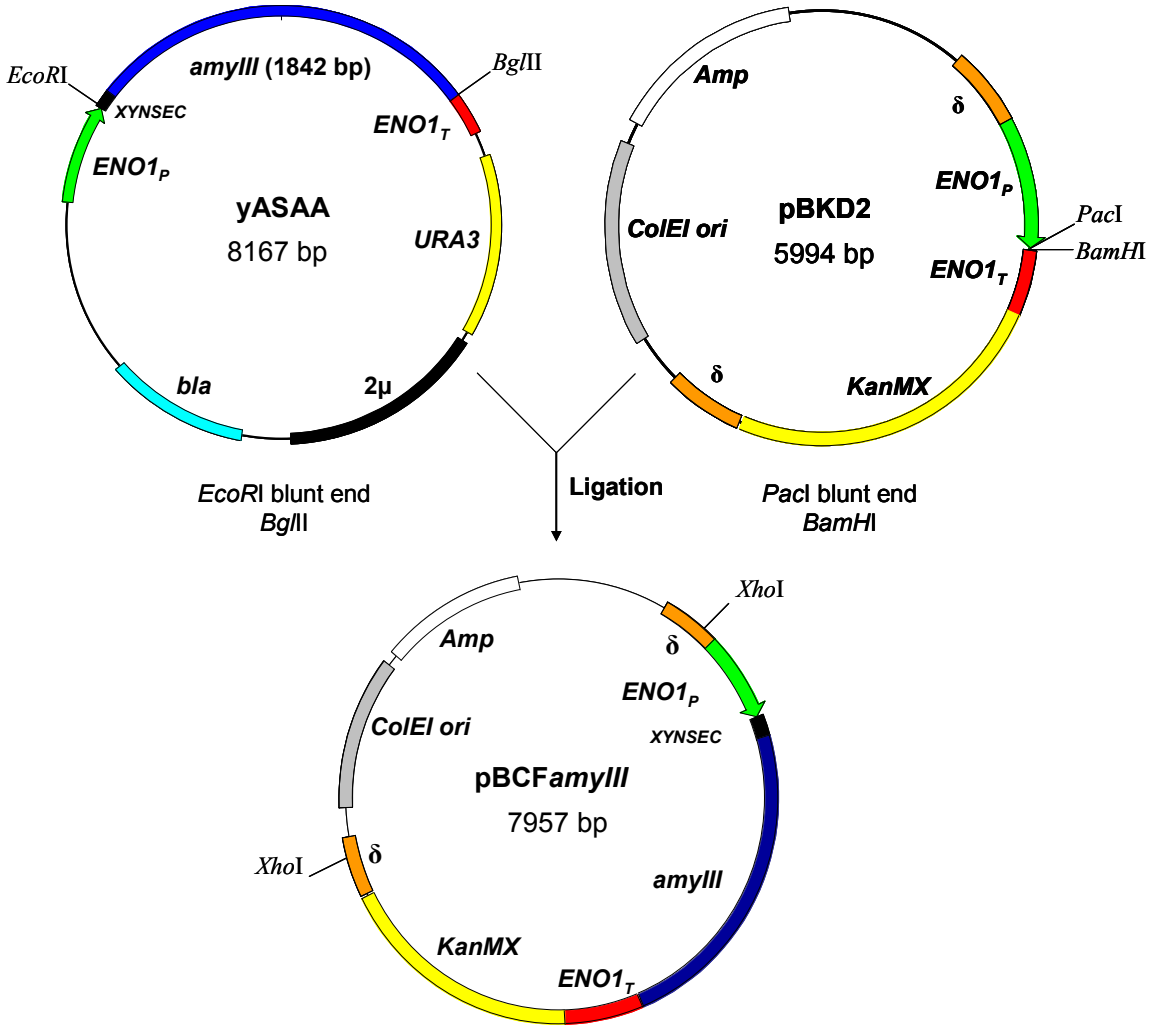

Figure 3.31. Construction of the δ-integrative vector, pBCF*amyIII*, for *amyIII* constitutive expression in *S. cerevisiae.*

The *XYNSEC-sgaI* fragment was retrieved from ySYAG with an *Eco*RI and *Bgl*II digestion (Figure 3.32). The recessed 3' *Eco*RI site was filled in with Klenow enzyme and

the fragment was then ligated with pBKD1 vector (digested with *Pac*I, treated with T4 DNA polymerase and digested with *BamH*I) to generate plasmid pBK*sgaI*.

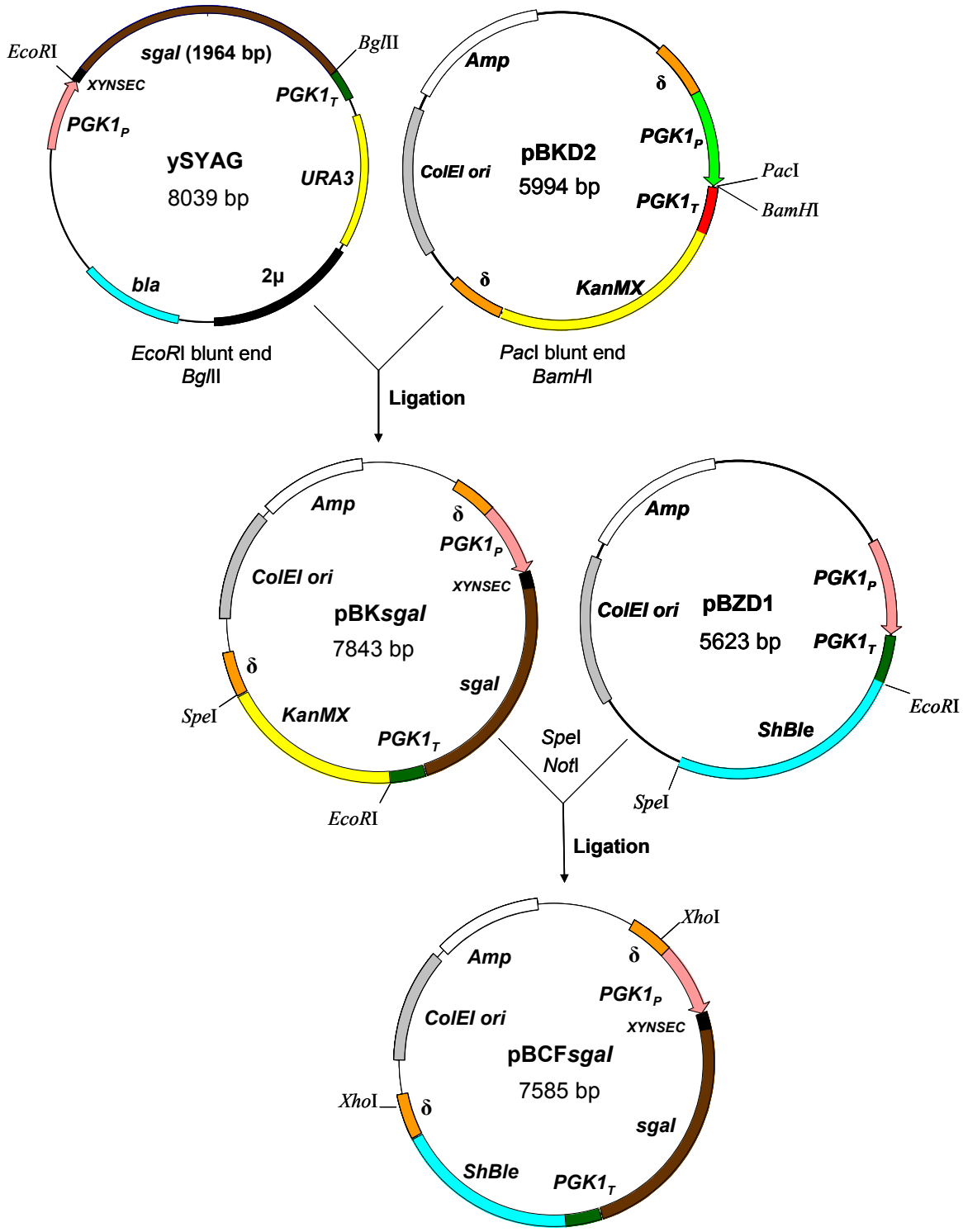

Figure 3.32. Construction of the δ-integrative vector, pBCF*sgaI*, for *sgaI* constitutive expression in *S. cerevisiae.*

The *KanMX* gene (G418 resistance) of the vector pBK*sgaI* was replaced with the *Shble* gene (Zeocin resistance) removed from the plasmid pBZD1 digested with *Eco*RI and *Spe*I. The final integrative plasmid was named pBCF*sgaI* (Figure 3.32).

To obtain a unique δ-vector for both glucoamylase and α-amylase expressions in *S. cerevisiae*, the pBCF*amyIII* was digested with *Spe*I and *Not*I. The resulting fragment was ligated into the *Spe*I site of pBK*sgaI* to obtain the final vector construct, pBGA (Figure 3.33).

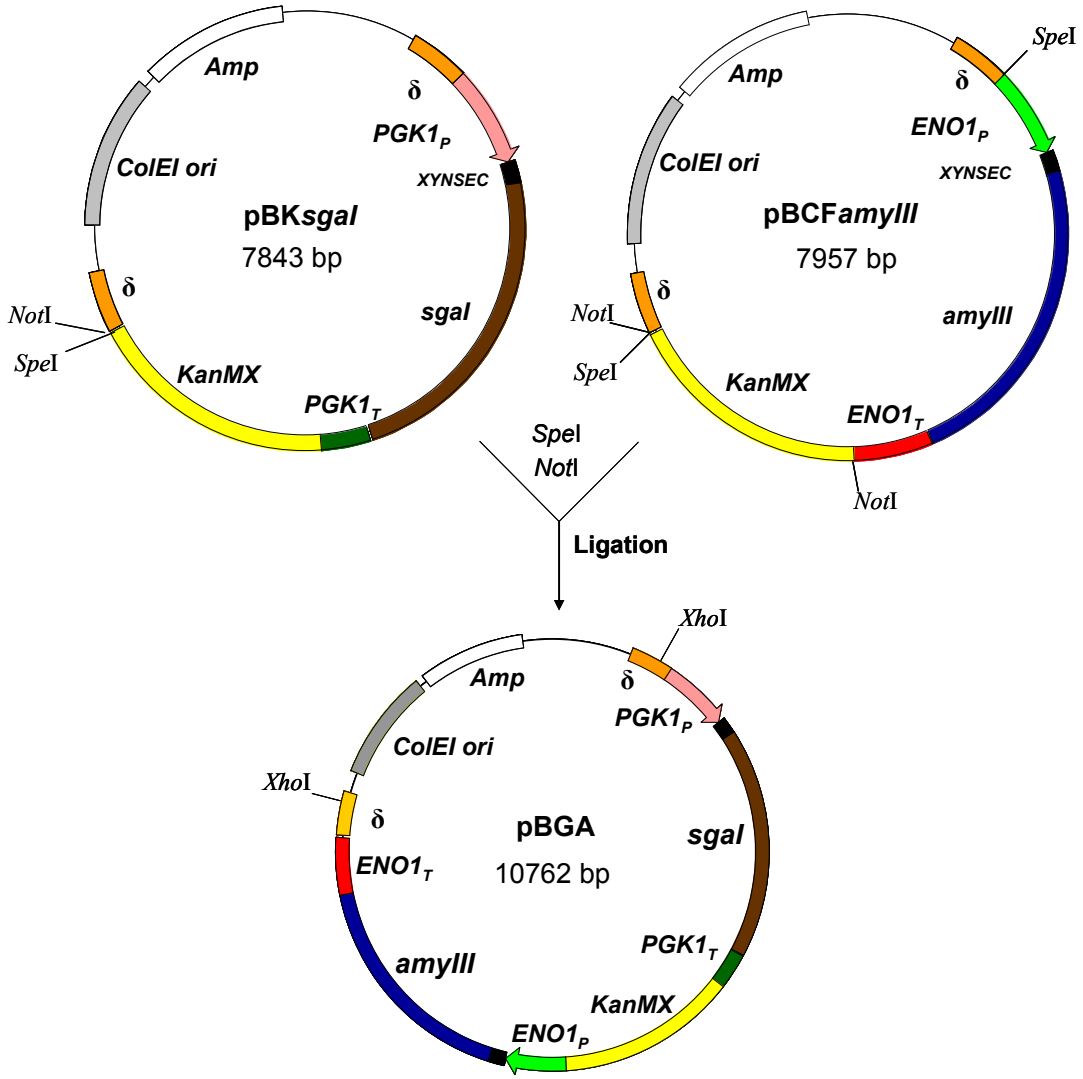

Figure 3.33. Construction of the δ-integrative vector, pBGA, containing the *amyIII* and *sgaI* cassettes for constitutitive expression in *S. cerevisiae.*

3.5.3 Amylolytic yeast strain generation.

The integrative plasmids constructed contain a unique *Xho*I site in the δ-sequence for an efficient homologous recombination into yeast chromosomes. However, since the *amyIII* gene contains a *Xho*I site, partial *Xho*I digestion were conducted with plasmids pBCF*amyIII* (Figure 3.31) and pBGA (Figure 3.33). All vectors were digested with *Xho*I and used to transform *S. cerevisiae* s1, s2, F6 and H1 previously selected as the most promising fermentative yeasts (Paragraph 3.5.2).

Unlike laboratory haploid strains of *S. cerevisiae*, wild type isolates lack selective genetics markers and thus could only be transformed with vectors containing dominant selection markers such as zeocin gene (*Shble*, in pBCF*sgaI* plasmid) and geneticin gene (*KanMX*, in pBCF*amyIII* and pBGA constructs). The resistance to these antibiotics were determined for the *S. cerevisiae* strains and is reported in Table 3.18.

***S. cerevisiae* strains**	**s1**	**s2**	**F6**	**H1**
Geneticin (μg mL^{-1})				
0	++++	++++	++++	++++
50	++	++++	++++	++++
100	+	+++	+++	++
150	ng	+	++	+
200	ng	ng	ng	ng
300	ng	ng	ng	ng
Zeocin (μg mL^{-1})				
0	++++	++++	++++	++++
50	ng	ng	ng	ng
100	ng	ng	ng	ng
150	ng	ng	ng	ng
200	ng	ng	ng	ng

Table 3.18. Dominant selection marker resistance of *S. cerevisiae* strains s1, s2, F6 and H1 grown on YPD plate supplemented with increasing concentration of geneticin and zeocin. (++++: consistent growth; ng: no growth).

The concentration of 200-300 (μg mL^{-1}) and 75-100 (μg mL^{-1}) of geneticin and zeocine, respectively, were chosen for the selection of the recombinants. Yeast cells were prepared as described in Paragraph 2.6.2 and transformed through electroporation. The electroporated cells were plated on selective YPD agar, supplemented with zeocin or geneticin, and then tested for amylolytic activity on soluble and raw starch agar media. The integrated yeasts with the largest starch hydrolysis halos were selected and maintained on agar plates for further analysis. The number of the obtained recombinants from each wild type host strain is reported in Table 3.19.

	pBCF*sgaI*		**pBCF*amyIII***		**pBGA**	
***S. cerevisiae* strains**	**n. obtained**	**n. stable**	**n. obtained**	**n. stable**	**n. obtained**	**n. stable**
s1	32	-	6	-	-	-
s2	25	2	21	-	6	-
F6	16	-	36	-	3	-
H1	87	3	27	-	4	-

Table 3.19. Recombinant strains obtained with electro-transformation of wild type *S. cerevisiae* strains with pBCF*sgaI*, pBCF*amyIII* and pBGA integrative plasmids. Stable transformants maintained both antibiotic resistance and amylolytic activity after 120 growth generations in non selective YPD.

To study their mitotic stability, all mutants were grown in sequential batch cultures using non-selective YPD broth. The majority of the yeasts lost the phenotype of both resistance to antibiotic and amylolytic activity as the number of generations increased. After 120 generations, only five engineered strains were found to be mitotically stable. They displayed both resistance to zeocin and hydrolytic ability on soluble starch. As reported in Table 3.19, all stable recombinants were engineered for the multiple integration of the codon-optimised synthetic gene *sgaI*. The mutant strains of *S. cerevisiae* s2 were named sBCF2 and sBCF6 while the integrants of *S .cerevisiae* H1 were named LH3, LH4 and LH18.

3.5.4 Expression of *sgaI* gene in engineered yeasts.

The ability of the amylolytic strains to produce functional amylases was confirmed as hydrolysis halos in both raw and soluble starch agar plates (data not shown). The enzymatic activity of engineered yeasts was then detected in liquid assays. Firstly, the strains were evaluated for the production of the recombinant SgaI in three different broths: 2xSC, 2xSC-modified and YPD. Cultivation media and mainly their nitrogen sources, indeed, could influence the production of heterologugus proteins by engineered microbial strains.

The glucoamylolytic activities of sBCF2 and LH4, reported here as representative of the others recombinant strains, are shown in Figure 3.34 and 3.35. The enzymatic assays were conducted at 50°C in citrate-phosphate buffer (pH 4.5) with 0.1% soluble starch.

As expected, the *sgaI* gene fused to the *PGK1* promoter was constitutively expressed since the engineered yeasts constantly showed significant enzymatic activity; highest values were obtained after 72 hour incubation.

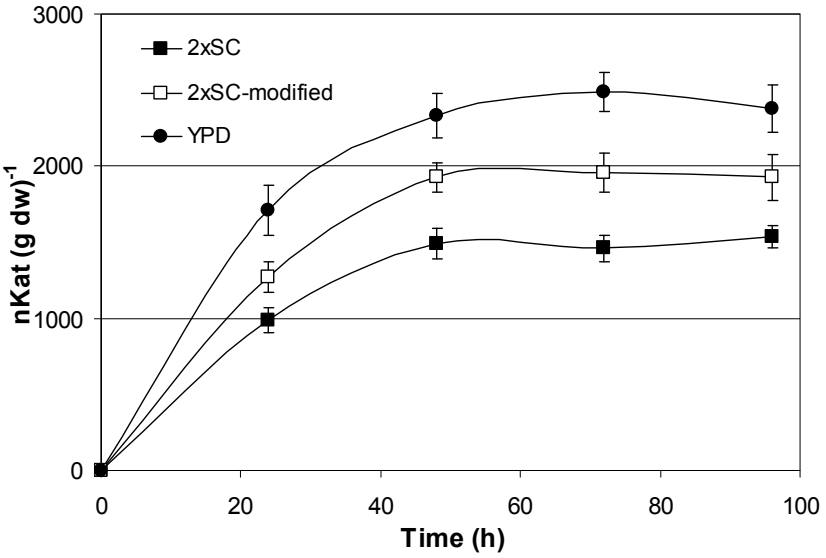

Figure 3.34. Glucoamylolytic activity of *S. cerevisiae* sBCF2, recombinant of *S. cerevisiae* s2 with multiple integrations of *sgaI*, grown in YPD (●) 2xSC (■) and 2xSC-modified broth (□) supplemented with 0.75% yeast extract. The activity, detected at 50°C in buffer at 4.5 pH, is expressed as nKat(g dw cells)$^{-1}$ that is the enzyme activity needed to produce 1 nmol of glucose per second per gram dry cell weight. The experiment was conducted in triplicate (± SD).

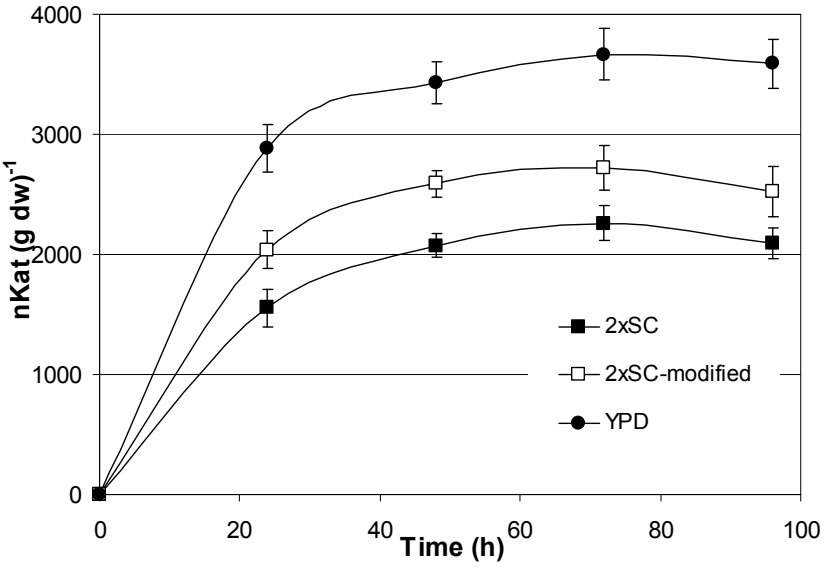

Figure 3.35. Glucoamylolytic activity of *S. cerevisiae* LH4, recombinant of *S. cerevisiae* H1 with multiple integrations of *sgaI*, grown in YPD (●) 2xSC (■) and 2xSC-modified broth (□) supplemented with 0.75% yeast extract. The activity, detected at 50°C in buffer at 4.5 pH, is expressed as nKat(g dw cells)$^{-1}$. The experiment was conducted in triplicate (± SD).

Media supplements influenced the production of SgaI by recombinant yeasts. All strains produced the maximum enzymatic activities once grown in rich YPD broth, containing both yeast extract and peptone. Therefore, the medium was selected for further enzymatic studies. The glucoamylolytic activity of the stable transformants secreting SgaI was monitored each 24 hour in three buffers at pH 4.5, 6.0 and 7.5. Highest values, reported in Table 3.20, were obtained after 72 hour incubation. The strains sBCF2 and LH4 produced the most efficient soluble starch hydrolysing ability.

The SgaI protein works better in acid conditions: the enzyme produced significantly lower values at pH 6.0, while at higher pH value no detectable hydrolytic activity was recorded.

On the basis of the preliminary enzymatic assays reported above, the glucoamylolytic activity of SgaI was tested at pH values of 4.5-5.0 and 6.0 (Figure 3.36). The 5.4 pH was also investigated since it has been reported as optimal for the SgaI of *A. awamori* (de Villiers, 2008).

	Glucoamylolytic activity at 50°C (nKat mL^{-1})		
	pH 4.5	**pH 6.0**	**pH 7.5**
S. cerevisiae H1	ND	ND	ND
S. cerevisiae LH3	0.72 ± 0.05	0.52 ± 0.06	ND
S. cerevisiae LH4	2.35 ± 0.18	1.42 ± 0.15	ND
S. cerevisiae LH18	0.63 ± 0.06	0.41 ± 0.05	ND
S. cerevisiae s2	ND	ND	ND
S. cerevisiae sBCF2	1.79 ± 0.15	1.20 ± 0.12	ND
S. cerevisiae sBCF6	1.08 ± 0.09	0.65 ± 0.08	ND

Table 3.20. Glucoamylolytic activity (nKat mL^{-1}) of engineered strains LH3, LH4, LH18, sBCF2, sBCF6 and their respective wild type yeast *S. cerevisiae* H1 and s2. The enzymatic activity was measured on cell-free supernatants after 72 h incubation in YPD broth. The assays were performed at 50°C in citrate-phosphate buffer (0.1% soluble starch) at 4.5-6.0-7.5 pH. ND: not detectable. The experiment was conducted in triplicate (± SD).

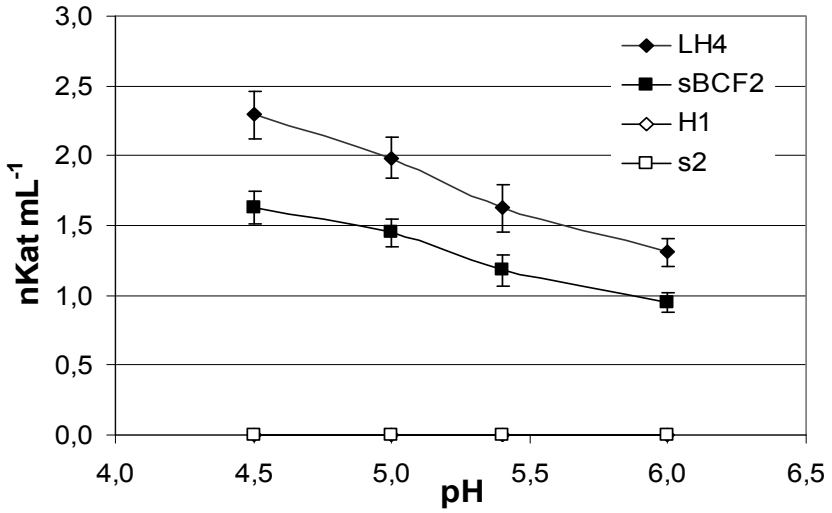

Figure 3.36. Glucoamylolytic activity in cell-free culture supernatant (nKat mL^{-1}) of engineered strains LH4, sBCF2 and their respective wild type yeast *S. cerevisiae* H1 and s2, grown for 72 h in YPD. The assays were performed at 50°C in citrate-phosphate buffer (0.1% soluble starch) at 4.5-5.0-5.4-6.0 pH. The experiment was conducted in triplicate (± SD).

In Figure 3.36, the glucoamylolytic activity of SgaI secreted by the strains LH4 and sBCF2 is reported. An optimal pH of 4.5 was measured and the enzymatic activity decreased as the pH increased. Only about 72% and 58% of the maximum glucoamylolytic activity was still detectable at pH 5.4 and 6.0, respectively.

Raw starch and soluble starch activity was determined at the optimal pH and temperature incubation for SgaI (pH 4.5, 50°C). The assays were also conducted at 30°C, growth temperature preferred by the yeast (Table 3.21).

	Soluble starch		**Raw starch**	
S. cerevisiae strains	50°C	30°C	50°C	30°C
H1	ND	ND	ND	ND
LH3	993.5 ± 87.5	281.5 ± 26.0	594.1 ± 127.5	174.2 ± 16.5
LH4	3218.3 ± 327.2	834.2 ± 77.4	1232.0 ± 137.3	360.1 ± 40.0
LH18	1151.7 ± 120.5	317.2 ± 32.1	694.4 ± 97.3	196.9 ± 19.9
s2	ND	ND	ND	ND
sBCF2	2122.4 ± 245.4	624.7 ± 35.6	1040.8 ± 65.9	315.3 ± 38.1
sBCF6	1778.8 ± 122.1	489.1 ± 36.4	855.6 ± 75.4	224.4 ± 17.2

Table 3.21. Glucoamylolytic activity (nkat (g dw cells)$^{-1}$) of the engineered *S. cerevisiae* strains and their respective wild type yeasts (s2 and H1) grown in YPD broth for 72 hours. The assays were performed at 30° and 50°C in citrate-phosphate buffer at 4.5 with either 0.1% soluble starch or 2% raw starch. The values are the means of the results obtained from two experiments conducted in triplicate (± SD). ND: not detectable.

The yeast sBCF2, engineered strain of *S. cerevisiae* s2, showed remarkable soluble as well as raw starch hydrolysing activity. Among H1 recombinants, the highest enzymatic activity was produced by the strain LH4 with a glucoamylolytic activity of 3218 and 1232 nkat(g dw cells)$^{-1}$ on soluble and raw starch, respectively.

3.5.5 Fermentation studies.

The two recombinant yeasts sBCF2 and sBCF6 were selected for fermentation studies from glucose, soluble and raw starch. Although the strains produced enzymatic activity lower than LH4, they originated from the parental yeast s2, much more efficient fermentative strain that H1 as reported in Paragraph 3.5.1.

The fermentation experiments were conducted in two different systems described in Paragraph 2.6.2. The ethanol yield displayed by the yeasts were similar in both fermentation procedures but the magnetic multi-stirring significantly enhanced the yeast ethanol productivity. Therefore, the results obtained with multi-stirring method will be presented.

The anaerobic growth rate and the fermentative performance from glucose were compared between sBCF2 and sBCF6 strains and the wild type yeast s2. As shown in Figure 3.37, no notable differences were observed when the yeasts were grown in GFM medium (glucose 20.25 gL^{-1}).

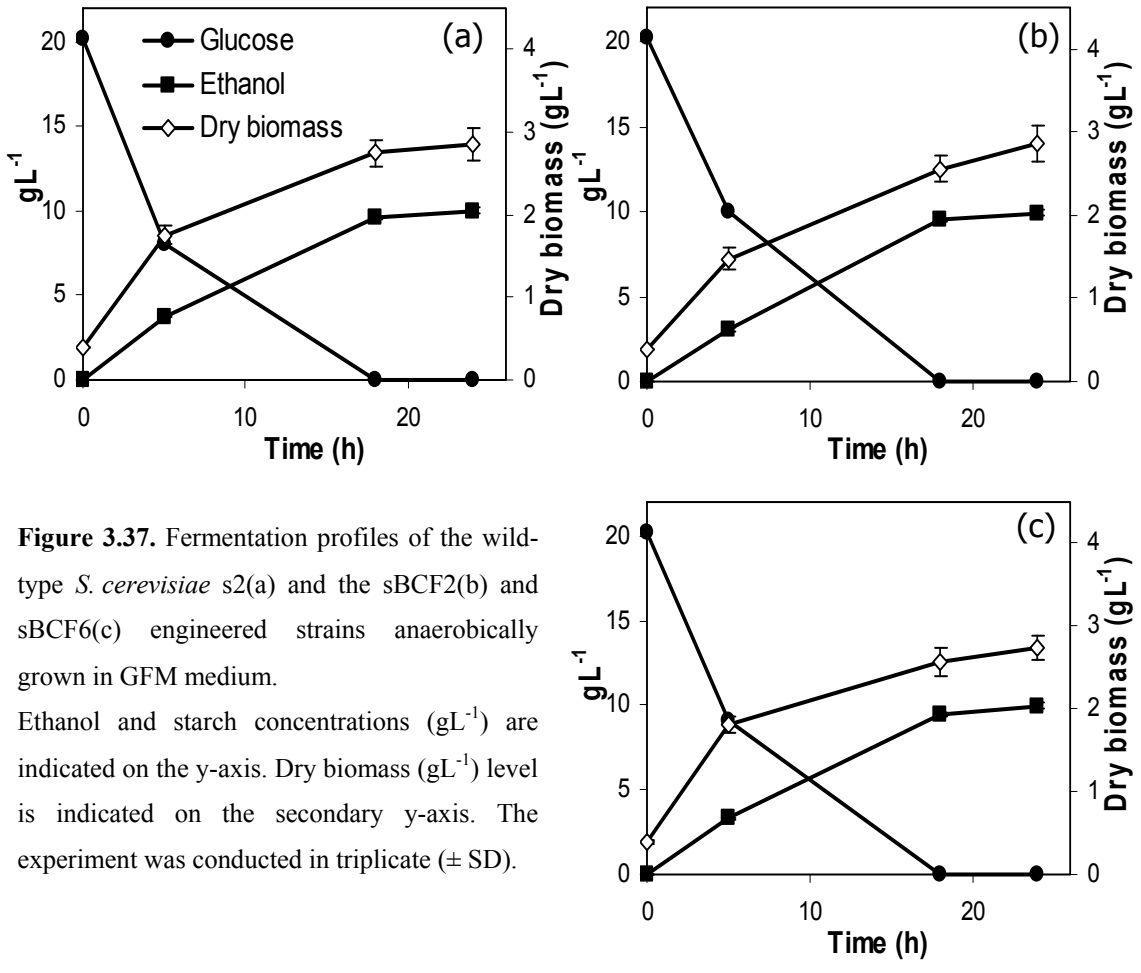

Figure 3.37. Fermentation profiles of the wild-type *S. cerevisiae* s2(a) and the sBCF2(b) and sBCF6(c) engineered strains anaerobically grown in GFM medium.
Ethanol and starch concentrations (gL^{-1}) are indicated on the y-axis. Dry biomass (gL^{-1}) level is indicated on the secondary y-axis. The experiment was conducted in triplicate (± SD).

Glucose was rapidly depleted and the strains produced up to 9.9 gL^{-1} ethanol after 24 h. Their ethanol yield of about 0.49 gg^{-1} corresponded to 96% of the theoretical maximum yield from glucose. As indicated in Table 3.22, the major fermentative parameters considered in this study were similar in all strains.

The engineered strains were also used for direct ethanol fermentation from soluble starch (Figure 3.38 and Table 3.22). The stable transformants, sBCF2 and sBCF6, hydrolysed 69% and 63% of the soluble starch and produced 5.4 and 4.8 gL^{-1} of ethanol after 48 h, respectively (Figure 3.38b and 3.38c). As expected, the parental yeast s2 did not convert starch into ethanol after long incubation at 30°C (Figure 3.38a).

The sBCF2 strain showed an ethanol yield of 0.44 g ethanol per gram of consumed starch (79% of theoretical maximum) while strain sBCF6 produced a yield of 0.42, corresponding to 76% of the theoretical yield (0.56 gg^{-1}).

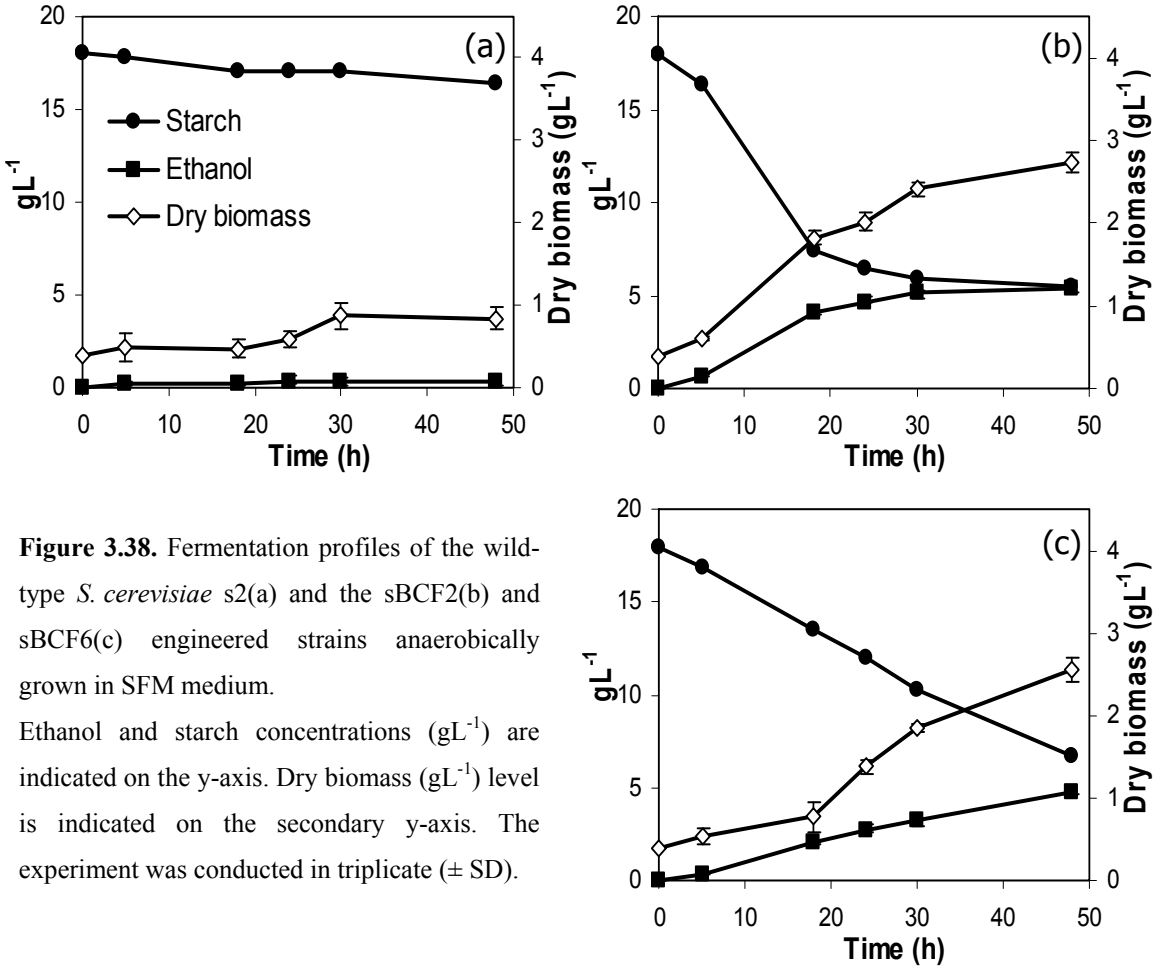

Figure 3.38. Fermentation profiles of the wild-type *S. cerevisiae* s2(a) and the sBCF2(b) and sBCF6(c) engineered strains anaerobically grown in SFM medium.
Ethanol and starch concentrations (gL^{-1}) are indicated on the y-axis. Dry biomass (gL^{-1}) level is indicated on the secondary y-axis. The experiment was conducted in triplicate (± SD).

S. cerevisiae strains	Sugar[a] gL^{-1}	Ethanol gL^{-1}	*Q* (volumetric productivity) $(gL^{-1})h^{-1}$	Q_{max} $(gL^{-1})h^{-1}$	*q* (specific productivity) $g(g\ dw\ cell)^{-1}h^{-1}$	q_{max} $g(g\ dw\ cell)^{-1}h^{-1}$	Ethanol Yield
Raw starch medium							
sBCF2	20.25	2.4 after 336 h	0.007	0.016 (45 h)	0.011	0.035 (45 h)	0.41 (72%)[b]
sBCF6	20.25	1.8 after 336 h	0.005	0.011 (45 h)	0.010	0.025 (45 h)	0.40 (72%)[b]
Soluble starch medium							
sBCF2	20.25	5.4 after 48 h	0.11	0.23 (18 h)	0.040	0.12 (18 h)	0.44 (79%)[b]
sBCF6	20.25	4.8 after 48 h	0.10	0.11 (18 h)	0.037	0.08 (18 h)	0.42 (76%)[b]
Glucose medium							
s2	20.25	9.9 after 24 h	0.41	0.70 (6 h)	0.136	0.32 (6 h)	0.49 (96%)[c]
sBCF2	20.25	9.8 after 24 h	0.41	0.64 (6 h)	0.135	0.29 (6 h)	0.49 (95%)[c]
sBCF6	20.25	9.9 after 24 h	0.41	0.66 (6 h)	0.135	0.25 (6 h)	0.49 (96%)[c]

Table 3.22. Ethanol production by the engineered *S. cerevisiae* strains (sBCF2 and sBCF6) and their wild type yeast (s2). [a] Sugar equivalent amounts determined from the sum of starch and glucose in medium. [b] Ethanol yield as g (g consumed sugar)$^{-1}$ and % of theoretical maximum (0.56 g g^{-1} from starch) indicated in brackets. [c] Ethanol yield as g g^{-1} and % of theoretical maximum (0.51 g g^{-1} from glucose) indicated in brackets.

As reported in Table 3.22, the final volumetric productivity (Q) was similar for both engineered yeasts but the maximum ethanol productivity (Q_{max}) of the sBCF2 strain (0.23 $gL^{-1}h^{-1}$) was approximately two-fold that of the sBCF6 yeast (0.11 $gL^{-1}h^{-1}$).

The conversion rate of starch to ethanol was also found to be much more efficient in the case of sBCF2 (Figure 3.38b), especially up to 18 h of fermentation. The comparison of the residual starch deposits seems to confirm this finding (Figure 3.39).

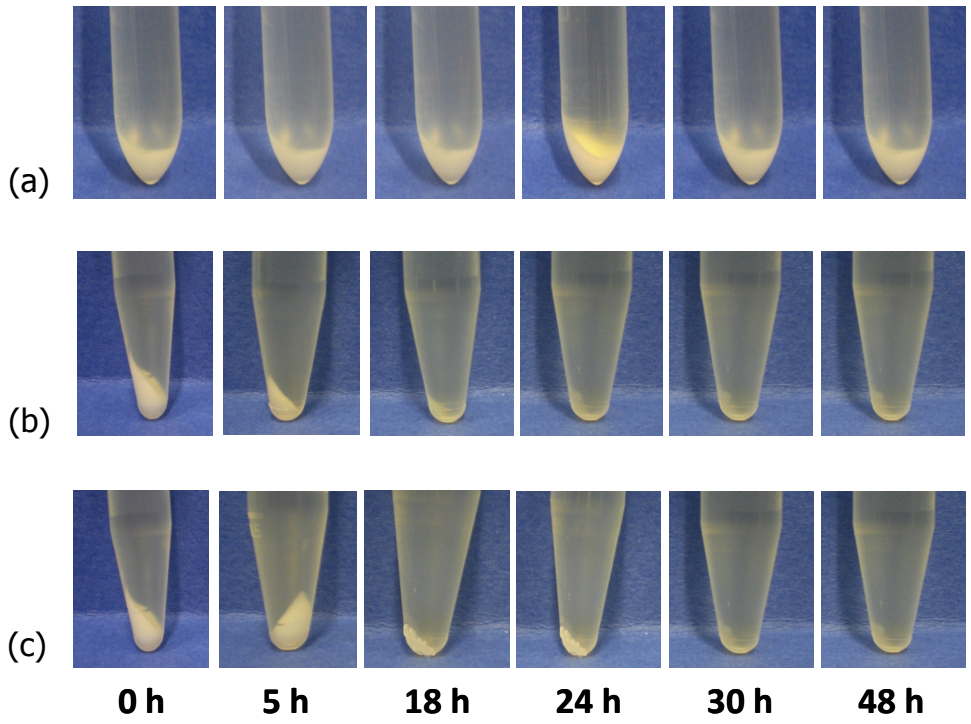

Figure 3.39. Soluble starch deposit (after -20°C storage) in SFM medium inoculated with wild type yeast *S. cerevisiae* s2 (a) and the sBCF2(b) and sBCF6(c) engineered strains.

The yeast sBCF2 hydrolysed the major amount of starch within 24 h incubation (Figure 3.39b), while sBCF6 showed slower starch hydrolysing ability (Figure 3.39c). As expected, the wild type strain did not use the polysaccharide for growth: the amount of starch was constant in the SFM medium during the entire fermentation (Figure 3.39a).

Each recombinant yeast was also evaluated for direct ethanol production from raw starch in anaerobic conditions (Figure 3.40). The strains, sBCF2 and sBCF6, consumed 32% and 25% of the raw starch and produced 2.4 and 1.8 gL^{-1} of ethanol, respectively.

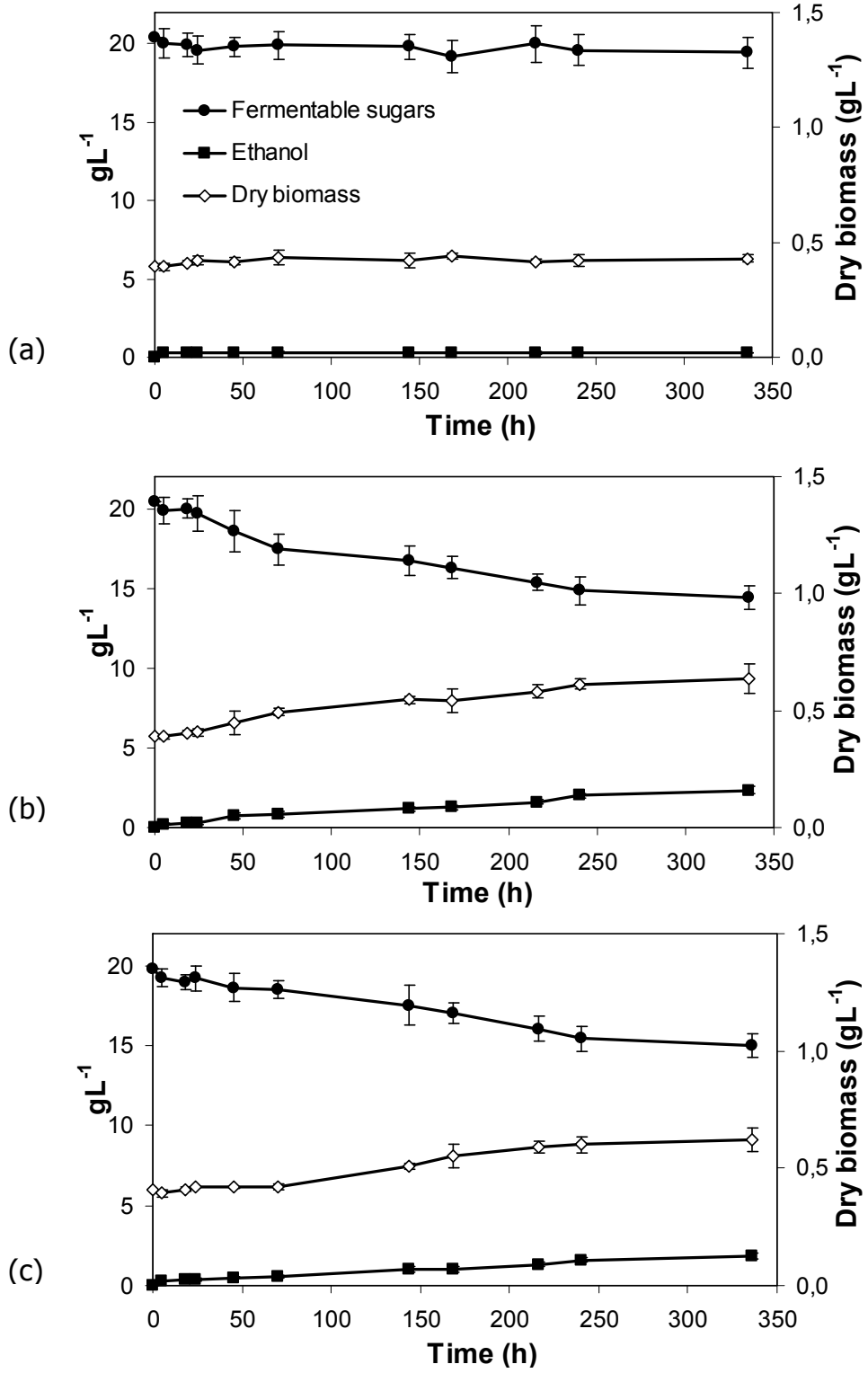

Figure 3.40. Fermentation profiles of the wild type *S. cerevisiae* s2(a) and the sBCF2(b) and sBCF6(c) engineered strains anaerobically grown in RSFM medium. Ethanol and fermentable sugars concentrations (gL^{-1}) are indicated on the y-axis. Dry biomass (gL^{-1}) level is indicated on the secondary y-axis. The experiment was conducted in triplicate (± SD).

The alcohol yields were similar, corresponding to 72% of the theoretical maximum yield (Table 3.22). Moreover, both strains displayed similar fermentative profiles.

However, the fermentation with sBCF2 was faster than that of sBCF6: the maximum ethanol productivity (Q_{max}) of sBCF2, (0.016 gL^{-1})h^{-1}, was approximately 1.5-fold higher than sBCF6 (Table 3.22). Considering the maximum ethanol specific productivity (q_{max}) the sBCF2 displayed a value of 0.035 g(g dw cell)$^{-1}h^{-1}$ after 45 h of incubation which was 1.4-fold higher than sBCF6 value.

3.5.6 Discussion

The wild type yeasts, showing interesting amylolytic activities (Paragraph 3.3), have distinct physiological properties rendering them suitable for large scale fermentation.

Firstly, they originated from different fermentation plants and are specifically adapted to the oenological environment. In addition, wild type strains are generally adapted for efficient fermentation in grape musts with high sugar content (up to 260 gL^{-1}), and/or in environments with high alcohol content (up to 15% v/v), low pH (3.0-3.5), often with limiting amounts of nitrogen, lipids and vitamins (Fleet and Heard, 1993; Hahn-Hägerdal et al., 2005; Pretorius, 2000).

The availability of such isolates as candidates for metabolic engineering programs is crucial in order to assure successful introduction of novel recombinant strains into industrial ethanol processes. Whereas strain development by recombinant techniques is performed in genetically defined laboratory yeasts in particular media, the typical industrial production microbe is genetically undefined and adapted to perform in poor, toxic and nutrient-limited broths (Sauer, 2001).

The potentially amylolytic yeasts were further evaluated for their fermentative vigour in defined medium with high sugar levels. MNS broth, used in this study, was designed in order to simulate natural musts with defined supplements and additives (Delfini, 1995). The broth could be considered quite similar to several poor industrial media (Dahod, 1999; Miller and Churchill, 1986). In Table 3.23, MNS composition is compared with that of two commonly used defined broths in the recombinant yeast development: defined mineral

medium (DMM; Verduyn et al., 1992) and synthetic complete (SC) medium equivalent to supplemented YNB broth (Difco).

Components (gL^{-1})	MNS	DMM	SC (YNB+Suppl)
$(NH_4)_2SO_4$	0.3	5	5
$(NH_4)_2HPO_4$	0.3	-	-
KH_2PO_4	1	3	1
$MgSO_4 \cdot 7H_2O$	0.5	0.5	0.5
NaCl	0.1	-	0.1
Malic Acid	2	-	-
Tartaric Acid	3	-	-
Vitamins (mgL^{-1})			
Biotin	0.02	0.05	0.002
D-Pantothenic Acid	0.4	1	0.4
myo-Inositol	2	25	2
Nicotinic Acid	0.4	1	0.4
Thiamine	0.4	1	0.4
Pyridoxine	0.4	1	0.4
p-Aminobenzoic Acid	0.2	0.2	0.2
Riboflavin	-	-	0.2
Folic Acid	-	-	0.002
Trace elements (mgL^{-1})			
H_3BO_3	0.5	1	0.5
$CuSO_4 \cdot 5H_2O$	0.04	0.3	0.04
KI	0.1	0.1	0.1
$NaMoO_4 \cdot 2H_2O$	0.2	0.4	0.2
$ZnSO_4 \cdot 7H_2O$	0.4	4.5	0.4
$FeSO_4 \cdot 7H_2O$	-	3	-
$FeCl_3 \cdot 6H_2O$	0.4	-	0.2
$MnCl_2 \cdot 2H_2O$	-	-	-
$MnSO_4 \cdot 4H_2O$	-	-	0.4
EDTA	-	15	-
$CaCl_2 \cdot 2H_2O$	100	4.5	100
Supplements (mgL^{-1})			
Adenin (hemisulfate salt)	-	-	40
L-arginine	-	-	20
L-aspartic acid	-	-	100
L-glutamic acid (hemisulfate salt)	-	-	100
L-histidine	-	-	20
L-leucine	-	-	60
L-lysine (mono-HCl)	-	-	30
L-methionine	-	-	20
L-phenylalanine	-	-	50
L-serine	-	-	375
L-threonine	-	-	200
L-tryptophan	-	-	40
L-tyrosine	-	-	30
L-valine	-	-	150
Uracil	-	-	20

Table 3.23. Composition of defined media used in the development of yeast strains for industrial applications.

YNB medium is a chemically defined broth that can be supplemented to satisfy auxotrophic requirements of yeast mutants used in metabolic engineering, then referred to as SC medium.

DM medium contains almost all components of YNB medium (Table 3.23), however, some nutrients are present in higher concentration than in YNB broth. The DMM medium is commonly used to obtain quantitative physiological data for yeast strains. The broth has been designed to assure that concentrations of vitamins and trace elements do not exercise growth limitation (Verduyn et al., 1992).

As reported in Table 3.23, MNS broth is characterized by the lowest amounts of components, macro and micro-nutrients. Yeast strains able to grow and efficiently ferment with limiting nutrients could be very interesting for industrial scale applications.

From this point of view, the fermentation kinetics of the tested wild type *S. cerevisiae* strains (Paragraph 3.5.1) are promising. Yeasts, selected for their potentially amylolytic abilities, displayed high fermentative vigour in all MNS media tested.

However, the strains showed some genetic variability in the fermentative phenotype. Once incubated in 20% glucose, few isolates converted rapidly glucose into ethanol at high levels: the strains s2 and F6 exhibited consistent glucose consumption rate and ethanol yield (Figure 3.28). Their efficiency was even more consistent than that of *S. cerevisiae* H1, used as reference industrial strain.

All yeasts were influenced by the xylose addition in MNS broth (Figure 3.29 and 3.30). Glucose consumption rates and ethanol yields decreased as the xylose concentration increased. Since *S. cerevisiae* is unable to utilise xylose as fermentative substrates (Hahn-Hägerdal et al., 2007), this finding could be explained considering that the yeast uptakes xylose by facilitated diffusion even though the sugar is not a natural substrate (Hamacher et al., 2002; Jojima et al., 2010).

S. cerevisiae indeed takes up xylose mainly through non-specific hexose transporters encoded by the HXT gene family (Kruckeberg, 1996; Saloheimo et al., 2007; Sedlak and Ho, 2004). However, their affinity for xylose is much lower than that for glucose and the xylose uptake through the transporters is strongly inhibited by glucose (Matsushika et al., 2009; Saloheimo et al., 2007).

Therefore, all yeast strains were affected by xylose which, present in high concentrations, may have acted as alternative substrate for specific and non-specific hexose transporters. In addition, xylose was initially reduced to xylitol, as indicated by the limited amounts of xylitol detected in MNS supplemented with either 5 or 10% xylose (Table 3.16 and 3.17). The first enzyme in the xylose-utilising pathway is xylose reductase (XR), which converts xylose to xylitol. Thus, the yeast strains should have XR enzyme(s) rendering them able of converting xylose into xylitol. The suggestion is consistent with previous works that have described xylose reductase activities in wild type *S. cerevisiae* strains (Kuhn et al., 1995; van Zyl et al., 1993).

Since one of the main properties for an industrial strain is the ability to ferment in the presence of a mixture of unusual sugars and under nutrient limitation, the yeasts performing the highest ethanol yields in MNS broths were selected. Thus the isolates s1, s2, F6 and H1 were used as host strains for the development of industrial amylolytic yeasts.

To successfully express amylolytic sequences in the selected *S. cerevisiae* strains, the construction of new delta vectors was necessary subcloning *sgaI*, sinthetic glucoamylase gene from *A. awamori* and/or *amyIII* α-amylase gene from *A. oryzae*, into integrative plasmids.

Chromosomal integration is an effective method for introducing heterologous genes in *S. cerevisiae* (Akada, 2002; Kang et al., 2008; Latorre-Garcia et al., 2008). The segregational instability of plasmid vectors is avoided and, for structurally stable insertions, the cloned gene copy number can be maintained at its optimum value (Lee and Silva, 1997).

In this study, genes integration was targeted to δ-sequences of *S. cerevisiae.* This elements are the long terminal repeats of retrotransposons Ty1 and Ty2 (Boeke and Sandmeyer, 1991). In addition to their close association with Ty1 and Ty2, δ-sequences also occur as isolated elements (Boeke, 1989). Because there exist about 30 copies of Ty1 and 425 copies of δ-sequences dispersed throughout the haploid yeast genome (Dujon, 1996), this δ-integration system makes possible to integrate more copies of genes into yeast chromosomes than other conventional integration procedures.

Three integrative delta vectors were constructed for the expression of *amyIII* α-amylase gene (Figure3.31), *sgaI* syntethic glucoamylase (Figure 3.32) and both amylolytic sequences (Figure 3.33). However, the majority of the integrants, obtained by electroporation method, revealed mitotically unstable (Table 3.19) and only five stable yeasts secreting SgaI were selected.

The maintenance of both zeocin resistance and amylolytic activity phenotype does not necessarily mean that these recombinant strains should be efficient amylolytic yeasts. For this reason, the stable integrants were tested for their glucoamylolytic activities and fermentative performance from glucose, soluble and raw starch.

Firstly, the engineered strains were evaluated for the production of the heterologous protein in different broths. Media supplements were shown to have the major influence on the production of SgaI. In particular, yeast extract and peptone enhanced the glucoamylase secretion by recombinant strains (Figure 3.34 and 3.35). In YPD broth supplemented with both additives the enzymatic activity was about 1.4 and 1.8-fold higher than those detected in the supernatant of cultures grown in 2xSC-modified and 2xSC media, respectively.

The result is in accordance with Hahn-Hägerdal et al. (2005) who illustrated that the choice of nitrogen source in media for the production of heterologous proteins is crucial. For instance, inconsistency in complex components such as yeast extract can limit the reproducibility of industrial fermentation performance, resulting in 2-3 fold differences in heterologous protein production levels (Zhang et al., 2003).

The remarkable enzymatic activity produced by the engineered yeasts secreting SgaI indicated that the choice of cloning *sgaI* in frame with *T. reesei* xylanase 2 secretion signal was effective. The *XYNSEC* signal avoided the inefficient secretion of the recombinant enzyme, one of the main factors negatively affecting the adequate production of heterologous extracellular proteins. The phenomenon has been observed in yeast (Lee et al., 1999), and de Moraes et al. (1995) showed that when using the native leader peptide from *A. awamori* glucoamylase, 5-12% of the activity was left within cells.

The enzymatic studies on SgaI activity revealed an optimal pH value of 4.5 (Figure 3.36). The glucoamylolytic activity was also influenced by temperature incubation and substrate (Table 3.21): at 30°C incubation, the enzymatic activity was nearly 28% of the maximum value obtained at 50°C, optimal temperature for the enzyme. As expected, on

unmodified corn starch, the integrated strains produced about 50% of their enzymatic activity performed on soluble starch.

Among the recombinant strains, sBCF6 and LH4 exhibited the most important activity on both raw and soluble starch (Table 3.21). Their efficient enzymatic abilities should be related to the high number of integrated gene copies as compared to those inserted in the other yeasts. However, further genetic studies are required to confirm this hypothesis.

The engineered strains sBCF2 and sBCF6, once grown in GFM medium (glucose 20.25 gL^{-1}), produced ethanol with yield similar to that of the parental yeast s2 (Table 3.24). This result could indicate that multiple gene integrations did not significantly affect the yeast fermentative performance.

The recombinant strains efficiently convert soluble starch into ethanol (Figure 3.38). The maximum ethanol concentration was 5.4 and 4.8 gL^{-1} after 48 hours for sBCF2 and sBCF6, respectively. Their fermentative abilities was compared to that of previously engineered strains (Table 3.24) and their starch conversion capacity resulted similar. A higher ethanol concentration has been measured in a previous work, where up to 14.3 gL^{-1} ethanol was produced after 140 hours in a controlled batch fermentation with *S. cerevisiae* SR93 secreting Sta1 glucoamylase (Nakamura et al., 1997). However, the yeasts developed in this study showed comparable volumetric productivity levels (Table 3.24). Moreover, the yields of ethanol per gram of consumed starch were similar to the yield of *S. cerevisiae* SR93, constructed by integrating *sta1* glucoamylase gene of *S. diastaticus*.

At the end of the fermentation, only about 66% of starch was hydrolysed by the recombinant yeasts (Figure 3.37). In addition, after 20 h, the starch to ethanol conversion rate of both strains decreased notably. This result may be explained considering that SgaI glucoamylase could efficiently cleave only α-1,4 linkages.

This suggestion seems to be confirmed by the fermentation kinetics of the recombinant strains grown in raw starch medium. The yeast sBCF2 produced the maximum ethanol concentration of 2.4 gL^{-1} after 336 hours of fermentation (Figure 3.39). As reported in Table 3.24, its volumetric ethanol productivity, 0.007 $(gL^{-1})h^{-1}$, was much lower than the productivity determined for previously generated strains, 0.31-0.46 $(gL^{-1})h^{-1}$. However, the strains showed ethanol yield comparable with those exhibited by yeasts recently engineered with glucoamylase and α-amylase for raw starch conversion (Yamada et al., 2009).

S. cerevisiae strains	Sugar[a] gL^{-1}	Ethanol gL^{-1}	*Q* (volumetric productivity) $(gL^{-1})h^{-1}$	Ethanol Yield	Reference
Raw starch medium					
MT8-1SS	110.00	26.0 after 84 h	0.31	0.45 (80%)[b]	Yamada et al., 2009
NBRC1440SS	110.00	28.0 after 84 h	0.33	0.52 (93%)[b]	Yamada et al., 2009
MN8140SS	110.00	39.0 after 84 h	0.46	0.44 (79%)[b]	Yamada et al., 2009
sBCF2	20.25	2.4 after 336 h	0.007	0.41 (72%)[b]	This study
sBCF6	20.25	1.8 after 336 h	0.005	0.40 (72%)[b]	This study
Soluble starch medium					
SR93	55.00	14.3 after 140 h	0.10	0.48 (85%)[b]	Nakamura et al., 1997
sBCF2	20.25	5.4 after 48 h	0.11	0.44 (79%)[b]	This study
sBCF6	20.25	4.8 after 48 h	0.10	0.42 (76%)[b]	This study
Glucose medium					
s2	20.25	9.9 after 24 h	0.41	0.49 (96%)[c]	This study
sBCF2	20.25	9.8 after 24 h	0.41	0.49 (95%)[c]	This study
sBCF6	20.25	9.9 after 24 h	0.41	0.49 (96%)[c]	This study

Table 3.24. Ethanol production by *S. cerevisiae* strains engineered for the multiple integration of amylolytic genes.[a] Sugar equivalent amounts determined from the sum of starch and glucose in medium. [b] Ethanol yield as g (g consumed sugar)$^{-1}$ and % of theoretical maximum (0.56 g g^{-1} from starch) indicated in brackets. [c] Ethanol yield as g g^{-1} and % of theoretical maximum (0.51 g g^{-1} from glucose) indicated in brackets.

Although the recombinant strains sBCF2 and sBCF6 secreted the sole glucoamylase enzyme, their raw starch fermentative capacity should be considered promising. Indeed, the yeasts, described in Yamada et al (2009), were developed by mating two integrated haploid strains expressing the α-amylase or glucoamylase gene.

The limited amount of ethanol produced by the *sgaI* expressing yeasts seemed to confirm that the codon optimised glucoamylase could efficiently hydrolyse only α-1,4 linkages. Indeed, the strains consumed low amount (up to 32%) of the available raw starch (Figure 3.39).

Since the engineered yeasts were able to ferment all glucose available in the GFM medium (Figure 3.37), the main factor limiting ethanol fermentation from both soluble and raw starch seems to be the inability of SgaI to hydrolyse α-1,6 linkages.

The co-expression of *sgaI* and other amylolytic genes in the constructed strains is in progress in order to increase their starch conversion efficiency. Furthermore, this study reported the first multiple integration of a codon optimised glucoamylase gene into wild type *S. cerevisiae* strains. The use of the synthetic *sgaI* gene should have increased gene expression making it less laborious for the host strain, since codons not frequently used by *S. cerevisiae* (Sharp et al., 1988; Sharp and Cowe, 1991) are removed from the glucoamylase sequence.

The constructing strategy adopted in this work proved effective and could be applied to other genes encoding efficient extracellular enzymes in order to achieve high expression levels in wild type yeasts.

4. CONCLUSIONS.

Polysaccharides of plant biomass represent a sustainable source of fuel ethanol. The main obstacle hampering the utilisation of biomass is the lack of low-cost technology. In this respect, the Consolidated Bioprocessing (CBP) is gaining increasing recognition as a potential breakthrough for cost-effective biomass conversion relying on a single microbial step.

Microorganisms suitable for CBP applications that possess the combination of polysaccharides utilisation (e.g., high-level production of hydrolytic enzymes and consumption of resulting sugars) and ethanol production properties have not been described.

In this study, the development of such microorganisms was started mainly via two distinct strategies, (1) the selection and isolation of strains having excellent hydrolytic abilities for their future improvement of desired production properties and (2) the engineering of microbes, with optimal fermentative traits, for the production of efficient hydrolytic enzymes.

The collection of new cellulolytic strains isolated from a forestry ecosystem could be considered a good microbial and gene-pool source for the development of a CBP microbe. Few isolates, indeed, produced efficient cellulose-degrading activities and may have improvable ethanol production properties. Nevertheless, further analyses using molecular and physiological approaches are required to study their fermentative abilities and other technologically related characteristics.

This work revealed the potential for oenological yeasts to produce extracellular enzymes of interest for future bioethanol application (particularly pectinases, cellulases and amylases). Twelve *S. cerevisiae* strains showed pectolytic activity. The non-*Saccharomyces* yeast, identified as *A. schoenii*, proved effective for the secretion of cellulose-degrading enzymes both on plates and in enzymatic assays.

Since in literature no yeast isolated from oenological environment has been described for such cellulolytic activity, the saccharolytic ability of *A. schoenii* seems promising. On the basis of the preliminary results of this study, the non-*Saccharomyces* yeast could be

efficiently used in a microbial CBP consortium together with a high fermentative *S. cerevisiae* strain.

Furthermore, thirteen wild type *S. cerevisiae* isolates were selected for their ability to grow on soluble starch on both complete and minimal media. Their weak growth on starch minimal plates was unexpected as the common dogma is that wild-type *S. cerevisiae* cannot grow on starch (Pretorius, 1997).

However, the multi-disciplinary study conducted on their potentially amylolytic activity confirmed that the yeasts utilise starch as sole carbon source. To date, the enzymatic mechanism at the basis of their phenotype is not completely understood and additional genetic and physiological analyses are in progress. Moreover, a new approach using natural selection will be applied to the amylolytic strains in order to develop yeasts with improved ability to use and ferment starch.

The potentially amylolytic *S. cerevisiae* yeasts were further evaluated for their fermentative vigour in defined medium with high sugar levels. Their ability to ferment was tested also in a SHF process for the conversion of wheat bran into ethanol.

The results obtained in the SHF system are interesting. Wheat bran was efficiently pre-treated and hydrolysed through the combination of mild physical-chemical methods and optimised enzyme dosages. As a result, high sugar yields were achieved and no furan derivates were formed. In addition, the low amounts of released inhibitors did not affect the downstream fermentation phase. Ethanol production reached high levels during the fermentation of wheat bran hydrolysates both using *S. diastaticus* and *S. cerevisiae* s1, selected as the most efficient amylolytic *S. cerevisiae* isolate.

This study demonstrated that *S. diastaticus*, producing glucoamylolytic enzymes, could be used for the conversion of wheat bran into ethanol without adding commercial glucoamylase enzymes. From the other hand, *S. cerevisiae* s1 showed the highest ethanol yield from glucose available at the beginning of the fermentation. The yeast exhibited its outstanding fermentative performance also during the static fermentation of unfiltered and heterogeneous hydrolysates.

Once incubated in defined medium with increasing sugar concentrations (glucose and xylose), the potentially amylolytic *S. cerevisiae* strains produced high ethanol yields. The yeasts utilised only glucose and seemed affected by the presence in the broth of increasing

xylose levels. Nevertheless, the strains fermented at high rate in the MNS broth, used in this study to simulate the typical poor industrial media.

The tested wild type *S. cerevisiae* yeasts showed several physiological properties rendering them suitable for large scale fermentation. Therefore, the strains performing the highest ethanol yields in MNS broths were selected as candidates for a metabolic engineering program in order to develop an amylolytic yeast for industrial bioethanol production.

A codon-optimised glucoamylase gene (*sgaI*) was successfully multi-copy integrated in two *S. cerevisiae* strains. The stable recombinants secreting SgaI produced remarkable hydrolysing activities on both soluble and raw starch.

The fermentation kinetics monitored for two engineered yeasts showed their ability to ferment soluble and raw starch. In order to reach a more efficient and practical fermentation process, it would be required to investigate on the co-expression of other specific amylolytic enzymes such as α-amylase and pullulanase. Nonetheless, the preliminary fermentations indicated that the recombinant strains are capable raw starch converters paving the way for the Consolidated Bioprocessing of starchy materials (e.g., corn, wheat, industrial residues).

In conclusion, the adopted strategies provided the basis for the development of a CBP microbe. Wild-type microrganisms with desired properties for both lignocellulose hydrolysis and fermentation were selected. Traditional microbiological and biochemical techniques were applied to evaluate their hydrolytic and technologically related characteristics. Moreover, a metabolic engineering approach was conducted in order to improve and enhance the starch hydrolysing activity of few yeasts with industrial fermentative traits.

Further studies are required to define a properly evolved CBP strain. However, this multi-disciplinary work seems to be a promising platform to achieve the one step bioconversion of biomass into ethanol.

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APPENDIX.

SCUOLA DI DOTTORATO IN

SCIENZE DELLE PRODUZIONI VEGETALI

(Agrobiotecnologie)

XXII CICLO

Giudizio di Ammissione all'Esame finale del Dottorando

Lorenzo FAVARO

Il Dott. Lorenzo Favaro ha conseguito la Laurea Specialistica in Scienze e Tecnologie Agrarie nell'anno accademico 2005-2006 presso la Facoltà di Agraria dell'Università di Padova.

Dal gennaio 2007 ha svolto attività di ricerca nell'ambito del XXII ciclo del Dottorato di Ricerca in "Scienze Delle Produzioni Vegetali" presso i laboratori di Microbiologia del Dipartimento di Biotecnologie Agrarie, Università di Padova.

L'attività di ricerca ha riguardato lo sviluppo di una tecnologia a basso costo per la conversione di residui agro-industriali in bioetanolo. Tra i processi tecnologici finora proposti, il Consolidated BioProcessing è riconosciuto come la strategia più promettente dal momento che prevede la conversione di biomassa in etanolo ad opera di un unico microrganismo o consorzio microbico. La ricerca condotta ha avuto come scopo principale lo sviluppo di un ceppo CBP tramite due distinte strategie a) l'isolamento di nuovi ceppi microbici cellulosolitici e il miglioramento delle loro performances fermentative b) la ricerca, o il conferimento mediante tecniche di biologia molecolare, di efficienti attività idrolitiche in organismi con comprovato vigore fermentativo, quali, ad esempio, *Saccharomyces cerevisiae*.

Durante l'attività di ricerca, sono state impiegate tecniche di microbiologia di base ed avanzata per l'isolamento e la caratterizzazione di nuovi ceppi microbici fortemente cellulosolitici ed amilolitici. Inoltre, un'ampia collezione di isolati di *S. cerevisiae* e non-*Saccharomyces* sp. selezionati sulla base del loro elevato potere fermentante, sono stati saggiati per numerose attività idrolitiche extracellulari.

I ceppi di *Saccharomyces* sp. con le caratteristiche enzimatiche e fermentative più interessanti sono stati impiegati per lo sviluppo di un processo di Separated Hydrolysis and Fermentation (SHF) per la conversione di crusca di grano in etanolo. La resa in alcool del processo è risultata promettente.

Al fine di ottenere un ceppo CBP per la conversione su scala industriale di residui amidacei, è stato messo a punto un programma di biologia molecolare al fine di conferire la capacità di idrolizzare amido grezzo a ceppi di *S. cerevisiae* wild-type selezionati per le loro elevate capacità fermentative.

Tre costrutti integrativi sono stati realizzati per l'integrazione cromosomica in lievito di sequenze fungine codificanti per glucoamilasi (*sgaI* da *Aspergillus awamori*) ed α-amilasi (*amyIII* da *A. oryzae*). I trasformati stabili ottenuti hanno dimostrato la capacità di idrolizzare l'amido e di convertirlo in etanolo in un unico passaggio. I risultati preliminari delle cinetiche di fermentazione eseguite su amido grezzo indicano che i ceppi mutanti potrebbero essere efficacemente utilizzati in un processo industriale CBP a partire da residui agricoli amidacei.

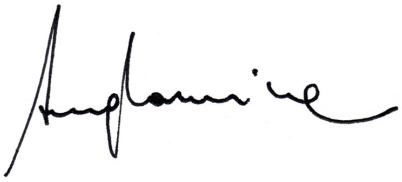
Durante il 2° anno di dottorato il Dott. Favaro ha trascorso un periodo di sei mesi presso il laboratorio di Microbiologia dell'Università di Stellenbosch (Sud Africa) sotto la supervisione del prof. van Zyl.

Durante il periodo di dottorato il Dott. Lorenzo Favaro ha partecipato a numerosi meetings nazionali ed internazionali connessi con lo svolgimento dei Progetti di ricerca nei quali il suo lavoro di dottorato era inserito, presentando lui stesso i propri risultati.

Il Dott. Favaro ha svolto con grande entusiasmo le attività di ricerca dimostrando iniziativa e originalità nell'impostazione dei propri esperimenti, spirito critico, nonché capacità di interpretare i risultati ottenuti.

Il Collegio dei Docenti del Dottorato in "Scienze Delle Produzioni Vegetali", indirizzo Agrobiotecnologie, esprime ampio e unanime apprezzamento per l'attività svolta dal Dott. Lorenzo Favaro e lo ammette a sostenere la difesa della Tesi di Dottorato per il conseguimento del titolo.

Il Coordinatore
Prof. Angelo Ramina



Agripolis 26.11.2009

PH.D SCHOOL IN

CROP SCIENCE (Agrobiotechnology)

XXII CYCLE

Lorenzo FAVARO

Dr. Lorenzo Favaro graduated in 2005/06 on Agricultural Sciences and Technologies at the Agricultural Faculty of the University of Padova. From January 2007 he entered the XXII cycle of the PhD School in Crop Science (Agrobiotechnology) at the University of Padova, Faculty of Agriculture, Department of Agricultural Biotechnology.

The research activity focused on the development of a strategy for the conversion of agricultural residues into ethanol. Among the new technologies developed for the lignocellulose-to-ethanol conversion, CBP (Consolidated BioProcessing) was selected since it is gaining increasing recognition as a potential breakthrough for low-cost biomass processing.

The experimental activity aimed at the development of a CBP organism through two distinct strategies (a) the improvement of fermentation properties of naturally hydrolytic organisms with excellent hydrolytic capabilities and (b) the selection for hydrolytic properties in an organism having optimal ethanol performance such as *Saccharomyces cerevisiae.*

Firstly, the isolation of new hydrolytic microbes were carried out with traditional and modern microbiological tecniques. In the meantime, a wide collection of *S. cerevisiae* and non-*Saccharomyces* strains, selected for their high fermentative performance, were screened for several depolymerising activities. Therefore, *Saccharomyces* strains, selected for their fermentative vigour and promising extracellular activities, were used in a Separated Hydrolysis and Fermentation (SHF) process for the conversion of wheat bran into ethanol. The alchool yield of the SHF system was promising.

To obtain an efficient CBP amylolytic yeast for industrial ethanol production, a molecular biology approach was planned for endowing raw starch hydrolytic enzymes in wild type *S. cerevisiae* strains with promising industrial fermentative performance. Three integrative plasmids were constructed and used for the stable chromosomial integration of two fungal amylases: *sgaI* glucoamylase gene form *Aspergillus awamori* and *amyIII* α-amylase gene from *A. oryzae*. The stable transformants obtained were able to perform the one step starch-to-ethanol conversion. Further preliminary fermentation studies on raw corn starch indicate that the engineered yeasts could be efficiently used for the Consolidated Bioprocessing of different starchy industrial residues.

In his second PhD year, Lorenzo Favaro spent a long research period at the Microbiology Department of the Stellenbosch University under the supervision of Prof. van Zyl.

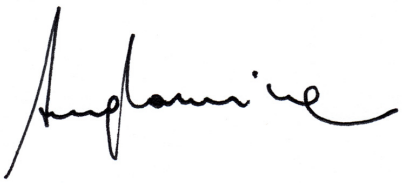
During his activity Lorenzo Favaro participated, and presented his results, to national and international meetings related to the research Projects his research work is part of.

Dr. Lorenzo Favaro enthusiastically went through his research activity showing in the meantime personal initiative and originality in setting out the experiments, a fine critical approach and the ability to gather the results obtained.

The teaching body of the PhD School in Crop Science (Agrobiotechnology) express a broad and unanimous appreciation on the activities performed by Dr. Lorenzo Favaro and is glad to admit him to defend his PhD thesis.

The Coordinator

Prof. Angelo Ramina



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Other Activities and publications

SEMINARS

- Prof. Francesco Canganella, *Dagli abissi allo spazio: un viaggio microbiologico a costo zero...*, 14 March 2007, Legnaro (PD).
- Prof. Borrini, *La protezione brevettuale delle nuove varietà vegetali*, 18 April 2007, Legnaro (PD).
- Prof. Michael Costello, *Plant-Insect interaction* (4 seminars), April 2007, Legnaro (PD).
- Bioenergie: "Filiera del biodiesel"; "Filiera dell'olio grezzo"; "Legno ed energia, quadro normativo", May 2007, Legnaro (PD).
- Prof. Lucia Colombo, *Controllo molecolare dello sviluppo dell'ovulo di Arabidopsis*, 30 May 2007, Legnaro (PD).
- Dr. Steve Van Sluyer, *Botrytis interaction with grape pathogenesis-related proteins*, 15 October 2007, Legnaro (PD).
- Dr Elena Barbieri, *Tartufo bianco pregiato: aspetti biologici e merceologici verso l'istituzione di un marchio di identità regionale*, 12 February 2008, Legnaro (PD).
- Prof. Steven Taff, *What are the economics underlying the U.S. enthusiasm for cellulosic ethanol?*, 8 April 2008, Legnaro (PD).
- Prof. Steven Taff, *Farm-level economics of biofuels production systems: an analysis for the White Earth Indian Reservation*, 9 April 2008, Legnaro (PD).
- Dr. Riaan den Haan, *You'll eat what I'll tell you to eat*, 13 June 2008, Stellenbosch (South Africa).
- Prof. Barbel Hahn Hagerdal, *Mixed sugar utilisation by recombinant Saccharomyces cerevisiae*, 24 July 2008, Stellenbosch (South Africa).
- Prof. Willem van Zyl, *Biomass to biofuels: is it food versus fuel or could it be food and fuel?*, 13 August 2008, Stellenbosch (South Africa).

- Dr Shaunita Rose, *An alternative to pushing*, 03 September 2008, Stellenbosch (South Africa).
- Dr Jim Flat, *Challenges in developing cellulosic biofuels through bioconversion processes*, 06 October 2008, Stellenbosch (South Africa)
- Dr Eki Ado, *Characterization of genus Fructobacillus*, 15 October 2008, Stellenbosch (South Africa).
- Dr Daniël la Grange, *Fuel for the future*, 29 October 2008, Stellenbosch (South Africa).
- Prof. W.H. (Emile) van Zyl, *From Biomass to Sustainable Biofuels: Construction of cellulolytic Saccharomyces cerevisiae strains for consolidated bioprocessing*, 20 May 2009, Legnaro (PD).
- Prof. Nicholas M. Dickinson, *Using plants to clean-up contamination (a broad perspective of phytoremediation, based on studies across the world)*, 8-9 June 2009, Legnaro (PD).
- Prof. Svetoslav D. Todorov, *Probiotic: the two faces of the story*, 23 June 2009, Legnaro (PD).
- Prof. Stig Larsson, *Future Forests - a Swedish research initiative to meet the uncertainties of tomorrow's forests and forestry*, 27 October 2009, Legnaro (PD).
- Accademia dei Georgofili - Sezione Nord Est, *Le bioenergie: una strategia non più rinviabile*, 27 October 2009, Legnaro (PD).
- Prof. Alan Bakalinsky, *Mannoproteins are enriched in model wine aged 9 months on the yeast lees*, 29 October 2009, Legnaro (PD).

COURSES

- Scientific writing
- English B2
- REF-WORKS Bibliography
- Statistics courses

CONGRESS

- Participation at the 13th Meeting of Denitrification - COST 856 - 9-11 September 2007, Ljubljana, Slovenia.
- Participation at "*Bio-Ethanol Conference*", 5 February 2008, Tortona.
- Participation at "*Bioenergy World Europe 2008*" 7-10 February 2008, Verona.
- Participation at "Venice 2008, Second International Symposium on Energy from Biomass and Waste". 17-20 November 2008, Venezia.
- Participation at "*Second Bio-Ethanol Conference*", 4 March 2009, Milano.
- Participation at "*FEMS 2009, Microbes and Man-Interdipendence and future challenges*", 3rd Congress of European Microbiologist, 28 June-02 July, 2009, Goteborg, Sweden.
- Participation at The Second National Conference SIMTREA *"The role of Microbiology in Agricultural, Food and Environmental Fields"*, 10-12 June 2009. Sassari.
- Participation at "*SARDINIA 2009, Twelfth International Waste Management and Landfill Symposium*", 5-9 October 2009, S. Margherita di Pula (Cagliari).

AWARD

Best poster award in Agricultural Microbiology for:

Favaro L., Basaglia M., Casella S. (2009), *Oenological yeasts as a source of extracellular enzymes for future applications in bioethanol production*. The Second National Conference SIMTREA *"The role of Microbiology in Agricultural, Food and Environmental Fields"*, 10-12 June 2009. Sassari.

PUBLICATIONS

- Bottegal M., Basaglia M., **Favaro L**., Baldan E., Toffanin A., Shapleigh J.P., Casella S.. (2007). *How nirK of R. sullae HCNT1 is involved in selenite reduction.* In: Denitrification: a challenge for pure and applied science. COST 856. 25-28 March. Aberdeen, UK.
- Bottegal M., Baldan E., **Favaro L.**, Toffanin A., Basaglia M., Casella S. (2007). *Can nitrite reductase of R. sullae work as selenite reductase?*. In: Denitrification - basic research and its application in wetlands. COST 856. 9-11 September. Ljubliana, Slovenia.
- **Favaro L.**, Basaglia M., Casella S. (2008). *Bioethanol from agricultural wastes: an Italian lab-scale case study*. Bioenergy World Europe 2008. 7-10 February. Verona.
- **Favaro L.**, Basaglia M., Casella S. (2008). *Biocarburanti: nel serbatoio i residui dell'agro-industria*. Agroenergia 2008. 6-8 March. Tortona.
- **Favaro L.**, Basaglia M. and Casella S., (2008), *Could Saccharomyces cerevisiae convert agricultural by-products?* 9 pp. Venice 2008, Second International Symposium on Energy from Biomass and Waste. 17-20 November, Venezia.
- **Favaro L.**, Basaglia M., Berta G., Casella S. (2009). *Bioetanolo: criticità e strategie di ricerca.* Agroenergia 2009. 5-7 March. Tortona.
- **Favaro L.**, Todesco S., Basaglia M., Zanirato S., Casella S. (2009), *Direct microbial conversion of cellulose into ethanol.* Convegno Azione Biotech IIbis e III. 21 April. Padova.
- **Favaro L.**, Bezzo F., Bolzonella C., Balestrieri M., Rossi E., De Maria M., Basaglia M., Sartori L., Bertucco A., Casella S., Boatto V. (2009), *Optimization of the energy production processes from bioethanol.* Convegno Azione Biotech IIbis e III. 21 April. Padova.
- **Favaro L.**, Bezzo F., Bolzonella C., Balestrieri M., Basaglia M., Sartori L., Bertucco A., Casella S., Boatto V. (2009), *Optimization of the energy production processes from bioethanol.* II Convegno della Società Italiana di Bioenergie e Agroindustria (SIBA). 4-5 May. Roma.

- **Favaro L.**, Basaglia M., Casella S. (2009), *Oenological yeasts as a source of extracellular enzymes for future applications in bioethanol production*. Annals of Microbiology 59:27. The Second National Conference SIMTREA "The role of Microbiology in Agricultural, Food and Environmental Fields", book of abstracts, 10-12 June 2009. Sassari.
- **Favaro L.**, Basaglia M., Casella S., (2009), *Extracellular enzymes profiling in oenological yeast strain for future biofuel applications*. FEMS 2009, 3rd Congress of European Microbiologist, Microbes and Man-Interdipendence and future challenges, 28 June - 02 July, 2009. Goteborg, Sweden.
- **Favaro L.**, Basaglia M., Casella S., (2009), *Biotechnological approach to bioethanol: substrates, microbes and processes.* in Biofuels, Zeroemission 2009. 30 September - 02 October. Roma.
- Alibardi L., **Favaro L.**, Lavagnolo M.C., Basaglia M., Casella S., Cossu R., (2009), *Microbiological analyses in batch test for hydrogen production*. 11 pp. SARDINIA 2009, Twelfth International Waste Management and Landfill Symposium 5-9 October 2009, S. Margherita di Pula (Cagliari) (Extended proceedings: In Press).
- **Favaro L**., Basaglia M., Saayman M., Rose S., van Zyl W.H. and Casella S., *Engineering amylolytic yeasts for industrial bioethanol production.* Chemical Engineering Transactions, (In Press)