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Selection and genetic improvement of yeasts for the conversion of lignocellulose into 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.

Alberto Trento Padova, 31/01/2013

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RIASSUNTO.

Il bioetanolo di origine lignocellulosica rappresenta una delle alternative più promettenti tra i biocarburanti. Dal punto di vista industriale, la produzione di bioetanolo da biomassa vegetale non è ancora sostenibile. Una delle strategie più interessanti proposte è la costruzione di un microganismo CBP (Consolidated BioProcessing) capace di idrolizzare i polimeri complessi della biomassa cellulosica e di convertirli efficacemente in etanolo.

In questa prospettiva, questo lavoro di tesi si è focalizzato sullo sviluppo di un microbo CBP di tipo industriale per la conversione di cellobiosio in alcol etilico. A tal scopo, è stato necessario mettere a punto un nuovo metodo per la selezione di un ceppo di lievito idoneo alla produzione di bioetanolo su scala industriale caratterizzato da elevate performance fermentative e da una notevole capacità di tollerare gli inibitori normalmente presenti negli idrolizzati lignocellulosici. La selezione di tale microrganismo è partita da una collezione di ceppi di lievito di origine enologica. I ceppi enologici saggiati, pur dimostrando elevate capacità fermentative, non si sono purtroppo rivelati tolleranti nei confronti di inibitori quali furfurale, acido acetico, acido formico ed acido lattico.

È stato quindi necessario eseguire un programma di isolamento mirato ad ottenere ceppi di lievito altamente fermentanti e capaci di tollerare elevate concentrazioni di inibitori. L'isolamento, eseguito in condizioni selettive per la presenza di un cocktail di inibitori, ha consentito di ottenere una ampia ceppoteca di lieviti con caratteristiche promettenti per la loro futura applicazione nel campo del bioetanolo di seconda generazione. Tra di essi, alcuni lieviti *S. cerevisiae* si sono distinti per vigore fermentativo ad elevata temperatura e per una consistente tolleranza agli inibitori. In particolare, il ceppo *S. cerevisiae* T2 è stato selezionato come host strain per lo sviluppo di un ceppo ricombinante capace di secernere la betaglucosidasi BglI di *Saccharomycopsis fibuligera*, specie di lievito tra le più efficienti per l'idrolisi del cellobiosio. Per la prima volta in questo lavoro di tesi è stato descritto un ceppo di lievito industriale betaglucosidasico. In ogni caso, l'attività idrolitica del ceppo ricombinante dovrà essere necessariamente incrementata al fine di ottenere un efficiente microrganismo CBP cellulosolitico.

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

ABSTRACT.

Bioethanol produced from lignocellulosic biomass represents a promising alternative among biofuels. To date a cost-effective method for the industrial production of bioethanol from vegetal biomass has not been developed. One of the most attractive strategies is the construction of a CBP (Consolidated BioProcessing) microbe able both to hydrolyze the complex polymers of lignocellulosic biomass and to convert these into ethanol.

In this context, the present study focused on the development of an industrial CBP microbe for the conversion of cellobiose into ethanol. To this purpose, it was necessary to define a new screening method for the selection of a yeast strain, suitable for the industrial bioethanol production having high fermentative abilities and considerable tolerance to inhibitors commonly present in lignocellulosic hydrolysates. The selection started from a collection of oenological yeasts. These strains, although showing interesting fermentative abilities, did not exhibit a good tolerance to inhibitors such as furfural, acetic acid, formic acid and lactic acid.

Therefore, a new isolation programme was necessarily conducted in order to select efficient fermenting yeast strains able to tolerate high concentrations of inhibitory compounds. The isolation procedure, conducted in the presence of an inhibitors cocktail, allowed to obtain a wide collection of yeasts with interesting features for their future applications in the field of second generation bioethanol. Among them, few *S. cerevisiae* yeasts exhibited remarkable fermenting vigour at high temperature and promising inhibitors tolerance. In particular, *S. cerevisiae* T2 was selected as host for the development of a recombinant strain able to produce the BglI β-glucosidase of *Saccharomycopsis fibuligera*, one of the most efficient cellobiose hydrolyzing yeast species. For the first time, in this study, an industrial yeast strain secreting β-glucosidase BglI was described. However, the hydrolytic activity of the recombinant strain must be necessarily increased in order to produce an efficient cellulolytic CBP microbe.

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 lignocellulosic biomass to ethanol.

AIM OF THE WORK.

The aim of this work was to develop an industrial *S. cerevisiae* yeast able to convert cellobiose into bioethanol. Such microorganism should possess great inhibitor tolerance, high-level production of hydrolytic enzymes, efficient utilization of glucose and proper ethanol production performances.

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

1. the selection of robust yeast strains, previously isolated and/or newly isolates, having both excellent fermenting abilities and inhibitor tolerance.
2. the engineering of the selected yeasts for the secretion of the β-glucosidase BglI of *S. fibuligera*.

1. INTRODUCTION

1.1 Bioethanol.

In recent years, increasing attention has been devoted to the conversion of biomass into bioethanol, considered the cleanest liquid fuel, as alternative to fossil fuels. Bioethanol is a liquid fuel which can be produced from several different biomass feedstocks and conversion technologies. It is an attractive alternative fuel because it is a renewable bio-based resource and it is oxygenated thereby provides the potential to reduce particulate emissions in compression–ignition engines (Hansen et al., 2005).

Moreover it can be blended with gasoline or used as neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization (Hahn-Hägerdal et al., 2006). In 2006, global production of bioethanol reached 13.5 billion gallons, up from 12.1 billion gallons in 2005. Bioethanol currently accounts for more than 94% of global biofuel production, with the majority coming from sugar cane. Brazil and the United States are the world leaders, which exploit sugar cane and corn, respectively, and they together account for about 70% of the world bioethanol production. The top ten bioethanol producers are presented in Table 1.1.

Country	2011	2010	2009
USA	13,900	13,231	10,938
Brazil	5,573.24	6,921.54	6,577.89
European Union	1,199.31	1,176.88	1,039.52
China	554.76	541.55	541.55
Thailand			435.20
Canada	462.3	356.63	290.59
India			91.67
Colombia			83.21
Australia	87.2	66.04	56.80
Others			247.27

Table 1.1. Annual fuel ethanol production by country (2009-2011). Top 10 countries/regional blocks. (Millions of U.S. liquid gallons per year).

Nearly, all bioethanol fuel is produced by fermentation of corn glucose in the United States or sucrose in Brazil, but any country with a significant agronomic-based economy can use current technology for bioethanol fermentation. In Europe, the feedstocks used for bioethanol are predominately wheat, sugar beet and waste from the wine industry (Balat et al., 2008).

Biological feedstocks that contain appreciable amounts of sugar can be fermented to produce bioethanol to be used in gasoline engines. Feedstocks can be conveniently classified into three types: sucrose-containing feedstocks (e.g. sugar beet, sweet sorghum and sugar cane), starchy materials (e.g. wheat, corn, and barley), and lignocellulosic biomass (e.g. wood, straw and grasses). Different feedstocks that can be utilized for bioethanol production and their comparative production potential are given in Table 1.2 (Kumar et al., 2009).

	Bioethanol production potential (L/ton)
Sugarcane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other lignocellulosic biomass	280

Table 1.2. Different feedstocks for bioethanol production and their comparative production potential.

Biomass resources for bioethanol are essentially comprised of sugarcane and sugar beet. Two-third of world sugar production is from sugarcane and one-third is from sugar beet. These two are produced in geographically distinct regions. Sugarcane is grown in tropical and subtropical countries, while sugar beet is only grown in temperate-climate countries. Since bioethanol trade is mainly from the South, feedstocks may eventually impact cane sugar trade. Brazil is the largest single producer of sugar cane with about 27% of global production.

In European countries, beet molasses are the most utilized sucrose-containing feedstock (Cardona et al., 2007). Sugar beet crops are grown in most of the EU-25 countries, and yield substantially more bioethanol per hectare than wheat. The advantages with sugar beet are a lower cycle of crop production, higher yield, and high tolerance of a wide range of climatic variations, low water and fertilizer requirement.

Sweet sorghum (*Sorghum bicolor* L.) is one of the most drought resistant agricultural crops as it has the capability to remain dormant during the driest periods. Of the many crops being investigated for energy and industry, sweet sorghum is one of the most promising candidates, particularly for bioethanol production principally in developing countries.

Another type of feedstock, which can be used for bioethanol production, is starch-based materials. Starch is a biopolymer and defined as a homopolymer consisting only one monomer, D-glucose. To produce bioethanol from starch it is necessary to break down the chains of this carbohydrate for obtaining glucose syrup, which can be converted into bioethanol by yeasts.

Starch can also be converted to fermentable sugar by a method called ‘‘the hydrolysis technique’’. Hydrolysis is a reaction of starch with water, which is normally used to break down the starch into fermentable sugar. There are two types of hydrolysis: enzymatic hydrolysis and acid hydrolysis. The hydrolysis of starch by amylases at relatively high temperatures is a process known industrially as liquefaction. The factors that affect the enzymatic hydrolysis of starch include substrates, enzyme activity, and reaction conditions (temperature, pH, as well as other parameters) (Neves 2006). The starch-based bioethanol industry has been commercially viable for about 30 years; in that time, tremendous improvements have been made in enzyme efficiency, reducing process costs and time, and increasing bioethanol yields. This type of feedstock is the most utilized for bioethanol production in North America and Europe. Corn and wheat are mainly employed with these purposes. The United States has a large corn-based bioethanol industry with a capacity of over 15 billion L per year; production capacity is anticipated to continue rising to about 28 billion L per year by 2012 (Mabee et al., 2006). For example other starchy materials, by-products of industrial processes, such as wheat bran and potatos peels are interesting low-cost substrates for ethanol production (Favaro et al., 2012b, 2012c and 2013a).

To make bioethanol a sustainable commodity, not in competition with food sources, it is necessary to move away from sugar cane or corn (first generation bioethanol) toward lignocellulosic biomasses such as corn stover or other agricultural wastes, wood by-products, or dedicated fuel crops such as *Miscanthus* or switchgrass (second generation bioethanol). However, to achieve this result, there are technical challenges that must be overcome.

1.2 Bioethanol from lignocellulosic biomass.

Lignocellulosic feedstocks are renewable, largely unused, and abundantly available source of raw materials for the production of fuel ethanol. Lignocellulosic substrates can be obtained at low cost from a variety of resources, e.g. forest residues, municipal solid waste, waste paper, and crop residue resources. This biomass contains sugars polymerized in form of cellulose and hemicellulose, which can be liberated by hydrolysis and subsequently fermented to ethanol by microorganisms (Palmqvist and Hahn-Hägerdal, 2000).

Lignocellulosic biomass could produce up to 442 billion L per year of bioethanol (Bohlmann et al., 2006). Rice straw is one of the abundant lignocellulosic waste materials in the world. It is annually produced about 731 million tons and can potentially produce 205 billion liters bioethanol per year, which is the largest amount from a single biomass feedstock (Karimi et al., 2006).

Lignocellulosic biomass predominantly contains a mixture of carbohydrate polymers (cellulose and hemicellulose), lignin, extractives and ashes. Cellulose fibers provide wood's strength and comprise 40-50 wt% of dry wood. Cellulose is a homopolysaccharide composed of β-D-glucopyranose units linked together by (1-4)-glycosidic bonds. The cellulose molecules are linear; glucose anhydride, which is formed via the removal of water from each glucose, is polymerized into long cellulose chains that contain 5,000-10,000 glucose units. The basic repeating unit of the cellulose polymer consists of two glucose anhydride units, called a cellobiose units (Mohan et al., 2006). The length of cellulose polymer depends on the type of plants of origin.

A second major wood chemical constituent is hemicellulose. Hemicelluloses belong to a group of heterogeneous polysaccharides and its amount is usually between 11% and 37% of the lignocellulosic dry weight. Hemicellulose is a mixture of various polymerized monosaccharides such as glucose, mannose, galactose, xylose, arabinose, 4-O-methyl glucuronic acid and galacturonic acid residues. Xylose is the predominant pentose sugar derived from the hemicellulose of most hardwood feedstocks, but arabinose can constitute a significant amount of the pentose sugars derived from various agricultural residues and other herbaceous crops, such as switchgrass, which are being considered for use as dedicated energy crops.

Lignin is a very complex molecule constructed of phenylpropane units linked in a three-dimensional structure. Lignins are often bound to adjacent cellulose fibers to form lignocellulosic complexes that are extremely resistant to chemical and enzymatic degradation (Palmqvist and Hahn-Hägerdal 2000, Taherzadeh et al., 1999). The lignin contents on a dry basis in both softwoods and hardwoods generally range from 20% to 40% by weight and from 10% to 40% by weight in

various herbaceous species, such as bagasse, corncobs, peanut shells, rice hulls and straws (Yaman, 2004).

Many lignocellulosic substrates have been tested for bioethanol production. In general, lignocellulosic materials for bioethanol production can be divided into six mains groups: crop residues (cane bagasse, corn stover, corn fiber, wheat straw and bran, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olives tone and pulp), hardwood (aspen and poplar), softwood (pine, spruce), cellulose wastes (newsprint, waste office paper, recycled paper sludge), herbaceous biomass (switchgrass, reed canary grass, coastal Bermudagrass, thimoty grass), and Municipal Solid Wastes (MSW). The composition of some of these materials is reported in Table 1.3 (Favaro, 2010).

Feedstock	**Glucan (cellulose)**	**Xylan (hemicellulose)**	**Lignin**
Corn stover	37.5	22.4	17.6
Corn fiber	14.28	16.8	8.4
Pine wood	46.4	8.8	29.4
Poplar	49.9	17.4	18.1
Wheat straw	38.2	21.2	23.4
Switch grass	31.0	20.4	17.6
Office paper	68.6	12.4	13.3

Table 1.3: Percent dry weight of lignocellulosic feedstocks (modified from Mosier, 2005)

There are several options for a lignocellulose-to-bioethanol process, but regardless of which is chosen, the following features must be assessed in comparison with established sugar- or starch-based bioethanol production (Hahn-Hägerdal et al., 2000):

- Efficient de-polymerization of cellulose and hemicelluloses to soluble sugars.
- Efficient fermentation of a mixed-sugar hydrolysate containing six-carbon (hexoses) and five-carbon (pentoses) sugars as well as fermentation inhibitory compounds.
- Advanced process integration to minimize process energy demand.
- Lower lignin content of feedstock decreases of the cost of bioethanol.

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

1.2.1 Pretreatment of lignocellulosic biomass

Processing of lignocellulosics to ethanol consists of four major unit operations: pretreatment, hydrolysis, fermentation, and product separation/purification. Pretreatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure so that hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose (Fig. 1.1).

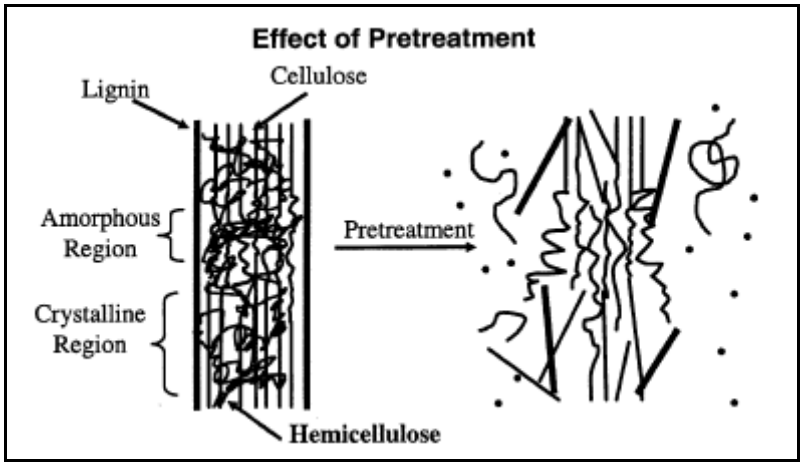


Fig. 1.1. Schematic representation of goals of pretreatment on lignocellulosic materials (Hsu, 1980)

A successful pretreatment must meet the following requirements (Silverstein et al., 2004): (i) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis, (ii) avoid the degradation or loss of carbohydrate, (iii) avoid the formation of by-products inhibitory to subsequent hydrolysis and fermentation processes, and (iv) be cost effective. These properties, along with others including low pretreatment catalyst cost or inexpensive catalyst recycle, and generation of higher-value lignin co-product form a basis of comparison for various pretreatment options. Pretreatment results must be balanced against their impact on the cost of the downstream

processing steps and the trade-off between operating costs, capital costs, and biomass costs (Lynd et al., 1996).

Pretreatment categories.

Pretreatment methods are either physical or chemical; some methods incorporate both effects (Hsu et al. 1996). Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size), steam explosion, and hydrothermolysis. Comminution, including dry, wet, and vibratory ball milling (Millett et al., 1979; Rivers and Emert, 1987; Sidiras and Koukios, 1989), and compression milling (Tassinari et al., 1980, 1982) is sometimes needed to make material handling easier through subsequent processing steps.

Acids or bases promote biomass hydrolysis and improve the yield of glucose recovery from cellulose by removing hemicelluloses or lignin during pretreatment. The most commonly used acid and base are H_2SO_4 and NaOH, respectively. Cellulose solvents are another type of chemical additive. Solvents that dissolve cellulose in bagasse, cornstalks, tall fescue, and orchard grass resulted in 90% conversion of cellulose to glucose (Hamilton et al., 1984) and showed enzyme hydrolysis could be greatly enhanced when the biomass structure is disrupted before hydrolysis. Alkaline H_2O_2, ozone, organosolv (uses Lewis acids, $FeCl_3$, $(Al)_2SO_4$ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis. Concentrated mineral acids (H_2SO_4, HCl), ammonia-based solvents (NH_3, hydrazine), aprotic solvents (DMSO), metal complexes (ferric sodium tartrate, cadoxen, and cuoxan), and wet oxidation also reduces cellulose crystallinity and disrupt the association of lignin with cellulose, as well as dissolve hemicelluloses.

The effects of various pretreatment methods are summarized in Table 1.4. Steam explosion, liquid hot water, dilute acid, lime, and ammonia pretreatments, have potential as cost-effective pretreatments and are discussed below.

Tab 1.4:
Effect of various pretreatment methods on the chemical composition and chemical/physical structure of lignocellulosic biomass

	Increases accesible surface area	**Decrystalizes cellulose**	**Removes hemicellulose**	**Removes lignin**	**Alters lignin structure**
Uncatalyzed steam explosion	■		■		□
Liquid hot water	■	ND	■		□
pH controlled hot water	■	ND	■		ND
Flow-through liquid hot water	■	ND	■	□	□
Dilute acid	■		■		■
Flow-through acid	■		■	□	■
AFEX	■	■	□	■	■
ARP	■	■	□	■	■
Lime	■	ND	□	■	■

■: Major effect.
□: Minor effect.
ND: Not determined.

Uncatalyzed steam explosion.

Uncatalyzed steam explosion refers to a pretreatment technique in which lignocellulosic biomass is rapidly heated by high-pressure steam without addition of any chemicals. The biomass/steam mixture is held for a period of time to promote hemicellulose hydrolysis, and terminated by an explosive decompression (Brownell and Saddler, 1984).

Hemicellulose is thought to be hydrolyzed by the acetic and other acids released during steam explosion pretreatment. Water, itself, also acts as an acid at high temperatures (Weil et al., 1997). Steam provides an effective vehicle to rapidly heat cellulosics to the target temperature without excessive dilution of the resulting sugars. Rapid pressure release rapidly reduces the temperature and quenches the reaction at the end of the pretreatment. The rapid thermal expansion used to terminate the reaction opens up the particulate structure of the biomass but enhancement of digestibility of the cellulose in the pretreated solid is only weakly correlated with this physical effect (Biermann et al., 1984).

The major chemical and physical changes to lignocellulosic biomass by steam explosion are often attributed to the removal of hemicellulose. This improves the accessibility of the enzymes to the cellulose fibrils.

Liquid hot water pretreatment.

Flow-through processes pass water maintained in the liquid state at elevated temperatures through cellulosics. This type of pretreatment has been termed hydrothermolysis (Bobleter et al., 1981), aqueous or steam/aqueous fractionation (Bouchard et al., 1991), uncatalyzed solvolysis (Mok and Antal, 1992, 1994), and aquasolv (Allen et al., 1996).

Solvolysis by hot compressed liquid water contacts water with biomass for up to 15 min at temperatures of 200–230 °C. Between 40% and 60% of the total biomass is dissolved in the process, with 4–22% of the cellulose, 35–60% of the lignin and all of the hemicelluloses being removed. Over 90% of the hemicellulose is recovered as monomeric sugars when acid was used to hydrolyze the resulting liquid. The variability in pretreatment results was related to the biomass type with high lignin solubilization impeding recovery of hemicellulose sugars.

There are three types of liquid hot water reactor configurations: Co-current, countercurrent, and flow through (Fig. 1.2).

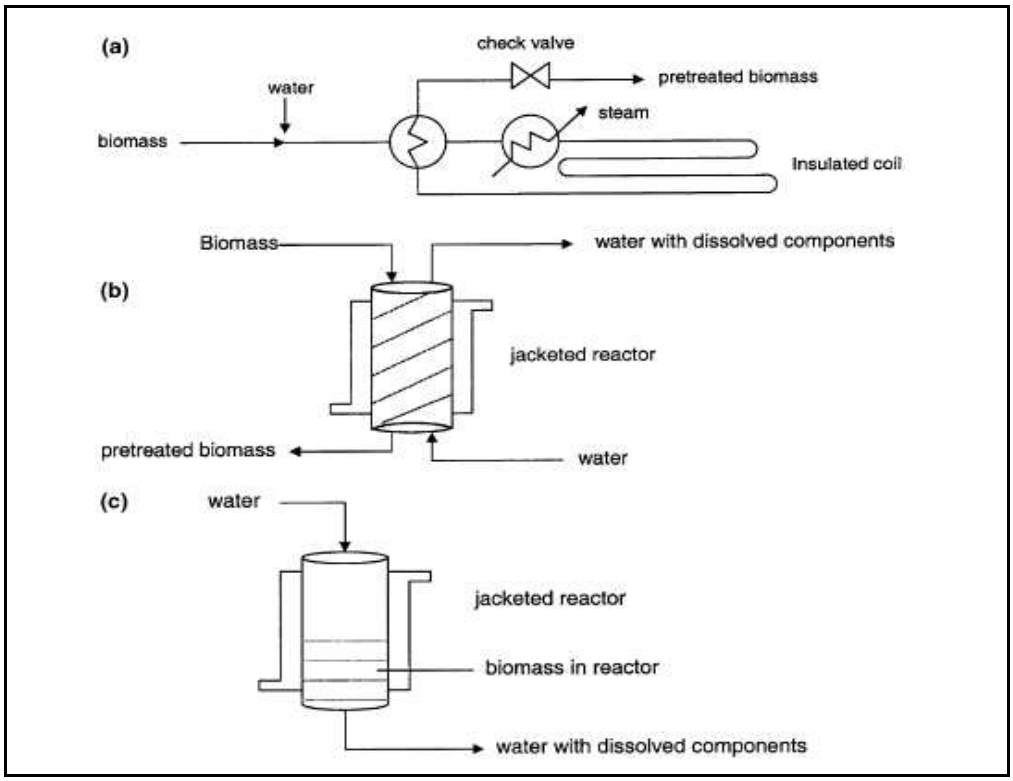


Fig 1.2. Schematic illustration of co-current, counter-current, and flow-through pretreatment methods: (a) Co-current liquid hot water pretreatment, (b) counter-current reactor, (c) flow-through reactor

In the co-current pretreatment, liquid slurry of biomass (16% undissolved solid) passes through heat exchangers, is heated to the desired temperature (140-180 °C) and then held at temperature for

15–20 min as the slurry passes through an insulated plug-flow, snake-coil. The slurry is cooled and heat recovered by countercurrent heat exchange with the incoming slurry. The resulting pretreated fiber is devoid of starch, and the cellulose is completely digestible.

In a flow-through reactor, hot water (180-220 °C and about 350-400 psi pressure) passed over a stationary bed of lignocelluloses hydrolyzes and dissolves lignocellulose components and carries them out of the reactor. Flow-through technologies achieve overall sugar yields of up to 96% but suffer from low concentration of sugars (of about 0.6–5.8 g/L) from hemicellulose. The solids that are left behind have enhanced digestibility and a significant portion of the lignin is also removed.

In countercurrent pretreatment the biomass slurry is passed in one direction while water is passed in another in a jacketed pretreatment reactor (Fig.1.2b). Temperatures, back pressures and residence times are similar to the flow-through technology.

Liquid hot water pretreatments are both helped and hindered by the cleavage of O-acetyl and uronic acid substitutions from hemicellulose to generate acetic and other organic acids. The release of these acids helps to catalyze formation and removal of oligosaccharides.

Acid pretreatment.

Acid pretreatment has received considerable research attention over the years. Dilute sulfuric acid has been added to cellulosic materials for some years to commercially manufacture furfural (Zeitsch, 2000).

In this method, the acid is mixed or contacted with the biomass and the mixture is held at temperatures of 160–220 °C for periods ranging from minutes to seconds. Hemicellulose is removed when sulfuric acid is added and this enhances digestibility of cellulose in the residual solids (Grous et al., 1985). The most widely used and tested approaches are based on dilute sulfuric acid (Kim et al., 2000). However, nitric acid (Brink, 1993, 1994), hydrochloric acid (Goldstein and Easter, 1992), and phosphoric acid (Israilides et al., 1978) have also been tested.

The mixture of acid and biomass can be heated indirectly through the vessel walls or by direct steam injection, the latter being operated in virtually the same manner as for uncatalyzed steam explosion. The acid is added to the liquid percolated through a bed, sprayed onto the residue after which the residue is heated, or agitated with the biomass in a reactor.

Dilute sulfuric acid has some important limitations including corrosion that mandates expensive materials of construction. The acid must be neutralized before the sugars proceed to fermentation. Formation of degradation products and release of natural biomass fermentation inhibitors are other

characteristics of acid pretreatment. Disposal of neutralization salts (Mes-Hartree and Saddler, 1983), as well as a 7-day reaction time with cellulase translate into added cost (Wooley et al., 1999) Nitric acid reduces containment costs relative to sulfuric, but the higher acid cost counterbalances this benefit.

Use of acid to remove hemicellulose has been tried on a wide range of feedstocks ranging from hardwoods to grasses and agricultural residues (Torget et al., 1990, 1991, 1992). Most species performed well, and corn cobs and stover were found to be particularly well suited to pretreatment by hemicellulose hydrolysis. Pretreatment of aspen wood and wheat straw were studied at higher solids concentrations with temperatures of 140 and 160 °C. The use of acid to hydrolyze oligomers released during uncatalyzed hydrolysis results in close to complete hydrolysis to monosaccharides but also the formation of aldehydes (Shevchenko et al., 2000).

There are primarily two types of dilute acid pretreatment processes: low solids loading (5-10% [w/w]), high-temperature (T>160 °C), continuous-flow processes and high solids loading (10-40% [w/w], lower temperature (T<160 °C), batch processes. In general, higher pretreatment temperatures and shorter reactor residence times result in higher soluble xylose recovery yields and enzymatic cellulose digestibility. Higher-temperature dilute acid pre-treatment has been shown to increase cellulose digestibility of pretreated residues. Depending on the substrate and the conditions used, between 80 and 95% of the hemicellulosic sugars can be recovered by dilute acid pre-treatment from the lignocellulosic feedstock (Jeffries et al. 2000).

Flow-through acid pretreatment.

Addition of very dilute sulfuric acid (about 0.07% versus the 0.7–3.0% typical for the dilute acid technology described) in a flow-through reactor configuration is effective at acid levels lower than 0.1%. Lower temperatures were applied to hydrolyze the more reactive hemicellulose in yellow poplar in a countercurrent flowthrough pretreatment. Fresh acid/water stream is first passed through the higher temperature zone and then the lower temperature region to reduce the exposure of sugars to severe conditions and improve yields.

Despite achieving excellent hemicellulose sugar yields and highly digestible cellulose with low acid loadings, equipment configurations and the high ratio of water to solids employed in flow-through systems require significant energy for pretreatment and product recovery. Practical systems that lend themselves to commercial applications have not been demonstrated.

Alkaline pretreatment.

Alkali pre-treatment processes utilize lower temperatures and pressures compared to other pre-treatment technologies. Unlike acid catalyzed pre-treatments, a limitation occurs because some of the alkali is converted to irrecoverable salts or incorporated as salts into the biomass by the pre-treatment reactions. The characteristic of alkaline pretreatment is that it can remove the lignin without having big effects on other components. NaOH treatment causes lignocellulosic biomass to swell, leading to an increase in the internal surface area, a decrease in the degree of crystallinity, and disruption of the lignin structure.

Alkali pretreatment reduces the lignin and hemicelluloses content in biomass, increases the surface area, allowing penetration of water molecules to the inner layers, and breaks the bonds between hemicellulose and lignin carbohydrate. Dilute NaOH is usually used for alkali pretreatment (Lee, 2005). Considering economic and environmental aspects, dilute NaOH treatment would be much more suitable than the concentrated NaOH pretreatment. Combination of dilute NaOH treatment and other treatments seems more efficient. For example, corn stover pretreatment of dilute NaOH (2%) combined with irradiation (500 kGy) caused the glucose yield to increase from just 20% for NaOH pre-treatment to 43%.

Lime pretreatment.

Recently, it was discovered that lime allow to have a good performance and great sugars recovery from lignocellulosic biomass. Lime (calcium hydroxide) has been used to pretreat wheat straw (Chang et al., 1998), poplar wood (Chang et al., 2001), switchgrass (Chang et al., 1997), and corn stover (Karr and Holtzapple, 1998, 2000). Playne treated sugarcane bagasse with lime at ambient conditions for up to 192 h to improve the enzyme digestibility of the cellulose from 20% before pretreatment to 72% after pretreatment. Higher temperatures and shorter reactions times were also shown to effectively pretreat lignocellulose with lime. Chang et al. (1998), obtained similar digestibility results by pretreating bagasse with lime at 120 °C for 1 h.

Lime has the additional benefits of low reagent cost and safety and being recoverable from water as insoluble calcium carbonate by reaction with carbon dioxide. The addition of air/oxygen to the reaction mixture greatly improves the delignification of the biomass, especially highly lignified materials such as poplar (Chang and Holtzapple, 2000).

The process of lime pretreatment involves slurrying the lime with water, spraying it onto the biomass material, and storing the material in a pile for a period of hours to weeks. The particle size of the biomass is typically 10 mm or less. Elevated temperatures reduce contact time.

In general, the major effect of the alkaline pretreatment is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, alkali pretreatments remove acetyl and the various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface.

Ammonia pretreatment.

Ammonia fiber/freeze explosion (AFEX) is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. Ammonia fiber explosion pretreatment yields optimal hydrolysis rates for pretreated lignocellulosics with close to theoretical yields at low enzyme loadings (Foster et al., 2001). Herbaceous and agricultural residues are well suited for AFEX. However, this method works only moderately well on hardwoods, and is not attractive for softwoods.

Pretreatment with aqueous ammonia in a flowthrough mode involves putting ammonia solution (5-15%) through a column reactor packed with biomass at elevated temperatures (160-180 °C) and a fluid velocity of 1 mL/cm^{2}min with residence times of 14 min. Under these conditions, aqueous ammonia reacts primarily with lignin (but not cellulose) and causes depolymerization of lignin and cleavage of lignin-carbohydrate linkages. This method is also known as ammonia recycled percolation (ARP) process since ammonia is separated and recycled.

This pretreatment simultaneously reduces lignin contents and removes some hemicelluloses while decrystallizing cellulose. Thus it affects both micro-and macro-accessibility of the cellulases to the cellulose. Modification of the process was attempted to further increase the extent of the delignification and to achieve fractionation of biomass (Kim et al., 2002). Since lignin is one of the key factors affecting the enzymatic hydrolysis (Lee and Yu, 1995), removal of lignin lowers the enzyme requirement.

The cost of ammonia and especially of ammonia recovery drives the cost of this pretreatment. However, biomass pretreatment economics are also strongly influenced by total sugar yields achieved, and by the loss in yield and inhibition of downstream processes caused by sugar

degradation products. The moderate temperatures (<90 °C) and pH values (<12.0) of the AFEX treatment minimize formation of sugar degradation products while giving high yields.

1.2.2 Inhibitors obtained from biomass pretreatment.

The pretreatment of lignocellulosic biomass aims to separate lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of lignocelluloses while minimizing chemical destruction of fermentable sugars required for ethanol production. Pretreatment of lignocellulosic biomass generate a broad range of compounds (Fig. 1.3). 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 d-glucuronic and 4-*O*-methylglucuronic acids are also produced during hydrolysis of hemicellulose. Hydrolysis treatments may result in further degradation of lignin and monomeric sugars to three major groups of compounds that inhibit fermentation: (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.

During dilute acid hydrolysis pretreatment, high temperature and pressure lead the degradation of xylose to furfural (Dunlop, 1948). Similarly, 5-hydroxymethyl furfural (HMF) is formed from hexose degradation (Ulbricht et al., 1984).

Formic acid is formed when furfural and HMF are broken down. Levulinic acid is formed by HMF degradation. Phenolic compounds are generated from partial breakdown of lignin and have also been reported to be formed during carbohydrate degradation (Suortti, 1983).

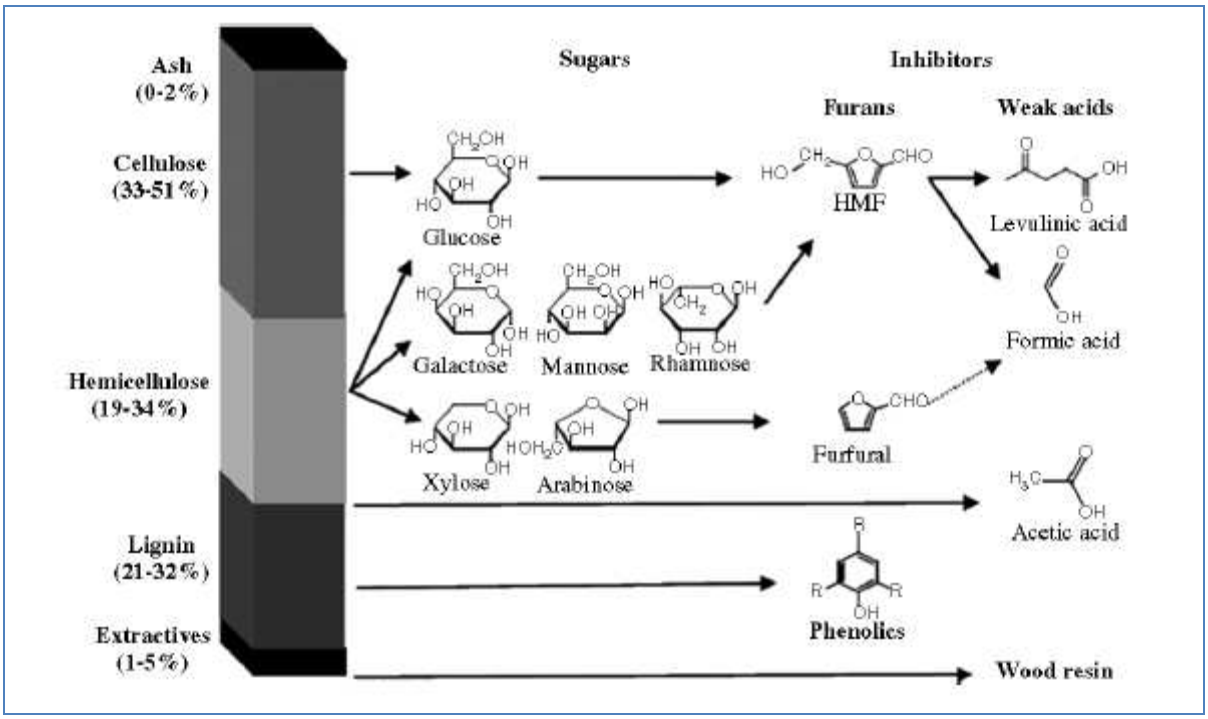
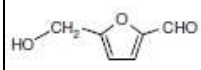
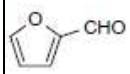
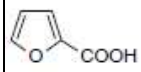
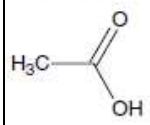
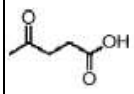
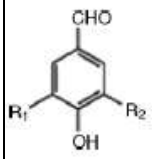
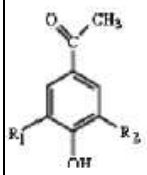
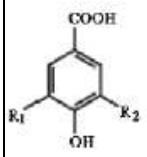
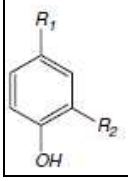


Fig 1.3. Average composition of lignocellulosic biomass and main derived hydrolysis products (Almeida et al., 2007)

Hibbert's ketones have been detected in the hydrolysate of pine (Clark and Mackie, 1984). Vanillic acid and vanillin, formed by the degradation of the guaiacylpropane units of lignin, have been detected in hydrolysates from willow, poplar (Ando et al., 1986), red oak (Tran and Chambers, 1985), and pine. In hardwood hydrolysates, syringaldehyde and syringic acid, formed in the degradation of syringyl propane units, have been reported. Hydroquinone (1,4-di-hydroxybenzene) has been identified in a hydrolysate of spruce and catechol (1,2-di-hydroxybenzene) has been identified in hydrolysates of willow, birch and spruce. 4-Hydroxybenzoic acid constitutes a large fraction of the lignin-derived compounds in hydrolysates from the hardwoods poplar, aspen, and willow. Trace amounts of the extractives caproic acid, caprylic acid, pelargonic acid, and palmitic acid have been reported in dilute-acid hydrolysate of red oak.

The hydrolysis temperature, time and acid concentration influence the generation of fermentation inhibitors. The severity of different pretreatment conditions can be compared by calculating a severity parameter, where the reaction temperature, T (°C), and residence time, t (min), are combined into a single reaction ordinate. The severity factor, logR_0, is defined by $R_0 = te^{(T-100/14.75)}$ (Overend and Chornet, 1987). The influence of hydrolysis pH (reflecting the amount of acid used), is taken into consideration by the combined severity, CS, defined as logR_0 - pH (Chum et al., 1990).

In Table 1.5, the major groups of inhibitory compounds that come from different biomass sources are listed.

Group of compounds		Concentrations (g/L)				
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.			

Tab 1.5. 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 pretreatment employed:
[a] upper values; two-steps 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

Inhibitors: effects and mechanism of action.

The compounds released during pretreatment and hydrolysis has been found to inhibit microorganism growth and ethanol production. The effect of furans, weak acids and phenolic compounds – as well as their synergistic effect – mainly on *S. cerevisiae* fermentation ability are represented in Figure 1.4 and summarized below.

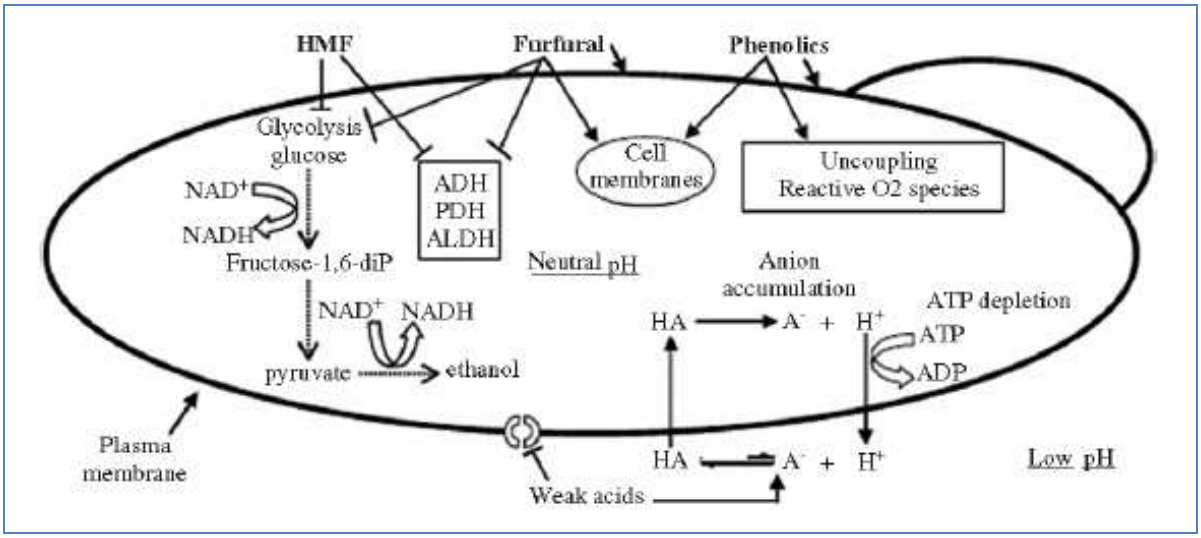


Fig 1.4. Schematic view of known inhibition mechanisms of furans, weak acids and phenolic compounds in *S. cerevisiae*. HMF: inhibition of ADH (alcohol dehydrogenase), (PDH) pyruvate dehydrogenase and ALDH (aldehyde dehydrogenase), inhibition of glycolysis (either enzyme and/or cofactors). Furfural: same as HMF, plus cell membrane damages. Weak acids: ATP depletion, toxic anion accumulation and inhibition of aromatic amino acids uptake. Phenolic compounds: uncoupling, generation of reactive O_2 species and membrane damage (Almeida et al., 2007).

Weak acids.

Acids are classified as either strong or weak, depending on their dissociation constant, Ka, the negative logarithm of which is denoted pKa. This value is the pH value at which the concentrations of the undissociated and dissociated form of the acid are equal, and the buffering capacity of the acid therefore is highest. The concentration of undissociated acid is a function of pH and pKa, and increases with decreasing pH (Henderson - Hasselbach equation).

Dilute acid hydrolysates of spruce have a high buffering capacity up to approximately pH 5.5, the normal fermentation pH, due to partial dissociation of acetic, formic, and levulinic acid. The

concentration of undissociated acids in lignocellulosic hydrolysates is therefore very sensitive to small pH deviations around pH 5.5.

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 (Verduyn et al. 1992). Consequently, less ATP is available for biomass formation.

According to the intracellular anion accumulation theory, the anionic form of the acid is captured inside the cell and the undissociated acid will diffuse into the cell until equilibrium is reached. Weak acids have also been shown to inhibit yeast growth by reducing the uptake of aromatic amino acids from the medium, probably as a consequence of strong inhibition of Tat2p amino acid permease (Bauer et al., 2003).

A clear difference in toxicity between acetic, formic, and levulinic acid at the same concentration of undissociated acid has been reported (Larsson et al., 1998). This may be due to differences in membrane permeability or in toxicity of the anionic form of the acids once they have entered the cell.

S. cerevisiae responds in different ways to weak acids and decreased intracellular pH. Growth in the presence of octanoic acid, sorbic acid, and low intracellular pH (Eraso and Gancedo, 1987) have been shown to activate the plasma membrane ATPase, and increase the proton pumping capacity of the cell. The production of succinic and acetic acid has been reported to decrease during cell growth in the presence of octanoic acid, and decrease the total acid stress experienced by the yeast (Viegas and Sá-Correia, 1995). The cell volume has also been shown to decrease with increasing concentration of octanoic acid in the medium, so that the buffering capacity of the cytoplasm increases due to a higher concentration of cellular compounds.

Furfural and HMF.

HMF and furfural decrease the volumetric ethanol yield and productivity, inhibit growth or give rise to a longer lag phase. These effects depend on the furan concentration and on the yeast strain used. Furfural is metabolised by *S. cerevisiae* under aerobic (Taherzadeh et al., 1998), oxygen-limited (Navarro, 1994) and anaerobic conditions (Palmqvist et al., 1999a). During fermentation furfural reduction to furfuryl alcohol occurs with high yields (Villa, 1992).

Inhibition of aerobic growth of *Pichia stipitis* by furfuryl alcohol has been reported (Weigert et al., 1988), whereas only slight inhibition of anaerobic growth of *S. cerevisiae* has been detected. Furfural oxidation to furoic acid by *S. cerevisiae* occurs to some extent, primarily under aerobic conditions.

Furfural has been shown to reduce the specific growth rate (Boyer et al., 1992), the cell-mass yield on ATP, the volumetric (Azhar et al., 1981), and specific ethanol productivities. Growth is more sensitive to furfural than is ethanol production.

NADH-dependent yeast alcohol dehydrogenase (ADH) is believed to be responsible for furfural reduction (Diaz de Villegas et al., 1992). Under anaerobic conditions, glycerol is normally produced to regenerate excess NADH formed in biosynthesis. Glycerol production has been shown to be significantly reduced during furfural reduction, suggesting that furfural reduction regenerates NAD^+.

The reduction of furans by yeast may also result in NAD(P)H depletion, which was suggested by the fact that increased levels of acetaldehyde were excreted when furfural was added to the medium. Furthermore metabolic flux analyses have shown that furfural affects glycolytic and TCA fluxes, which are involved in energy metabolism (Sarvari et al, 2003). In *S. cerevisiae* furfural causes reactive oxygen species to accumulate, vacuole and mitochondrial membranes damage, chromatin and actin damage. Adaptation of *S. cerevisiae* to furfural has been reported in batch (Banerjee et al., 1981a), fed-batch, and continuous culture (Fireoved and Mutharasan, 1986), leading to increased growth and volumetric ethanol productivity. The adaptation might be due to the synthesis of new enzymes or co-enzymes for furfural reduction (Boyer et al., 1992). Supporting this hypothesis, the ADH activity in anaerobic fermentation has been reported to increase by 78% after 48 h fermentation with an initial furfural concentration of 2 g/L (Banerjee et al., 1981b). HMF is also metabolised by *S. cerevisiae.* HMF has been reported to be converted at a lower rate than furfural, which might be due to lower membrane permeability, and cause a longer lag-phase in growth (Larsson et al., 1998). The main conversion product was 5-hydroxymethyl furfuryl alcohol, suggesting similar mechanisms for HMF and furfural inhibition.

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.

Phenolic compounds.

Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates. Phenolic compounds partition into biological membranes and cause loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Heipieper et al., 1994). 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 phenolic compounds are more inhibitory to *S. cerevisiae* than high molecular weight 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. 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 for *S. cerevisiae*.

Inhibition of fermentation has been shown to decrease when phenolic monomers and phenolic acids were specifically removed from a willow hemicelluloses hydrolysate by treatment with the lignin-oxidising enzyme laccase (Jönsson et al., 1998). 4-Hydroxybenzoic acid, vanillin, and catechol were major constituents in the untreated hydrolysate.

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.

1.2.3 Detoxification of lignocellulosic hydrolyzates.

During pretreatment and hydrolysis of lignocellulosic biomass, a great amount of compounds that can 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 are required. Biological, physical, and chemical methods have been employed for detoxification of lignocellulosic hydrolysates (Olsson and Hahn-Hägerdal, 1996).

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 literature (Amartey and Jeffries, 1996; Tran and Chambers, 1986; Yu et al., 1986).

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 280 nm, 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 molecular weight phenolic compounds.

The filamentous soft-rot fungus *Trichoderma reesei* has been reported to degrade inhibitors in a hemicelluloses hydrolysate obtained after steam pretreatment of willow, resulting in around three times increased maximum ethanol productivity and four times increased ethanol yield (Palmqvist et al., 1997). In contrast to the treatment with 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*.

Physical detoxification methods.

The most volatile fraction (10% (v/v)) of a willow hemicellulose hydrolysate obtained by roto-evaporation has been shown to only 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.

In fermentation of an acid hydrolysate of aspen with *P. stipitis* the ethanol yield has been reported to increase from 0 to 13% of that in a reference fermentation containing no inhibitors after roto-evaporation almost to dryness and subsequent resuspension of the residue in fermentation

medium (Wilson et al., 1989). The detoxification was ascribed to a decrease in the concentration of acetic acid, furfural and vanillin by 54, 100 and 29%, respectively, compared with the concentrations in the hydrolysate.

After continuous overnight extraction of a strongly inhibiting spruce hydrolysate with diethyl ether at pH 2, the ethanol yield (0.40 g/g) has been reported to be comparable to the value in a reference fermentation containing glucose and nutrients (Palmqvist and Hahn-Hägerdal, 2000). The ether extract contained acetic, formic, and levulinic acid, furfural, hydroxymethyl 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 *P. 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.

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 adjustment due to the precipitation of `toxic compounds' (van Zyl et al., 1988). The detoxifying effect of overliming is due both to the precipitation of toxic components and to the instability of some inhibitors at high pH. This has been demonstrated by the fact that preadjustment to pH 10 with NaOH of a strongly inhibiting dilute-acid hydrolysate of spruce prior to fermentation resulted in twice as high ethanol yield (and comparable to the yield in a reference fermentation containing glucose and nutrients) as after only adjustment to fermentation pH (5.5) (Palmqvist, 1998). Preadjustment to pH 10 with NaOH and $Ca(OH)_2$ has been reported to decrease the concentration of Hibbert's ketones in a dilute acid hydrolysate of spruce from 203 to 158 (22% decrease) and to 143 mg/L (30% decrease), respectively, and the concentration of both furfural and HMF by 20%.

In recent studies treatment of a dilute-acid hydrolysates of spruce with sodium sulphite (Larsson et al., 1999), or using a large cell inoculums (Palmqvist and Hahn-Hägerdal, 1999) have been shown to decrease the concentrations of furfural and HMF. A combination of sulphite and overliming has been shown to be the most efficient method to detoxify willow hemicellulose hydrolysate prior to fermentation by recombinant *Escherichia coli* (Olsson et al., 1995). Only 24% of the xylose was fermented in 40 h in the untreated hydrolysate, whereas complete depletion of monosaccharides was obtained in the same time after overliming. The effect of the combined treatment was probably due to decreased concentrations of Hibbert's ketones and aldehydes, and the removal of volatile compounds when a heat treatment was employed.

1.2.4 Hydrolysis of cellulose.

As the pre-treatment is finished, the cellulose is prepared for hydrolysis, meaning the cleaving of a molecule by adding a water molecule. This reaction is catalysed by dilute-acid, concentrated acid or enzymes (cellulase) and the latter has many advantages as the very mild conditions (pH 4.8 and temperature 45-50 °C) give high yields and the maintenance costs are low compared to alkaline and acid hydrolysis due to no corrosion problems.

Concentrated acids such H_2SO_4 and HCl have been used to treat cellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. Diluite-acid hydrolysis has been successfully developed for cellulose hydrolysis and high temperature is favorable. Although dilute-acid can significantly improve the cellulose hydrolysis, its cost is usually higher than some physic-chemical pretreatments because the neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. Moreover, diluite-acid hydrolysis provides a low sugar yields.

Another basic method for the hydrolysis of cellulose is enzymatic hydrolysis and this is carried out by cellulase enzymes which are highly specific. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50 °C) and does not have a corrosion problem (Sun, 2002). Enzymatic hydrolysis is attractive because it produces better yields than acid-catalyzed hydrolysis.

Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora,* and *Streptomyces* can produce cellulases. Although many cellulolytic

bacteria, particularly the cellulolytic anaerobs such as *Clostridum thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titres (Duff and Murray, 1996). Because the anaerobs have a very low growth rate and require an aerobic growth conditions, most research for commercial cellulase production has focused on fungi.

Fungi that have been reported to produce celllulases include *Sclerotium rolfsii, P. chrysosporium* and species of *Trichoderma, Aspergiullus, Schizophyllum* and *Penicillum. Trichoderma reseei* 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 polymerazation 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 cellobiose. For this reason, β-glucosidases from other microbial source needs to be added. Factorial optimization 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 take place. The three-dimensional structure of these particles in combination with their size and shape determines whether β-glucosidic linkages are or not are accessible to enzymatic attack (Zhang and Lynd, 2004). This makes cellulose hydrolysis to be slower compared to the enzymatic degradation of other biopolymers.

1.2.5 Fermentations 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.

Separate Hydrolysis and Fermentation (SHF).

Enzymatic hydrolysis performed separately from fermentation step is known as SHF. In the SHF configuration, which is represented in Figure 1.5, the joint liquid flow from both hydrolysis reactors first enters the glucose fermentation reactor. The mixture is then distilled to remove the bioethanol leaving the unconverted xylose behind. In a second reactor, xylose is fermented to bioethanol, and the bioethanol is again distilled (Grethlein and Dill, 1993).

The primary advantage of SHF is that hydrolysis and fermentation occur at optimum conditions; the disadvantage is that cellulolytic enzymes are end-product inhibited so that the rate of hydrolysis is progressively reduced when glucose and cellobiose accumulate.

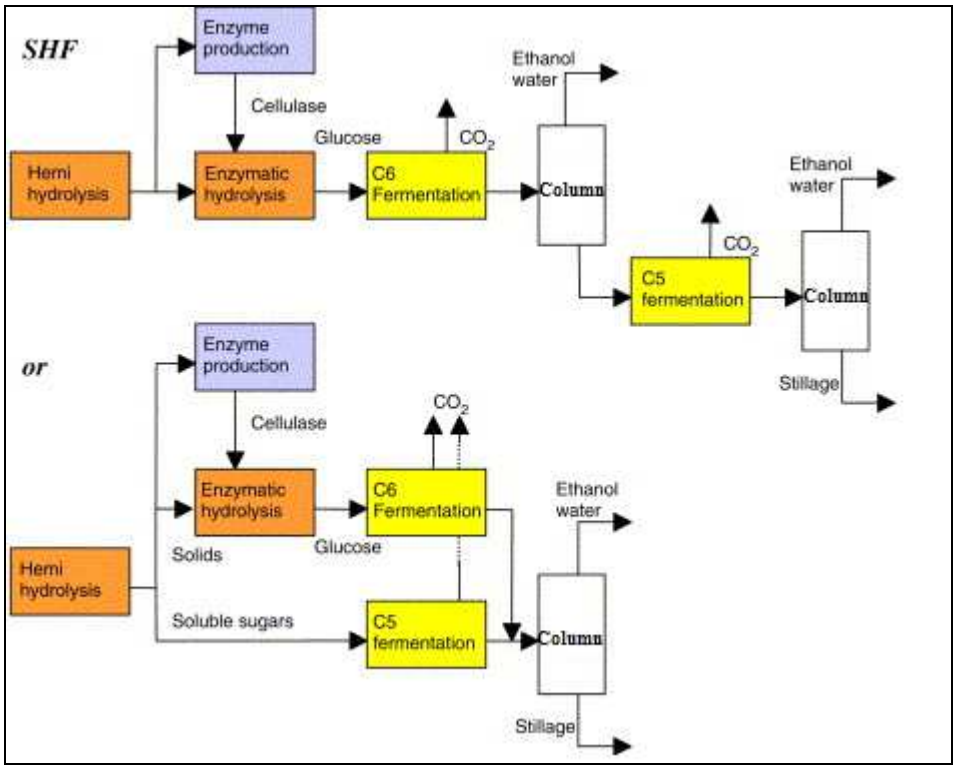


Fig. 1.5: Scheme of Separate Hydrolysis and Fermentation (SHF) process for lignocellulosic ethanol (Hemi: Hemicellulose)

The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load (Hamelinck, 2005). By testing lignocellulosic material from sugar cane leaves, Krishna et al. (2001) have found the best values of all these parameters. Cellulose conversion of about 65-70% was achieved at 50 °C and pH 4.5. Although enzyme doses of 100 FPU/g cellulose caused almost a 100% hydrolysis, this amount of enzymes is not economically justifiable. Hence, 40 FPU/g cellulose dosage was proposed obtaining only 13%

reduction in conversion. Regarding the substrate concentration, solid loads of 10% was defined as the most adequate considering arising mixing difficulties and accumulation of inhibitors in the medium.

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 non-starch polysaccharides determines its relative cellulase requirement. Therefore, the higher this ratio, the less enzyme is required after the pretreatment. Feedstocks with values of this ratio over about 0.39 are particularly well suited for cellulose-to-ethanol process as certain varieties of oat hulls and corn cobs.

Park et al. (2001) have studied the hydrolysis of waste paper contained in MSW (Municipal Solid Waste) obtaining significant sugars yield. Bioethanol production from cellulosic portion of MSW has been already patented (Timas, 1999). Moreover, some strategies for improving the fermentability of MSW acid hydrolysates has 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 FPU/g cellulose.

Simultaneous Saccharification and Fermentation (SSF).

The sugars from the pre-treatment and enzymatic hydrolysis steps are fermented by bacteria, yeast or filamentous fungi, although the enzymatic hydrolysis and fermentation can also be performed in a combined step, the so-called simultaneous SSF (Figure 1.6). In SSF, cellulases and xylanases convert the carbohydrate polymers to fermentable sugars. These enzymes are notoriously susceptible to feedback inhibition by the products - glucose, xylose, cellobiose, and other oligosaccharides.

SSF gives higher reported bioethanol yields and requires lower amounts of enzyme because end-product inhibition from cellobiose and glucose formed during enzymatic hydrolysis is relieved by the yeast fermentation (Dien et al. 2003). The efficiency of product formation increases with increasing bioethanol concentration up to about 5% on a w/w basis, so fermentation at high temperatures (>40 °C) and at or above 5% bioethanol are priorities for commercialization of this technology.

Major advantages of SSF as described by Sun and Cheng, include: (i) increase of hydrolysis rate by conversion of sugars that inhibit the cellulase activity, (ii) lower enzyme requirement, (iii) higher product yields, (iv) lower requirements for sterile conditions since glucose is removed immediately and bioethanol is produced, (v) shorter process time; and (vi) less reactor volume. SSF process has also some disadvantages. The main disadvantage of SSF lies in different temperature optima for saccharification and fermentation (Krishna et al., 2001).

In many cases, the low pH, e.g., less than 5, and high temperature, e.g., >40 °C, may be favorable for enzymatic hydrolysis, whereas the low pH can surely inhibit the lactic acid production and the high temperature may affect adversely the fungal cell growth (Huang et al., 2005). *T. reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and 50 °C. For *Saccharomyces* cultures SSF are typically controlled at pH 4.5 and 37 °C.

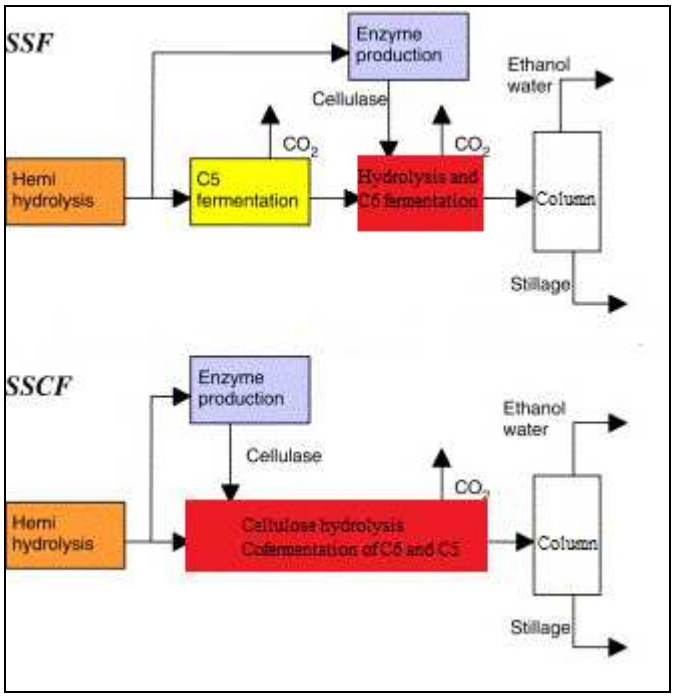


Fig. 1.6: Scheme of Simultaneous Saccharification and Fermentation (SSF) and Simultaneous Saccharification and CoFermentation of hexoses and pentoses sugars (SSCF) processes for lignocellulosic ethanol (Hemi: Hemicellulose).

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More recently, the SSF technology has proved advantageous for the simultaneous fermentation of hexose and pentose which is so-called simultaneous saccharification and co-fermentation (SSCF). In SSCF, represented in Figure 1.6, the enzymatic hydrolysis continuously releases hexose sugars, which increases the rate of glycolysis such that the pentose sugars are fermented faster and

with higher yield. SSF and SSCF are preferred since both unit operations can be done in the same tank, resulting in lower costs (Mosiet et al., 2005).

1.2.6 Fermentation of pentoses.

Complete substrate utilization 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 and hemicellulose must be converted to ethanol, and that microorganisms must be obtained that efficiently perform this conversion under industrial conditions.

Baker's yeast *Saccharomyces cerevisiae* is well established on large scale for the ethanolic fermentation of glucose, mannose, and galactose. But this microorganism is not able to assimilate cellulose and hemicelluloses directly. In addition, pentoses obtained during hemicelluloses 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; 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 1.6, 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 indicated.

Organism	Natural sugar utilization pathways					Major products		Tolerance			O_2 needed	pH range
	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

Tab. 1.6. Prons and cons of various natural microorganisms with regard to industrial ethanol production (Hahn-Hägerdal et al., 2007)

Bacteria.

Obligate anaerobic bacteria (Table 1.6) 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).

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 (Table 1.6) 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 *Escherichia coli* (Ingram et al. 1987; Hespell et al. 1996; Bothast et al. 1999)

In contrast to other bacteria, *Zymomonas mobilis* (Table 1.6) 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 materialized. 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 (Zhang et al. 1995; Mohagheghi et al. 2002). However, *Z. mobilis* would also need pathways for the metabolism of mannose and galactose, which constitute a considerable fraction of some lignocellulosic raw materials.

Yeasts.

Whereas a large number of yeast species metabolize 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 study identified four yeast species able to ferment arabinose to ethanol (Fig. 2b; 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 contain 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 (Skoog and Hahn-Hägerdal 1990). 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. 1994; Olsson et al. 1992; Hahn -Hägerdal and Pamment 2004; Klinke et al. 2004) 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 ethanolic fermentation of sugar- and starch-based raw materials and it is therefore well adapted to the 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 repeatedly demonstrated (Olsson et al. 1992; Hahn-Hägerdal et al. 1994, 2006; Hahn-Hägerdal and Pamment 2004). In favour of *S. cerevisiae* as the microorganism for fuel ethanol production speaks also the advantage of integrating large-scale lignocellulosic ethanol processes into the existing sugar cane and starch-based ethanol plants already using this yeast. Sugar- and starch-based ethanol plants today exclusively operate with *S. cerevisiae* as a production organism. The only, but major, inconvenience to use *S. cerevisiae* for lignocellulosic fermentation is its inability to metabolize and ferment the pentose sugars xylose and arabinose to ethanol.

While *S. cerevisiae* naturally harbors genes for xylose utilization (Kuhn et al. 1995; Toivari et al. 2004), these are expressed at such low levels that they do not support growth on xylose. Only a

limited number of industrial pentose-fermenting strains have been described in literature. The *Pichia stipitis* genes XYL1 and XYL2 encoding XR and xylitol dehydrogenase (XDH), respectively, have been introduced in *S. cerevisiae* (Kötter and Ciriacy 1993), which resulted in growth on xylose. It was recognized that also the endogenous XKS1 gene encoding xylulokinase (XK) had to be overexpressed for xylose fermentation to occur (Eliasson et al. 2000). Bacterial and fungal XI pathways have been also established in *S. cerevisiae* (Walfridsson et al. 1996). Only recently the development of industrial arabinose-fermenting *S. cerevisiae* strains has been initiated (Karhumaa et al., 2006). Moreover, the simultaneous cofermentation of hexose and pentose sugars constitutes the major strain engineering challenge.

1.3 Consolidated BioProcessing for bioethanol production from lignocellulose.

Lignocellulosic biomass is the only foresee able renewable feedstock for sustainable production of biofuels. The main technological impediment to more widespread utilization of this resource is the lack of low-cost technologies to overcome the recalcitrance of the cellulosic structure (Lynd et al., 2002). Four biological events occur during conversion of lignocellulose to ethanol via processes featuring enzymatic hydrolysis: production of saccharolytic enzyme (cellulases and hemicellulases), hydrolysis of the polysaccharides present in pretreated biomass, fermentation of hexose sugars, and fermentation of pentose sugars. 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. The ultimate objective would be a one-step "consolidated" bioprocessing (CBP) of lignocellulose to bioethanol, where all four of these steps occur in one reactor and are mediated by a single microorganism or microbial consortium able to ferment pretreated biomass without added saccharolytic enzymes (Figure 1.7).

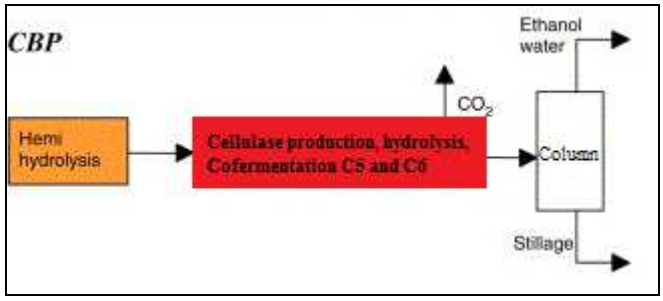

Fig. 1.7: Scheme of Consolidated Bioprocessing (CBP) for ethanol production as integration of the other systems developed for ligncellulosic biomass (Hemi: Hemicellulose).

CBP is gaining increasing recognition as a potential breakthrough for lowcost biomass processing. A fourfold reduction in the cost of biological processing and a twofold 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) finds CBP to be responsible for the largest cost reduction of all R&D-driven improvements incorporated into mature technology scenarios featuring projected ethanol selling prices of less than US $0.70 per gallon.

Finally, 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).

In addition to being desirable, 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*, exceed specific rates exhibited by the *Trichoderma reesei* cellulase system by approximately 20-fold; a substantial part of this difference resulted from "enzyme-microbe synergy", involving enhanced effectiveness of cellulases acting as part of cellulose-enzyme-microbe complexes.

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 broadly be divided into two groups: (1) native cellulolytic microorganisms that possess superior saccharolytic capabilities, but not necessarily product formation, and (2) recombinant cellulolytic microorganisms that naturally give high product yields, but lacking saccharolytic systems.

Examples of native cellulolytic microorganisms under consideration include anaerobic bacteria with highly efficient and complex saccharolytic systems, such as mesophilic and thermophilic *Clostridium* species (Demain et al. 2005) and fungi that naturally produce a large repertoire of saccharolytic enzymes, such as *Fusarium oxysporum* (Panagiotou et al., 2006) and a *Trichoderma* species. However, the anaerobic bacteria produce a variety of fermentation products, limiting the ethanol yield, whereas the filamentous fungi are slow cellulose degraders and give low ethanol yields. Candidates considered as potential recombinant cellulolytic microorganisms into which saccharolytic systems have been engineered, include the bacteria *Zymomonas mobilis* (Lawford et al., 2002), *Escherichia coli* (Tao et al., 2001) and *Klebsiella oxytoca* (Dien et al., 2003), and the

yeasts *S. cerevisiae* and the xylose-fermenting *Pachysolen tannophilus* (Slininger et al., 1987), *Pichia stipitis*, and *Candida shehatae* (Prior et al., 1989).

1.3.1 *S. cerevisiae* as CBP host.

Significant advances related to recombinant enzyme expression support the great potential for *S. cerevisiae* as a CBP host (van Zyl et al., 2007). However, the challenge of integrating all the different aspects of enzymatic hydrolysis and subsequence 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 co-expression of multiple genes in *S. cerevisiae*, for example in the case of the expression of tethered cellulolytic and xylanolytic enzymes (Fujita et al., 2004), xylose and arabinose utilizing enzymes (Becker and Boles, 2003), as well as xylose and oligosaccharides 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 (Van Rensburg et al., 1998).

However, the number of genes expressed is probably not important 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 the host cells are:

1. sequestering of transcription factors at highly expressed promoters used for heterologous gene expression,
2. impact of unfavourable codon bias on the translation of heterogous protein (can be overcome by the use of codon-optimized synthetic genes),
3. improper folding of foreing proteins.

Therefore the proper strategy would not be the sole overexpression of all the required genes to ensure a functional CBP yeast with desiderable 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 in laboratory strains. Much of this work has to be transferred to industrial strains that provide the fermentation capacity and robustness desired for industrial process.

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; Favaro et al., 2013b).

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 plasmid 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 pf 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* retrotrasposon 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 possible to integrate more copies of a gene into the yeast genome than the conventional integration system. Host strains and integrated gene size can significantly affect the transformation efficiency at δ-sequence; however, the transformation efficiency can be 10- to 100-fold those obtained when transforming with vectors that target a single gene on the yeast genome (Favaro et al., 2012a; Favaro et al., 2013b).

A more strategic approach would require 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 co-metabolizes

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 yeasts. Examples of such approaches in the past have been performed to enhance xylose fermentation in laboratory and industrial strains (Kuyper et al., 2005).

1.4 Reasons for developing a CBP microbe for cellulose conversion.

Current technology for conversion of cellulose to ethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by fermentation by a microrganism such as *Saccharomyces cerevisiae*. The large amounts of enzymes required for enzymatic conversion of cellulose to fermentable sugars impacts severely on the cost effectiveness of this technology. One-step CBP conversion of cellulose to ethanol with an organism capable of cellulose degradation and efficient fermentation would greatly enhance cost effectiveness of bioethanol production.

The development of a yeast strain capable of producing ethanol by fermenting cellulosic substrates has received a great deal of interest over recent years. The advantages of yeasts include (i) their high ethanol productivity and tolerance, (ii) larger cells size, which simplify their separation from the culture broth and (ii) resistance to viral infection.

Cellulases from bacterial and fungal sources have been transferred to *S. cerevisiae*, enabling the hydrolysis of cellulosic derivatives (Lynd et al., 2002), or growth on cellobiose (Van Rooyen et al., 2005). Most reports regarding the expression of cellulases and hemicellulases in yeast employed strong glycolytic (or other constitutively expressed) promoters to drive expression of the heterologous gene(s). Although the choice of promoter and leader sequences will undoubtedly have a great influence on expression levels attained, there are not enough data in the literature to suggest any general trends as to what are the best promoter and leader sequences to use when expressing cellulases and hemicellulases. Several researchers have sought to produce cellulases in an organism that would not yield interfering activities so as to gain insight into the mechanism of the original cellulolytic enzyme (Bailey et al., 1999), whereas others have sought to enable the yeast to hydrolyze non-native cellulolytic substrates (Fujita et al., 2004). Although most of the cellulases that have been successfully produced in *S. cerevisiae* were of fungal origin, there are reports of successful bacterial cellulases production (Van Rensburg et al., 1996).

1.4.1 Cellulose hydrolysis.

Full enzymatic hydrolysis of crystalline cellulose requires three major types of enzymatic activity (cellulase system): (1) endoglucanases (1,4-β-d-glucan 4-glucanohydrolases; EC 3.2.1.4); (2) exoglucanases, including d-cellodextrinases (1,4-β-d-glucan glucanohydrolases; EC 3.2.1.74) and cellobiohydrolases (1,4-β-d-glucan cellobiohydrolases; EC 3.2.1.91); and (3) β-glucosidases (β-glucoside glucohydrolases; EC 3.2.1.21) (Figure 1.8).

Endoglucanases randomly cut internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure (Teeri, 1997). β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose.

Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze β-1,4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1,4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base. The hydrolysis products can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end (Withers, 2001).

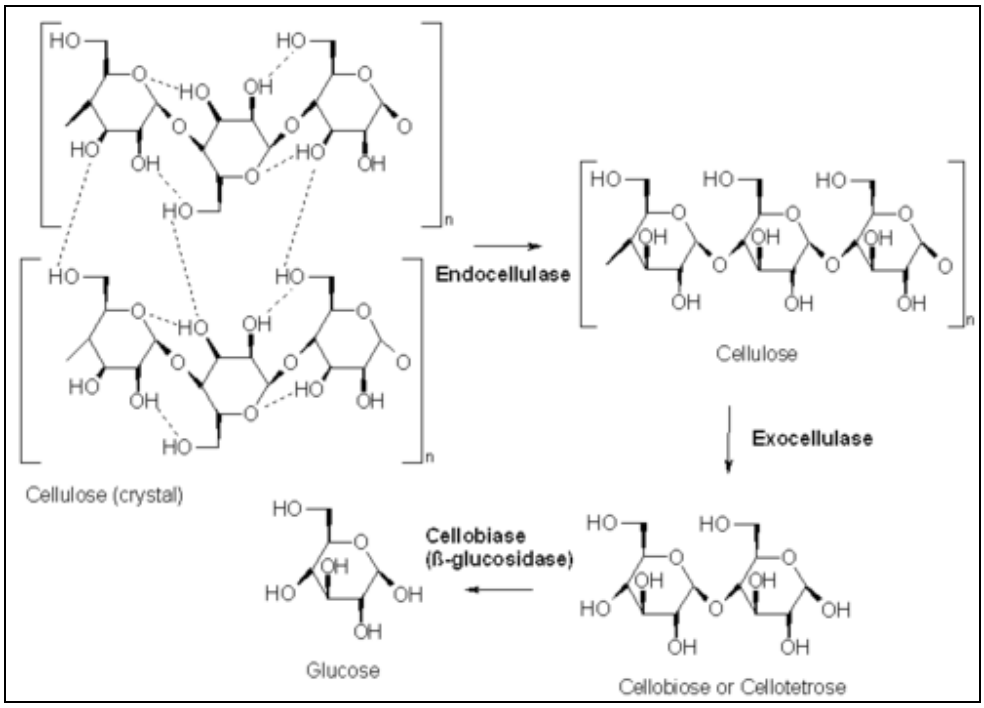

Fig. 1.8: Schematic representation of the mechanism of degradation of cellulose. The action of the three enzymes involved (Endocellulase, Exocellulases and β-glucosidase) are indicated.

A number of studies have expressed multiple cellulase enzymes in yeasts in attempts to recreate a fully cellulolytic, fermentative system (Katahira et al., 2006; Fujita et al., 2004; den Haan et al., 2006). Van Rensburg et al. (1998) constructed a yeast capable of hydrolyzing numerous cellulosic substrates and growing on cellobiose, while Cho et al. (1999) showed that decreased loadings of cellulases could be used for SSF experiments with their strain expressing a BGL enzyme and an enzyme with dual exo/endocellulase activity. Fujita et al. (2002, 2004) reported co-expression and surface display of cellulases in *S. cerevisiae*, and a recombinant strain displaying the *T. reesei* endoglucanase II, cellobiohydrolase II, and the *Aspergillus aculeatus* β-glucosidase 1 was built. High-cell density suspensions of this strain were able to directly convert PASC to ethanol with a yield of approximately 3 g/L from 10 g/L within 40 h. Den Haan et al. (2006) reported growth on and direct conversion of PASC to ethanol by a laboratory *S. cerevisiae* strain co-expressing the endoglucanase *T. reesei EG1* and the *Saccharomycopsis fibuligera BGL1*.

Since cellobiose (and longer chain cellooligosaccharides) is the major soluble by-products of cellulose hydrolysis, its efficient utilization is of primary importance to CBP development. Enzymatic hydrolysis of cellobiose requires the action of β-glucosidases. This heterogeneous group of enzymes displays broad substrate specificity towards cellobiose, cello-oligosaccharides and

different aryl- and alkyl-β-d-glucosides. β-Glucosidases occur widely in animals, plants, fungi and bacteria and they work synergistically with endoglucanases and exoglucanases on the degradation of cellulose. They not only catalyze the final step in the degradation of cellulose, but also stimulate the extent of cellulose hydrolysis by relieving the cellobiose-mediated inhibition of exoglucanase and endoglucanase (Sternberg et al., 1977).

2. MATERIAL AND METHODS

2.1 Strains and media

The genotypes, phenotypes and sources of yeast and bacterial strains used in this work are summarized in Table 2.1.

Strain	Relevant genotype or phenotype	Source or reference
Candida zemplinina	144 strains isolated from grape marcs containing an inhibitors cocktail	Trento et al., 2011
Candida glabrata	16 strains isolated from grape marcs containing an inhibitors cocktail	Trento et al., 2011
Escherichia coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F'proAB lacq ZΔM15 Tn10 (tet)]*	Stratagene (USA)
Issatchenkia orientalis	155 strains isolated from grape marcs containing an inhibitors cocktail	Trento et al., 2011
Saccharomyces cerevisiae	21 strains isolated from grape marcs containing an inhibitors cocktail	Trento et al., 2011
of which: T2	Strain with high fermentative vigour and inhibitor tolerance	
S. cerevisiae 27P	Yeast with industrial bioethanol traits	Favaro et al., 2012a
S. cerevisiae EC1118	Industrial wine strain	Padova Univ.
S. cerevisiae Fp96	Strain with high fermentative vigour and inhibitor tolerance	Favaro et al., 2012c
S. cerevisiae F12	Strain with high fermentative vigour and inhibitor tolerance	Favaro et al., 2012c
S. cerevisiae MH1000	Industrial distillery strain	Stellenbosch Univ.
S. cerevisiae YI30	Industrial distillery strain	Stellenbosch Univ.
S. cerevisiae Y294	*αleu2-3, 112 ura3-52 his3 trp1-289*	American Type Culture collection (ATCC)
S. cerevisiae S288c	*MATα SUC2 gal2 mal mel flo I flo8-1hap1 ho bio 1 bio6, MIP[S]*	ATCC

Tab 2.1. Summary of the yeast and bacterial strains used in this study

The media used in this work are reported in Table 2.2. All chemicals, media components and supplements were of analytical grade standard.

Medium	Reference or supplier
Luria-Bertani (LB)	DIFCO
Must Nutrient Synthetic (MNS)	Delfini, 1995
Yeast Nitrogen Base (YNB)	DIFCO
Yeast Peptone Dextrose (YPD)	OXOID
Yeast Peptone Dextrose Sorbitol (YPDS)	Favaro et al., 2012a

Tab 2.2. Summary of the media used in this study

Yeast strains pre-cultures were grown in YPD medium (g/L: yeast extract, 10; peptone, 20 and glucose, 20) at 30 °C on a rotatory shaker at 130 rpm unless otherwise stated.

2.2 Fermentative vigour evaluation

Fermentative vigour of *S. cerevisiae* strains was tested in MNS broth (Delfini, 1995). This medium was selected because it well simulates industrial conditions (Favaro et al., 2012b). *S.cerevisiae* MH1000, EC1118, 27P, Y294 were used as reference strains. Fermentation tests were performed as described by Delfini (1995). In short, MNS medium was supplemented with different concentrations of glucose and/or xylose (20% glucose, 10% glucose and 5% xylose) and glass serum bottles were 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 10^4 cells/mL and incubated in static condition at 25 and 40 °C. The experiments were carried out in triplicate. The fermentation vigour was daily monitored by measuring flasks weight loss in relation to CO_2 production. Every measure was reported as grams of glucose utilised per 100 mL of MNS medium, by a conversion factor of 2.118 (Delfini, 1995). Samples were withdrawn after 7 and 21 days and analyzed for glucose, xylose, xylitol, glycerol and ethanol by HPLC, as described in Favaro et al. 2012b.

2.3 Yeast isolation from grape marcs

Grape marcs were selected as promising ecological niche since it is a wide source of yeast strains having interesting fermentative abilities. Marcs were collected immediately after grape crushing, from a winery located in Melara (Rovigo). To set up the experiment, 5-kg aliquots of non-sulphited grape marcs were transferred into sterile plastic bags and closed with a spongy plug to allow excess gas release during fermentation.

The bags, with or without a cocktail of inhibitors more frequently present in the hydrolysates (g/L: Furfural 1.85, Acetic acid 4.8, Formic acid 1.63, Lactic acid 4.53, as defined in the next chapter), were incubated at 30 and 40 °C. Similarly, stalks bags were incubated at room temperature with or without the inhibitors cocktail.

For strains isolation, YPD-agar plates were prepared adding each single inhibitors at the same concentration of that used in the bags, and another series at half concentration (g/L: Furfural 0.92, Acetic acid 2.4, 0.81 g/L Formic acid 0.81, Lactic acid 2.26). YPD plates were also prepared with the addition of the entire inhibitors cocktail, both at the maximum and half concentration. All the media were supplemented with 100 µg/mL chloramphenicol in order to inhibit bacterial growth.

Twenty grams of grape marcs were collected randomly within each bag, dispersed in 180 mL of sterile NaCl solution (0.9%) and, after appropriate decimal dilutions, plated on YPD agar with and without inhibitors. The plates were aerobically incubated at the same temperature of the original bag (30 or 40 °C).

2.4 Yeast strains genetic identification

In order to proceed to a reliable identification of the isolates, most of the yeast strains available at the end of the isolation programme were analyzed by ITS (Internal Trascribed Spacer) amplification as described below.

For each isolate, a colony grown on YPD-agar plate was resuspended in 20 µL of sterile water and vortexed briefly. 3 µL of the suspension were used as template for PCR amplification, carried out in a thermalcycler gradient (BioRad Lab, Hercules, CA, USA).

For ITS region 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 3-Ml aliquot of cell suspension, prepared as described above, was heated at 94 °C for 5 minto allow cell lysis and then subjected to PCR amplification using 30 cycles with initial

denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. Amplification products were checked for purity by agarose gel electrophoresis.

The resulting amplification product was digested with *Hinf*I restriction endonuclease. The reaction was carried out in 15-µL volume and incubated at 37 °C for 4 h. Restriction fragments were separated by 1.8% (w/v) agarose gel electrophoresis in TBE buffer (0.5×) to obtain the relative restriction pattern.

Representative isolates from each *Hinf*I restriction pattern were then subjected to ITS region sequencing (BMR Genomics, University of Padova). Their 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 species identification.

2.5 Tolerance to inhibitors of *S. cerevisiae* strains

Strains genetically identified as *S. cerevisiae*, were evaluated for their fermentative abilities (see 2.2 Fermentative vigour evaluation) and for their inhibitors tolerance once grown in YNB and YPD broth supplemented with cocktails of inhibitory compounds commonly present in lignocellulosic hydrolysates (weak acids and furans). Inhibitors tolerance was also tested in YPD-agar plates. The concentration values of all the inhibitors comes from an extensive bibliographic research made in order to study the different concentrations of sugars and inhibitors that are present in the lignocellulosic hydrolysates. The final aim was to define a synthetic medium in order to simulate the industrial fermentation environments as well as the composition (i.e. sugars, nutrients, inhibitors) of the hydrolysates. Among a number of different cocktails tested, the following were adopted:

- *Cocktail B* (g/L: acetic acid 3.6; formic acid 1.2; lactic acid 3.4; furfural 1.4)
- *Cocktail C* (g/L: acetic acid 5.4; formic acid 1.8; lactic acid 5.2; furfural 2.1).

The pH of the medium, after the addition of the inhibitors, was set to 4.5 with KOH (5M). Yeast cells were inoculated at a concentration of about $1x10^6$ cells/mL in 0.9 mL of medium and incubated at 30°C (100 rpm). After 40 hours, optical density (OD_{600}) was measured. For each strain the tolerance was evaluated as relative growth by comparing the growth in the medium with and without the inhibitors, as OD value (%).

2.6 Growth of *S. cerevisiae* strains in medium with glucose

The *S. cerevisiae* strains were evaluated for their ability to grow in medium with glucose**.** To this purpose, minimal medium YNB (Yeast Nitrogen Base, 6.7 g/L) was used and 20 g/L of glucose and 5 g/L of NH_4-sulphate were added to the medium.

Yeast cells were inoculated at a concentration of about $1x10^6$ cells/mL in 35 mL of medium and incubated at 30 °C and 40 °C (100 rpm) in aerobic conditions. Samples were withdrawn at regular intervals for the evaluation of the growth by optical density at 600nm. At final sampling time, aliquots of the YNB cultures were collected for the evaluation of dry biomass and for the analysis of the glucose, xylose, xylitol, glycerol and ethanol content by HPLC.

2.7 Development of an efficient cellobiose hydrolyzing yeast strain for industrial bioethanol production.

Among the *S. cerevisiae* screened, the strain exhibiting the highest fermentative vigour and inhibitors tolerance was selected in order to develop an engineered *S. cerevisiae* yeast able to secreting the bglI β-glucosidase obtained from *Saccharomycopsis fibuligera*. Four *S. cerevisiae* strains (27P, F12, Fp96, Y130) were used in the experiment as benchmark strains.

2.7.1 Engineering *S. cerevisiae* yeasts by introducing the *bglI* β-glucosidase gene from *Saccharomycopsis fibuligera.*

Recombinant strains and plasmids.

The genotypes and sources of the plasmid, yeast and bacterial strains used in these experiments are summarised in Table2.3.

Plasmid/Strains	Relevant genotype or phenotype	Source
pBKD1_BGL1	*amp δ-sites PGK_P-XYNSEC-bglI-PGK_T-KanMX-δsites*	Stellenbosch Univ.
E. coli pBKD_BGL1	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F'proABlacq ZΔM15 Tn10 (tet)]*	Stellenbosch Univ
S. cerevisiae T2 [pBKD_BGL1]	*Recombinant strain of T2 for bglI multi copy integration*	This work
S. cerevisiae 27P[pBKD_BGL1]	*Recombinant strain of 27P for bglI multi copy integration*	This work

Table 2.3. Summary of plasmids and strains constructed for the development of an efficient recombinant *S. cerevisiae* strain able to utilize cellobiose.

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 for the selection of resistant bacteria.

DNA manipulations.

Restriction enzyme digestion, electrophoresis, DNA preparation from *E. coli* were performed using the standard methods according to Sambrook et al. (1989). The concentration and the purity of the DNA extracted from *E.coli* were evaluated with the Nanodrop instrument (Thermo Scientific Instrument Inc.). Restriction enzymes and buffers were supplied by either Roche or Fermentas.

Geneticin resistance tests.

To establish their dominant marker resistance, the wild type *S. cerevisiae* strains T2, 27P, F12, Fp96 and YI30 were grown in YPD broth at 30°C for 24h. Yeast cells were serially diluted in NaCl (0.9%) and plated onto YPD agar supplemented with increasing amounts of geneticin (0, 10, 20, 25, 30, 40, 50, 100 µg/mL). After 24h of incubation at 30 °C, each strain was evaluated for geneticin sensitivity.

Electrotrasformation of yeast strains with delta vectors.

The wild type *S. cerevisiae* strains T2, 27P, F12, Fp96 and YI30 were transformed with the *XhoI* digested pBKD_BGL1 integrative plasmid for chromosomal integration (Figure 2.1).

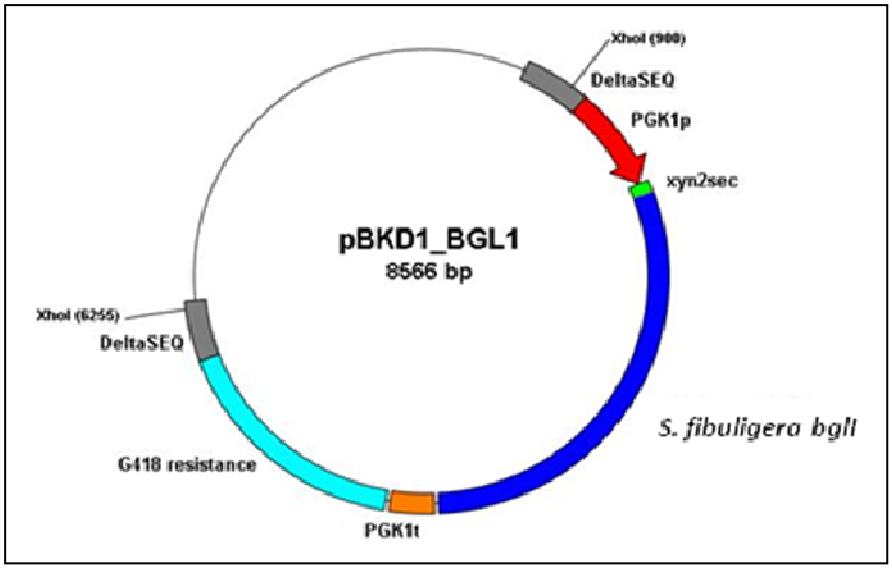

Fig 2.1. The δ-integrative vector, pBKD_BGL1, for the constitutive expression of *S. fibuligera bglI* in *S. cerevisiae*

This plasmid contains DNA sequences for the resistance to antibiotic geneticin (G418 resistance), the *bglI* gene from *S. fibuligera* for the expression the β-glucosidase enzyme, the *S. cerevisiae PGK1* (Phosphoglycerate Kinase) promoter and terminator sequences, and the Delta sequences for the recombination with the Delta sequences of the retrotrasposon Ty1 in the selected strain.

To obtain the chromosomal integration of *bglI* gene, selected strains were subjected to a electroporation protocol. Host cells, grown overnight in YPD broth, were harvested in Eppendorf tubes by centrifugation at 4000 rpm (Mikro 200, Hettic Laborzentrifugen) for 1 min, washed twice with distilled water and finally suspended in 1 mL of electroporation buffer containing 1 M sorbitol and 20 mM HEPES. After centrifugation at 4000 rpm (Mikro 200, Hettic Laborzentrifugen) for 1 min, the pellet was resuspended in 200 µL of electroporation buffer. The resuspended cells (50 µL) were transferred into electroporation cuvette (0.2 cm electrode, Bio-Rad). After adding 10 µg of linearized plasmid, an electric pulse of 1.4 kV and 200 ohms was applied with a capacitance of 25

µF by using Gene-Pulser (Bio-Rad Lab., Hercules, CA.,USA). In this pulsed cuvette, 1 mL of YPD supplemented with 1M sorbitol was added and the cuvette was incubated for 3h at 30 °C.

The recombinant cells were plated onto YPDS plates (containing 1 M sorbitol) supplemented with geneticin (25-35 µg/mL) and incubated at 30 °C for 3 days.

Detection of β-glucosidase activity on agar plates.

Once grown on YPDS plates, the recombinant cells were transferred onto fresh YPD with 4-methyl-umbelliferyl-β-D-glucopyranoside (4-MUG) as substrate. 4-MUG allows to detect a β-glucosidase activity as, once hydrolyzed, it produces fluorescence under the long-wave ultraviolet light.

A stock of 148 mmol/L 4–MUG was prepared in dimethylformamide (Sigma, ≥99.8%) and diluted to 37 mmol/L with sterile distilled water. Fifty µL of 37 mmol/L 4-MUG was spread onto the surface of the YPD agar plates and cultures were point-inoculated on plate. Every plate was inoculated with the relative wild type strain in order to evaluate the native background β-glucosidase activity. The plates were incubated at 30 °C and examined after 24 and 48 h under the long-wave ultraviolet light of a transilluminator. Strains with β-glucosidase activity hydrolyze the substrate giving a fluorescent halo and were further studied for their mitotic stability.

Evaluation of mitotic stability of the transformants.

To study mitotic stability of the obtained recombinants, the engineered strains producing an evident fluorescent halo were grown in sequential batch cultures as described in Favaro et al. (2010). In short, the integrants were cultivated in non-selective YPD broth (4 mL), at 30 °C on a rotary shaker set at 130 rpm, and transferred (1% v/v) to fresh YPD after glucose depletion.

After 120 generations the recombinant strains were plated onto YPD with 4-MUG and YPD with geneticin (25-35 µg/mL), then incubated at 30 °C for 24h. The stable trasformants remained resistant to geneticin and display hydrolytic activity on 4-MUG.

Enzymatic assays.

Stable mitotic trasformants were studied for their β-glucosidase activity with *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate. The enzymatic assays were conducted also with the wild type *S. cerevisiae* T2, 27P, F12, Fp96, YI30. Yeast cells were grown at 30 °C in 20 mL YPD medium (1% v/v) for 72h. Two mL samples were withdrawn at 24h intervals and stored in ice until thebeginning of the assay. Samples of cultures (50 µL) were mixed with 50 µL of the substrate (4mM pNPG in 0.1M citrate-phosphate buffer, pH 5.0). The hydrolyzing reaction was carried out at 55 °C for 20 minutes. Two-hundred µL of Na_2CO_3 1 M was added in order to raise the pH and stop the reaction. The samples were spin-down for 10 s at 13000 rpm (Mikro 200, Hettic Laborzentrifugen) and 50 µL of the supernatant of each sample were transferred in a 96-well flat transparent microplate for the evaluation of the absorbance at 405 nm.

At final sampling time, 10 mL aliquots of the YPD cultures were collected for the evaluation of dry biomass. To this purpose the aliquots were centrifuged (5000 rpm for 5 min, 3K15 Laborzentrifugen), then cell pellets were washed several times with distilled water and dried in an oven (80°C) to constant weight.

All enzymatic assays were done in duplicate and β-glucosidase activity was expressed in units per mg dry cell weight (Meinander et al., 1996) where one unit was defined as the amount of enzyme required to produce 1 µmol of a *p*-nitrophenol or reducing sugar per minute under the assay conditions.

2.8 Growth of the recombinants in medium with cellobiose.

Stable recombinant strains that presented the best enzymatic activities with pNPG were selected and tested for their ability to grow in medium with cellobiose using two media: minimal broth (YNB: 6.7 g/LYeast Nitrogen Base) and rich medium (YP 10 g/L Yeast extract, 20 g/L Peptone). For each medium, 3 different conditions were chosen: no sugar added, 10 g/L of glucose and 10 g/L of cellobiose added to the medium.

Yeast cells were inoculated at a concentration of about $1x10^6$ cells/mL in 35 mL of medium and incubated at 30°C (100 rpm) in aerobic conditions. Samples were withdrawn at regular intervals for the evaluation of the growth by optical density at 600nm.

2.9 Amplification of the integrated *bglI* gene of *S. fibuligera*.

Recombinant yeast cells were grown overnight in YPD broth at 30 °C and then the genomic DNA was extracted with the glass beads method as described by Sambrook et al., 1989. Three microliters of a 1:100 dilution of the DNA extracted were used for PCR amplification.

Primers, listed in Table 2.4, were designed from alignments of DNA sequences of *bglI* gene in *S. fibuligera*. Gene sequence was obtained from Gen-Bank and aligned using the CLUSTAL W software (Thompson et al., 1994). Primers BGL1-1fw and BGL1-1rw were designed within the 5' region of *bglI* gene of *S. fibuligera*; while primers BGL1-2fw and BGL1-2rw were derived from the 3' region of *bglI* gene.

The amplification reaction was performed in a total volume of 25 µL into 0.2 mL tubes with the following reagent concentrations: 1.25 mMdNTPs, 50 mM $MgCl_2$, 160 mM $(NH_4)SO_4$, 670 mMTris-HCl and 0.1% Tween20,EuroTaq polymerase (Euroclone, Milano, Italy), 0.005 mM (each) primers (Eurofins MWG Operon, Ebersberg, Germany; HPSF purified).

Name	**Sequence (5'-3')**	**T_m**	**Position**	**PCR product size (bp)**
BGL1-1 fw	TGCATAAATTGGTCAATGCAA	52.0 °C	60-81*	1534
BGL1-1 rw	TCAAAGCTGTGTCCTCCGTA	57.3 °C	1492-1512*	
BGL1-2 fw	AAATGGCGCTTTGTTTCAAG	53.2 °C	22-42*	1321
BGL1-2 rw	GCGCGCCTCAAATAGTAAAC	57.3 °C	1277-1297*	

Table 2.4. Primers used in this work (* relative to the two regionsof *S. fibuligera bglI* gene sequence)

The thermal protocol was designed as follows: initial incubation 95 °C for 2 min to allow the DNA denaturation, followed by 35 cycles composed of denaturation at 95 °C for 30s, annealing at 52 °C for 60 s and extension at 72 °C for 120 s. A final extension step was added at 72 °C for 5min.

Amplified samples were run on 1.2% agarose gel and the bands were visualized after Eurosafe nucleic acid stain (Euroclone) staining. Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

3. RESULTS AND DISCUSSION

3.1 Evaluation of the fermentative vigour of oenological *S. cerevisiae* yeasts.

Key feature in the development of an industrial yeast strain for the production of the second generation bioethanol is the high fermentative ability of the strain. To this purpose, fifty *S. cerevisiae* strains, previously isolated from different oenological environments, were tested for their fermentative ability at 25 and 40 °C in MNS broth according to the method described by Delfini (1995). The results, showed below in Figure 3.1 and 3.2, are reported as grams of glucose used by the strains when incubated at 25 and 40 °C, respectively.

At 25 °C in MNS medium with 20% of glucose, most of yeasts consumed almost all the glucose available (Figure 3.1), and this result confirms that 25 °C is the optimal temperature of fermentation for *S. cerevisiae*. This is even more evident observing the fermentative kinetics obtained with the same strains at 40 °C (Figure 3.2). In this condition, the strains consumed about half of the glucose available, and the fermentative kinetics stopped after nine days of fermentation.

The strains that presented the best fermentative vigour at 25 °C in MNS medium with 20% of glucose were *S. cerevisiae* 19, 45, 100 and 1.99, consuming 199.9, 200, 197.4 and 200 grams of glucose, respectively. In the same condition, the strains that presented the worst fermentative performances were 74, 56 and 1.42, consuming 134.1, 160.1 and 151.6 grams of glucose, respectively (Figure 3.1).

At 40 °C, in MNS medium with 20 % glucose, the strains that exhibited the best fermentative vigour were *S. cerevisiae* 6, 12, 17, 32 and 1.63, consuming 113.1, 116.6, 124.4, 112.5 and 120 grams of glucose, respectively. In the same condition, those showed the worst fermentative performances were strains 66, 85, 91, and 87, consuming 47.8, 40, 44.1 and 43.9 grams of glucose, respectively (Figure 3.2).

In this experiment three *S. cerevisiae* reference strains were used, strain 27P showing promising industrial traits (Favaro et al. 2012a), strain MH1000, a robust industrial yeast (van Zyl et al., 2011) and the commercial strain EC1118 used for wine production (Lallemand Inc., Canada). At 25 °C in MNS medium with 20% of glucose these strains presented good fermentative performances; under the same conditions reference strain EC1118 presented the best fermentative vigour with 193.4 grams of glucose consumed.

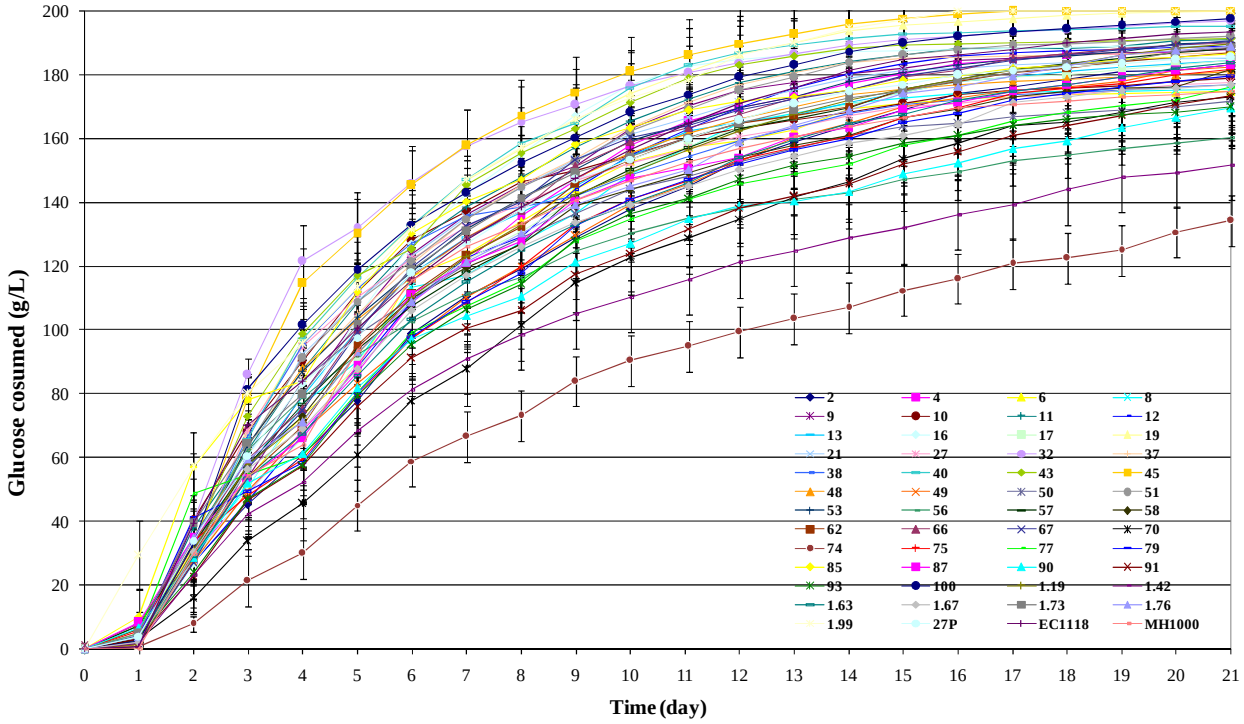

Fig. 3.1. Fermentative performance at 25 °C of *S. cerevisiae* strains in MNS medium with glucose (200 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

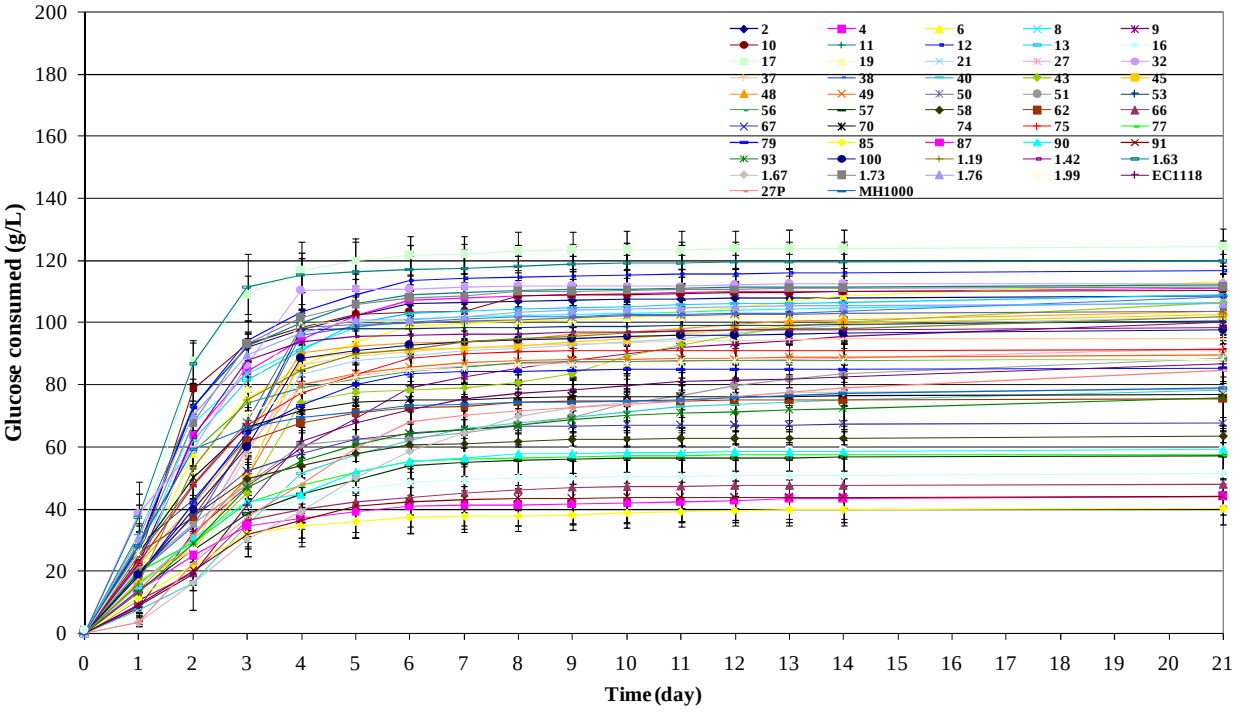

Fig. 3.2. Fermentative performance of *S. cerevisiae* strains at 40 °C in MNS medium with glucose (200 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

At 40 °C, the reference strains showed a fermentative vigour lower than that presented at 25 °C; and in this condition *S. cerevisiae* EC1118 presented the highest glucose consumption among the reference strains (86.8 grams).

The strains that presented the best fermentative vigour in MNS with 20% glucose at 25 °C *(S. cerevisiae* 19, 45, 100 and 1.99) showed high fermentative performances comparable to that exhibited by the reference strains (*S. cerevisiae* EC1118, 27P and MH1000). At 40 °C in the same broth, the strains that presented the best fermentative vigour (*S. cerevisiae* 6, 12, 17, 32 and 1.63) exhibited high fermentative performances compared to that showed by the reference strains (e.g., 124.4 grams of glucose consumed by *S. cerevisiae* 17 respect to the 86.8 grams consumed by the strain EC1118).

The obtained results allowed to select twenty-one strains, having the best fermentative ability in MNS glucose 20% at 25 and 40 °C, for further fermentation trails using MNS supplemented with xylose 5% and glucose 10% (Figure 3.3 and 3.4).

As reported below in Tables 3.2, the xylose content did not change, at least in the analysed samples. This suggested that all the strains did not ferment xylose. As shown in Figure 3.3, the strains 62, 1.19, 1.99 exhibited the best fermentative vigour at 25 °C by consuming the amount of sugar corresponding to the glucose content. As reported in Figure 3.4, *S. cerevisiae* strains 38, 45, 56, 1.63 exhibited the highest fermentative performance at 40 °C (84.9, 85.4, 82.5 and 82.7 grams of glucose, respectively).

Some strains showed good performance in both MNS media at 25 °C (i.e. *S. cerevisiae* 1.99) clearly consuming all the glucose available in the MNS broth. At 40 °C the strain showing the highest fermentative vigour both in MNS glucose 20% and in MNS glucose 10% with xylose 5% was *S. cerevisiae* 1.63.

To highlight the evidence that the temperature influences the fermentative ability of the strains, *S. cerevisiae* 32 was selected as representative of the other yeasts once grown in both MNS broths (Figure 3.5 and Figure 3.6). At 25 °C the strain showed the glucose consumption slower than at 40 °C in the first part of the fermentation (Figure 3.6 a). After 4 days at 40 °C, glucose consumption of the yeast stopped both in MNS and MNS with xylose. This result is in accordance with Mensonides et al (2002) reporting the negative metabolic response of *S. cerevisiae* to continuous heat stress (40-43 °C).

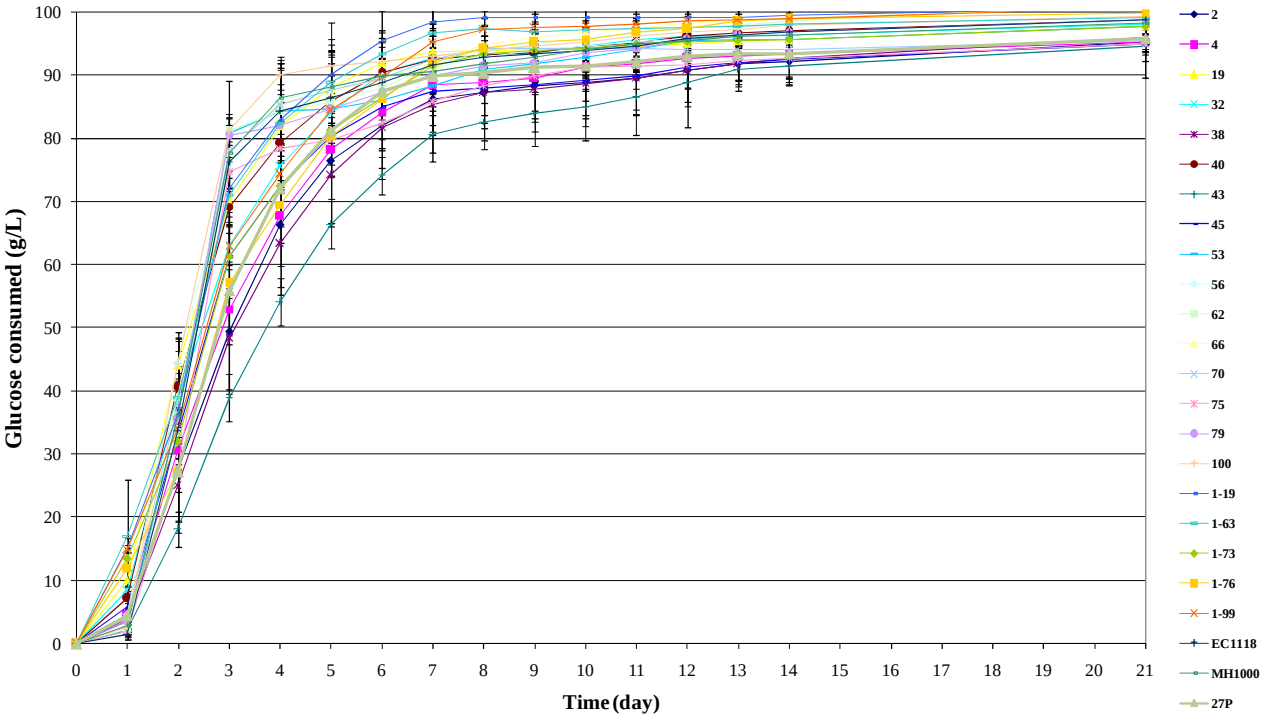

Fig. 3.3. Fermentative performance at 25 °C of *S. cerevisiae* strains in MNS medium with glucose (100 g/L) and xylose (50 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

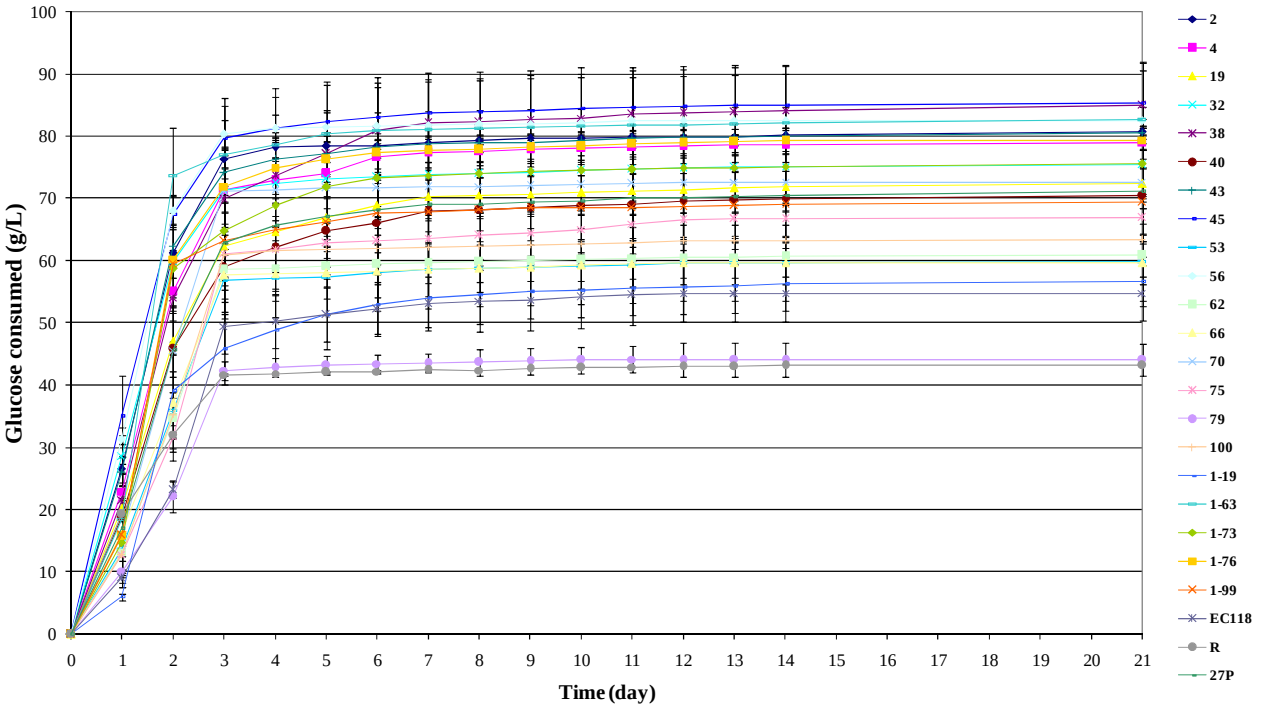

Fig. 3.4. Fermentative performance at 40 °C of *S. cerevisiae* strains in MNS medium with glucose (100 g/L) and xylose (50 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

Fig. 3.5. Effect of the temperature on the growth of *S. cerevisiae* 32 in MNS supplemented with glucose 20% after 21 days, at 25 °C (a) and 40 °C (b).

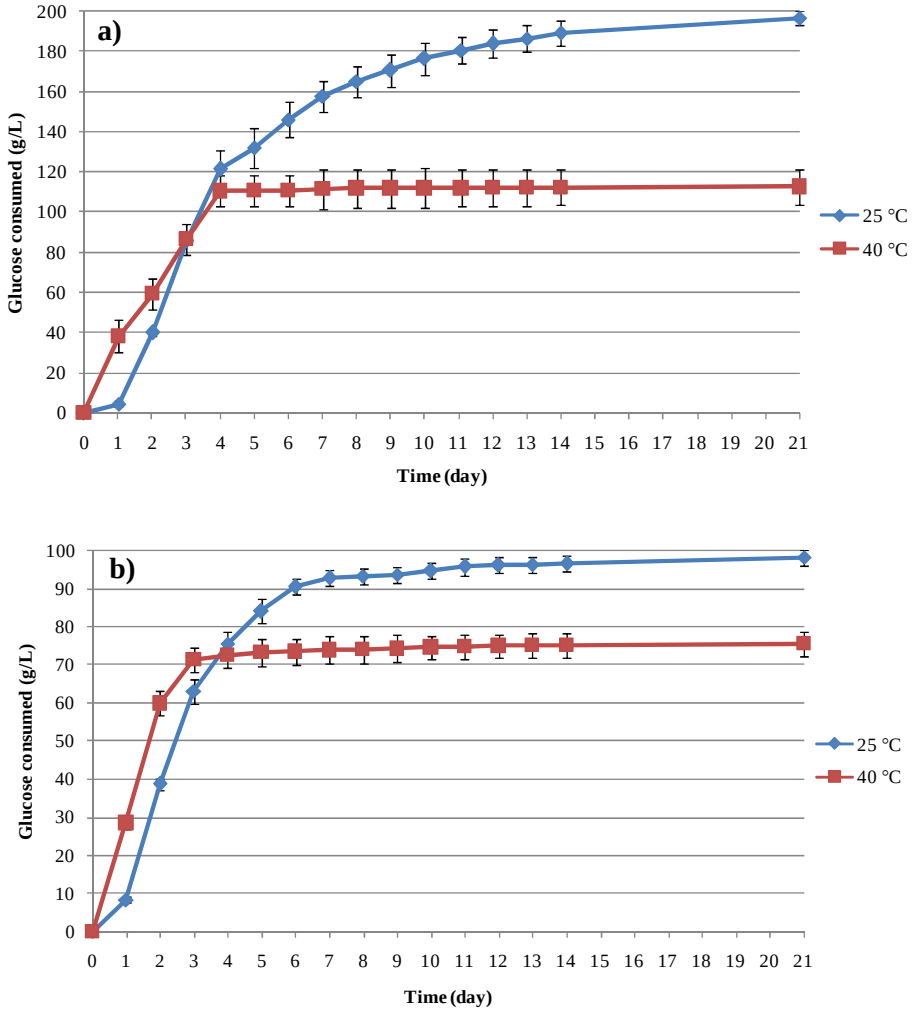

Fig. 3.6. Grams of glucose consumed by *S. cerevisiae*32 in MNS medium with glucose (200 g/L) (a), and glucose (100 g/L) with xylose (50 g/L) (b). The experiment was conducted in triplicate and vertical bars represent SD.

3.1.1 HPLC analysis.

The strains that exhibited the best fermentative vigour in MNS medium supplemented with glucose 10% and xylose 5% were selected for HPLC analysis of glucose, xylose, xylitol, glycerol and ethanol content after fermentation. To this purpose, samples were withdrawn after 21 days of fermentation and filtered through 0.22 µm. The samples were stored at -20 °C until use. The results are expressed as average value of three replicates (Tables 3.1 and 3.2).

S. cerevisae	**27P**	**EC1118**	**MH1000**	**2**	**19**	**32**	**38**	**43**	**45**	**56**	**62**	**66**	**70**	**100**	**1.19**	**1.63**	**1.73**	**1.76**	**1.99**
MNS with 20% glucose at 25 °C																			
Glucose (g/L)	5.8	13.3	7.7	11.7	5.3	10.4	10.2	5.2	5.6	34.4	6.9	5.1	23.8	6.4	9.6	27.4	15.2	11	7.7
Glycerol (g/L)	5.8	6.8	5.4	4.8	6.4	5.7	5.4	4.8	6.6	5.5	4.9	5.0	4.3	5.7	4.8	4.4	5	5.1	5.4
Acetic acid (g/L)	1.3	1.3	1.4	1.3	1.7	0.6	1.2	1.1	1.5	1.6	1.3	1.2	1.1	1.4	1.2	1.3	1.3	1.3	1.4
Ethanol (g/L)	93.7	84.2	92.7	90.9	97.2	90.6	90.5	93.9	99.1	74.9	91	94.5	80.7	92.2	90.0	79.2	87.3	90.2	86.8
Ethanol yield																			
g/g of glucose	0.48	0.4	0.45	0.48	0.50	0.48	0.48	0.48	0.51	0.45	0.47	0.48	0.46	0.48	0.47	0.46	0.47	0.48	0.45
% of the theoretical maximum	94	94	88	94	98	94	94	94	100	88	92	94	90	94	92	90	92	94	88
MNS with 20% glucose at 40 °C																			
Glucose (g/L)	110.8	105.6	110.8	79.6	96.3	93.5	106.0	86.8	95.6	93.9	119.0	127.0	102.0	106.0	88.8	79.5	85.7	96.6	107.0
Glycerol (g/L)	4.6	2.6	6.8	5.0	2.6	5.2	4.0	4.9	5.1	2.5	3.7	3.9	4.5	4.1	3.9	5.4	4.8	5.3	4.5
Acetic Acid (g/L)	1.5	0.8	1.3	1.3	1.3	1.3	1.3	1.3	1.4	0.7	1.3	1.3	0.7	1.3	1.3	1.5	1.5	1.3	1.4
Ethanol (g/L)	39	37.6	33.8	47	42.2	43.7	35.8	47.6	47.8	34.5	29.4	26.0	38.3	38.1	42.1	49.8	45.5	49.6	33.0
Ethanol yield																			
g/g of glucose	0.44	0.40	0.38	0.39	0.40	0.41	0.38	0.42	0.46	0.32	0.36	0.36	0.39	0.40	0.38	0.41	0.40	0.48	0.35
% of the theoretical maximum	86	78	74	76	78	80	74	82	90	63	70	70	76	78	74	80	78	94	69

Tab 3.1. Product formation by *S. cerevisiae* strains that presented the best fermentative abilities at 25 and 40 °C in MNS medium with glucose (200 g/L) as substrate. (ND Not Detected)

S. cerevisae	**27P**	**EC1118**	**MH1000**	**2**	**19**	**32**	**38**	**43**	**45**	**56**	**62**	**66**	**70**	**100**	**1.63**	**1.73**	**1.76**
MNS 10% glucose 5% xylose at 25 °C																	
Glucose (g/L)	5.1	5.1	5.1	2.5	2.5	ND	5.1	18.8	2.6	5.1	5.1	-	-	5.1	5.2	ND	5.1
Xylose (g/L)	49.2	49.5	49.7	49.7	49.3	ND	49.1	50	48.9	49.8	49.6	49.4	49.6	49.4	49.3	ND	48.7
Xylitol (g/L)	5.1	4.8	4.6	4.5	5.1	ND	5.7	4.3	5.0	4.6	4.6	5.0	5.2	4.6	5.3	ND	5.7
Glycerol (g/L)	3.6	3.7	4.1	3.6	4.3	ND	3.6	2.9	4.3	4.6	3.7	4.0	3.8	4.0	3.9	ND	3.3
Acetic acid (g/L)	1.2	1.2	1.3	1.2	1.6	ND	1.2	1.1	1.4	1.6	1.3	1.2	1.2	1.3	1.2	ND	1.2
Ethanol (g/L)	43.8	43.4	40.6	44.2	43.4	ND	44.3	37.4	43.0	43.5	43.9	43.0	43.4	43.1	44.0	ND	44.4
Ethanol yield																	
g/g of glucose	0.46	0.43	0.45	0.45	0.44	ND	0.47	0.46	0.44	0.46	0.46	0.43	0.43	0.48	0.45	ND	0.47
yield in % of the theoretical maximum	90	84	88	88	86		92	90	86	90	90	84	84	94	88		92
MNS 10% of glucose 5%xylose at 40 °C																	
Glucose (g/L)	29.3	43.4	49.2	24.4	ND	26.6	23.6	ND	16.0	22.6	ND	ND	37.7	ND	25.6	26.6	ND
Xylose (g/L)	48.2	49.7	49.2	49.5	ND	49	49	ND	48.7	49.3	ND	ND	49.2	ND	49.4	49.3	ND
Xylitol (g/L)	5.3	4.4	4.6	5.5	ND	5.0	6.4	ND	6.0	5.5	ND	ND	5.6	ND	6.3	5.5	ND
Glycerol (g/L)	3.7	3.5	2.8	3.3	ND	3.8	3.3	ND	4.0	3.8	ND	ND	3.3	ND	3.8	3.6	ND
Acetic Acid (g/L)	1.5	1.4	1.1	1.3	ND	1.2	1.3	ND	1.4	1.5	ND	ND	1.2	ND	1.4	1.4	ND
Ethanol (g/L)	29	22.1	12.4	32.9	ND	31.4	32.6	ND	36.8	33.3	ND	ND	25.9	ND	32.5	32	ND
Ethanol yield																	
g/g of glucose	0.41	0.39	0.32	0.43	ND	0.42	0.42	ND	0.44	0.43	ND	ND	0.41	ND	0.44	0.43	ND
% of the theoretical maximum	80	76	63	84		82	82		86	84			80		86	84	

Tab 3.2 Product formation by *S. cerevisiae* strains that presented the best fermentative abilities at 25 and 40 °C in MNS medium with glucose (100 g/L) and xylose (50 g/L) as substrate. (ND Not Detected)

At 25 °C, in MNS medium with 200 g/L of glucose, the isolates consumed at least 95% of the glucose available, with the exception of the strains 70 and 1.63. The formation of by-products, like glycerol and acetic acid, was very limited and variable between the different strains. In the same medium at 40 °C, the twenty-one strains consumed less glucose; however the ethanol yield was still high for all the strains tested. At this temperature in MNS medium with 20% of glucose, the strains that presented the best ethanol yield were *S. cerevisiae* 1.76, 45 and 43 with yields of 94, 90 and 82% of the theoretical maximum, respectively. This result indicates that a higher temperature influences the ability of the strains to consume glucose, but does not affect in the same extent the alcohol yield. The formation of glycerol and acetic acid is similar between the strains at 25 and 40 °C and this indicates that temperature does not influence the formation of these by-products.

The alcohol productions were not significantly influenced by the presence of xylose: at 25 °C, in MNS medium with 10% of glucose and 5% of xylose, *S. cerevisiae* 100 produced the same ethanol yield in both media (0.48 g ethanol per gram of glucose consumed, corresponding to 94% of the maximum theoretical yield). At 40 °C, in MNS medium with xylose, strains 45 and 1.63 produced ethanol with a slightly lower yield (0.44 g ethanol per gram of glucose consumed, corresponding to 86% of the maximum theoretical). This should indicate that the presence of this pentose in the fermentation medium does not influence their fermentative performance. This result is not in accordance with Favaro et al. 2012b, reporting that yeasts were influenced by the xylose addition. However, in that work the xylose concentration (100 g/L) was higher than that used here, and the uptake of this sugar by facilitated diffusion was favored by the greater concentration of this pentose in the medium. In fact, xylose in *S. cerevisiae* has been already described to be taken up mainly through non-specific hexose transporters encoded by the HXT (Hexose Transporters) 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).

3.2 Definition of a synthetic medium for the evaluation of the inhibitor tolerance in *S. cerevisiae* strains.

In order to study the different concentrations of sugars and inhibitors that could be present in the lignocellulosic hydrolysates, an extensive bibliographic research of works focused on pretreatments and fermentation of lignocellulosic substrates was made. In particular, the papers were collected on the basis of biomass or substrate used (such as spruce, wheat, corn fiber, corn stover, willow, aspen, pine, sugarcane bagasse, poplar, birch, energy crops) and the pretreatment conducted. The final aim

was to define minimum and maximum levels of concentration of sugars and inhibitors in order to have reference values for the next tolerance tests to inhibitors on selected *S. cerevisiae* strains.

For each type of lignocellulosic hydrolysate the concentration of sugars and inhibitors were summarized in the tables reported below (Tables 3.3-3.13).

Compounds	Two step diluite acid (Larsson etal. 1999)	Two step diluite acid (Nilvebrant et al.,2003)	Diluite acid Batch, Fed-Batch explosion (Gustafsson et al.)	Two stage diluite acid (Taherzadeh et al. 2007)	Diluite acid (Larsson et al., 2009)	Diluite acid (Taherzadehet al., 1998)	Two stage diluite-acid (Modig et al., 2008)	Two step diluite-acid (Almeida et al., 2009)	Two step diluite acid (Laarson et al., 2000)	Diluite acid + steam pretreatment (Taherzadehet al., 1997)	STEX with SO_2 (Boussaid et al., 1998)
Sugars											
Glucose	25.7	21.9	19.9,20.4	27.5	0-500	39.0	24.3	42.9	20.5	0.8-41.5	10-32
Xylose	3.5	8.5	2.6,3.0			3.3	5.6	10.4	7.0	1.7-12.4	2.5-6
Mannose	6.5	16.4	13.0,13.8	6.1		12.3	12.1	24.4	14.9	4.9-33.9	11.1-18.2
Galactose	3.7	3.3	5.3 , 6.7	1.6			2.9	7.7	2.9		2.4-3.6
Arabinose	0.6	1.7					1.4		1.4		1.0-1.95
Cellobiose	0.7										
Inhibitors											
Furans											
HMF	5.9	2.0	2.3, 2.2			7.3	1.9	3.6	2.3	1.5-8.4	0.9-3.6
Furfural	1.0	0.5	0.6, 0.5			2.2	0.5	2.1	1.4	0.4-1.3	0.8-4.1
Weak acids											
Acetic Acid	2.4	3.1	2.8, 2.2		2.8	3.2	2.0	6.2	2.8	2.0-3.2	
Formic Acid	3.1	0.9							0.7		
Levulinic Acid	0.9	1.1							1.1		
Lactic acid					87.0mg/L						
Phenolic compounds											
Vanillin	0.1	0.1									
Syringaldehyde	0.1										
4-hydroxybenzoic acid	5.0	39.2mg/L									
Vanillic acid	3.4mg/L	17.2 mg/L									
Cathecol	9.0mg/L	1.9mg/L									
Hydroquinone	17.0mg/L										
Coniferyl aldehyde	35.0mg/L	54.0mg/L									
Acetoguaiacone	7.0mg/L	0.1									
Cinnamic acid		1.1mg/L									

Table 3.3. Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of spruce. STEX: Steam Explosion

After a first analysis of Table 3.3, it is evident that the pretreatment of softwood biomass like spruce, especially with dilute-acid, leads to the formation of high concentrations of inhibitory compounds, in particular furans (Taherzadeh et al., 1997 and 1998), and fermentable sugars like

glucose (Larsson et al. 2009). In particular, there is a high prevalence of HMF respect to furfural and this evidence is justified by the fact that HMF comes from the degradation of hexose sugars, which are abundant in spruce hydrolysates.

Compounds	Diluite acid + steam pretreatment (Almeidaet al., 2007)	Two stage diluite acid pretreatment (Saha et al., 2005)	Diluite acid hydrolysis (Qi et al., 2010)	Diluite acid hydrolysis (Davis et al., 2005)	Alkaline wet oxidation (Schmidtet al., 1997)	Alkaline wet oxidation (Klinke et al., 2003)
Sugars						
Glucose	6.4	9.9-12	12.9	7.4	1.1	30.0
Xylose	35.4	12.4-13.4	130.0	19.6	7.4	6.0
Mannose	0.6					
Galactose	1.1	0.7-1.3				
Arabinose		4.7-5	21.3	11.8	1.3	1.2
Cellobiose				0.9		
Inhibitors						
Furans						
HMF	0.6	0.8	0.3	1.1		
Furfural	1.8	3.2-3.8	4.5	5.6		
Weak acids						
Acetic Acid	4.0	2.3-2.5	26.3	2.5	2.1	10.1
Formic Acid					1.5	9.1
Malic acid						5.6
Glycolic acid					1.1	5.6
Lactic acid				5.3		
Oxalic acid					0.7	
Maleic acid					0.2	
Phenolics compounds						
Vanillin						10 mM
Syringaldehyde						10 mM
Acetosyringone						10 mM
Syringic acid						10 mM
4-hydroxybenzoic acid						10 mM
Vanillic acid						1.68
Acetovanillone						10 mM
4-Hydroxybenzaldehyde						10 mM
4-Hydroxyacetophenone						10 mM

Table 3.4. Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of wheat straw.

Concerning the pretreatment and fermentation of wheat straw, in contrast to what was observed for spruce hydrolysates, there is a clear predominance in the release of pentose sugars (Table 3.4). Consequently, after the pretreatment, the furfural was present in a higher amount than HMF (Davis et al. 2005). As shown in Table 3.4, the production of acetic acid after pretreatment of wheat straw is higher compared to that produced after pretreatment of spruce biomass. Wheat straw contains an higher amount of hemicellulose, especially in acetylated form, and this, once exposed to the complete pretreatment, leads to high concentrations of acetic acid (Qi et al., 2005).

Compounds	Dilute acid + saccharification (Saha et al., 1999)	Dilute acid hydrolysate (O'Brienet al., 2000)	Water + steam pretreatment (Allenet al., 2001)
Sugars			
Glucose	41.7-52	48.5-52.3	0-7.5
Xylose	27.6-28.5	47.6-50.6	0-5.1
Mannose			
Galactose	4-4.3	10.4-11.4	0-1.0
Arabinose	18.9-20.7	26.9-29.5	0-2.9
Inhibitors			
HMF		0.1	0.0-0.3
Furfural		0.5	0.2-0.9
Acetic Acid		6.2	

Table 3.5 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of corn fiber

Table 3.5 and 3.6 show the data of concentrations of sugars and inhibitors collected from papers about the pretreatments of two different parts of the same plant, corn fiber and corn stover, respectively. Unlike to that observed for spruce and wheat straw hydrolysates, the pretreatments of this biomass lead to the formation of a lower concentration of inhibitory compounds, especially furans. As indicated by Saha et al (2005), this type of substrate is easily convertible to fermentable sugars in comparison to the hardwood materials. Therefore, the pretreatment conditions for corn are milder than those used for other types of lignocelluosic substrates.

Compounds	Alkaline, acid wet oxidation (Varga 2004)	Acqueous ammonia pretreatment (Chen et al., 2009)	Sodium hydroxide pretreatment (Chen et al., 2009)	Diluite-acid + steam pretreatment (Zacchi et al, 2006)	Diluite-acid steam pretreatment (Ohgren et al., 2006)	Diluite acid + steam explosion (Zimbardi et al., 2007)	Dilute-acid steam retreated (Ohgren et al., 2006)	Acid impregnated+steam pretreatment (Varga and Zacchi, 2004)
Sugars								
Glucose	11.4, 45.4	27.6	36.1	4.2	6.6	99.5	5.9-7.2	1-13.1
Xylose	2.5, 7.6	6.7	15.5	34.9	24.8	60.5	36.0-36.2	5.6-19.6
Mannose								
Galactose						2.5		
Arabinose	6.4, 17.9					5.7		1.1-3-4
Inhibitors								
Furans								
HMF				0.2	0.6	0-0.5	0.2	0.1-0.5
Furfural		ND	ND	1.3	0.7	0-4.7	1.3-1.5	0.2-2.7
Weak acids								
Acetic Acid		0.3	0.2		2.6	2.0-19.0	2.1-2.2	0.7-2.4
Formic Acid						1.0-4.2		
Lactic acid					2.0			
Phenolic compounds								
Vanillin	57.0, 74.0 mg/L							
Syringaldehyde	20.0, 38.0 mg/L							
Acetosyringone	18.0 mg/L							
Syringic acid	28.0, 42.0 mg/L							
4-hydroxybenzoic acid	51.0, 54.0 mg/L							
Vanillic acid	55.0, 57.0 mg/L							
Homovanillic acid	12.0, 28.0 mg/L							
Acetovanillone	13.0, 58.0 mg/L							
Guiacol	12.0, 13.0 mg/L							
Phenol	4.0, 6.0 mg/L							
Syringol	5.0 mg/L							
4-Hydroxybenzaldehyde	54.0, 102.0 mg/L							
4-Hydroxyacetophenone	8.0 mg/L							
p-Coumaric acid	32.0, 35.0 mg/L							
Ferulic acid	7.0, 9.0 mg/L							

Table 3.6 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of corn stover.

Table 3.7 show the concentrations of sugars and inhibitors released after the pretreatements of birch. The pretreatments released variable amounts of sugars and inhibitors, although the only type of reported pretreatments is steam explosion coupled with dilute-acid. As observed for wheat straw, also birch hydrolysates have high concentrations of xylose, derived from hemicelluloses, and this leads to the formation of considerable amounts of acetic acid (Taherzadeh et al., 1997).

Compounds	Diluite-acid + steam (Taherzadeh et al., 1998)	Diluite-acid + steam pretreatment (Taherzadeh et al., 1997)	Acid hydrolysis+steam pretreatment (Johansson et al., 2001)	Diluite-acid + steam pretreatment (Taherzadeh et al., 1997)	Dilute-acid STEX (Jasinskas et al., 2009)
Sugars					
Glucose	24.5	7.6-34.4	5.7	22.4-25.2	14.5-15.2
Xylose	15.4	2.9-37.3	39.0	11.1-28.1	20.7-21.7
Mannose	2.5	3.3-8.2	3.5	4.1-7.9	1.7-2.0
Galactose			3.1		
Arabinose			1.7		3.0-4.2
Cellobiose					
Inhibitors					
HMF	2.4	0.2-5.8	0.2	2.6-4.5	0.0-0.2
Furfural	5.7	0.5-3.9	0.7	2.7-3.3	2.0-2.7
Acetic Acid		2.0-11.5		9.1-11.2	

Table 3.7 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of birch.

Compounds	Diluite-acid + steam pretreatment (Taherzadeh et al., 1997)	Acid hydrolysis (Fenske et al., 1998)	Diluite-acid pretreatment (McMillan etal., 1999)	Diluite acid + enzymatic hydrolysis (Wyman et al., 2009)	SO_2 explosion + enzymatic hydrolysis (Wyman et al., 2009)	AFEX + enzymatic hydrolysis (Wyman et al., 2009)	Alkaline pretreatment + acid hydrolysis (Cho et al., 2010)*	LHW, STEX (Negro et al., 2003)	Steam pretreatment (Weil et al., 1997)
Sugars									
Glucose	19.4-45.4	1.9-2.4	4.0-6.5	41.4	33.2	62.3	57.0-59.5	2.1, 1.9	42.6
Xylose	4.7-36.6	14.3-17.4	17.2-23.4	22.3	25.8	16.2	27.8-29.7	10.8, 8.6	19.5
Mannose	3.8-11.6							1.8, 1.2	
Galactose								1.0, 1.1	
Arabinose								0.5, 0.8	
Inhibitors									
HMF	1.3-6.8						0.1-0.2	1.3, 0.2	1.6
Furfural	2.1-3.5						0.3	2.5, 0.9	
Acetic Acid	8.2-10.1	2.5-3.2	1.3-6.0	5.1	6.2	3.5	0.7-7.1	4.4, 2.9	
Formic Acid								0.6 , 0.5	0.4

Table 3.8 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of poplar. LHW Liquid Hot Water. AFEX Ammonia Fiber Explosion

Compounds	Steam explosion with NI, SO_2 e H_2SO_4 impregnation (Martin et al., 2002)	Acid hydrolysis (Chandel et al., 2007)	Acid hydrolysis (Alves et al., 1998)	Acid hydrolysate (Carvalho et al., 2002)	Acid hydrolysis (Neureiter et al., 2002)	Wet oxidation pretreatment (Martin et al., 2007)	Enzymatic hydrolysates (Martin et al., 2007)
Sugars							
Glucose	20.6, 22.8, 25.6	1.8-5.8	2.1	1.7	1.6	0.1-1.7	25.4-30.0
Xylose	8.1, 10.6, 4.4	16.5-21.5	15.7	20.3	7.6	0.7-8.0	4.9-11.0
Mannose					0.2		
Galactose					0.4		
Arabinose	0.8, 0.9, 0.4	1.8-2.9	2.3	1.6	0.4	0-0.8	
Cellobiose							
Inhibitors							
Furans							
HMF	0.4, 0.4, 1.4	Total Furans 0.9-3.4			0.1		Total Furans 0.7-4.5
Furfural	1.1, 1.2, 3.1			0.3	1.7	0.0-0.3	
Weak acids							
Acetic Acid	4.2, 4.4, 4.9	3.5-6.7	3.9	2.6	1.2	Total Carboxylic acids 1.4-3.8 (Succinic, Glycolic, Formic and Acetic)	Total Aliphatic acids 2.5-10.1 (Formic, Acetic and Levulinic)
Formic Acid	1.0, 1.1, 2.5						
Levulinic Acid	0, 0, 2.7						
Phenolic compounds							
Vanillin	50.0, 50.0, 58.0 mg/L	Total Phenolics 0.6-3.0				Total Phenolics 0.8-2.5	Total Phenolics 1.4-2.8
Syringaldehyde	26.0, 25.0, 35.0 mg/L						
4-hydroxybenzoic acid	11.0, 9.0, 10.0 mg/L						
Vanillic acid	20.0, 15.0, 36.0 mg/L						
Cathecol	17.0, 10.0, 14.0 mg/L						
Guiacol	4.0, 3.0, 16.0 mg/L						
Phenol	3.0, 3.0, 22.0 mg/L						
Benzoic acid	5.0, 5.0, 8.0 mg/L						
4-Hydroxybenzaldehyde	110.0, 100.0, 100.0 mg/L						
Protocatechuic acid	5.0, 3.0, 3.0 mg/L						
p-Coumaric acid	480.0, 410.0, 170.0 mg/L						
Caffeic acid	9.0, 5.0, 1.0 mg/L						
Ferulic acid	210.0, 190.0, 56.0 mg/L						

Table 3.9 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of sugarcane bagasse

As shown in Table 3.8 for the poplar biomass, the subsequent enzymatic saccharification of the material leads to a lower release of inhibitory compounds for the fermentation as compared to the others substrates previously reported (McMillian et al., 1999 and Wyman et al., 2009). Poplar biomass pretreatments allow to recover a good amount of fermentable sugars (Cho et al., 2010), while the pretreatments of sugarcane bagasse do not result in high sugars concentrations (Table 3.9). Concerning the production of inhibitors, the pretreatments of sugarcane bagasse produce a remarkable amount of weak acids, compared to the production of furans (Martin et al., 2007).

Compounds	Diluite-acid + steam pretreatment (Jonsson et al., 1998)	Diluite-acid + steam pretreatment (Taherzadeh et al., 1997)	Diluite-acid steam pretreatment (Sassner et al., 2005)	Diluite -acid steam pretreatment (Szengeyl et al., 1997)	Acid hydrolyzates (Bakker et al., 2004)	Diluite-acid steam pretreatment (Kovacs et al., 2008)
Sugars						
Glucose	50.0	4.1-6.4	22.8	0.3-1.3	58.7	0.3
Xylose		0.6-10.0	18.0	1.2-4.6	21.2	0.5
Mannose		1.8-5.9				0.1
Galactose			3.3			0.1
Cellobiose				0-0.1		
Inhibitors						
Furans						
HMF		0.3-3.9	0.5	0.0-0.2		
Furfural		0.3-3.2	2.0	0.3-1.0	0.3-1.0	
Weak acid						
Acetic Acid			6.8	0.7-2.6	4.4-8.2	0.2
Lactic acid						
Phenolic compounds						
Vanillin	430.0 mg/L					
Cathecol	440.0 mg/L					
Guiacol	615.0 mg/L					
Phenol	35.0 mg/L					
trans-isoeugenol	25.0 mg/L					
o-cresol	10.0 mg/L					
4-Hydroxybenzaldehyde	10.0 mg/L					

Tab 3.10 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of willow

Table 3.10 shows that the pretreatment of willow biomass allows to recover small amounts of fermentable sugars. For this type of biomass, dilute-acid is the only pretreatment used prior to fermentation.

Concerning energy crops (Table 3.11 below), variable amounts of fermentable sugars are produced. In particular *Sorghum* (Salvi et al. 2010) and *Triticale* (Jasinkas et al., 2010) pretreatments gave high glucose value while silvergrass (Guo et al., 2008) and switchgrass (Fenske et al., 1998) resulted in high xylose concentrations. Regardless of the type of pretreatment used (dilute acid or alkaline), energy crops release a very low amount of inhibitory compounds. The latter two evidences make energy crops an interesting biomass for the production of second generation bioethanol.

Compounds	Miscanthus alkaline treatment (de Vrije et al., 2009)	Silvergrass dilute-acid treatment (Guo et al., 2008)	Clover-ryegrass mixtures wet oxidation pretreatment (Martin et al., 2008)	Switchgrass acid hydrolysis (Fenske et al., 1998)	Sorghum diluite ammonia pretreatment (Salvi et al., 2010)	Triticale diluite-acid STEX (Jasinskas et al., 2009)
Sugars						
Glucose	33.0	3.7	4.1-5.3	5.7-5.8	45.8-47.9	0.9-40.2
Xylose	11.1	24.2	4.5-7.8	22.6-25.1	12.4-13.7	0.2-12.9
Mannose						0.1-1.3
Galactose	0.2					
Arabinose	1.6	1.2				0.2-4.6
Inhibitors						
Furans						
HMF			0.0-0.2		ND	0.0-2.2
Furfural		1.0	0.0-0.3		ND	0.6-2.5
Weak acids						
Acetic Acid	5.0-25.2	3.5	0.5-1.7	1.7-2.3	0-1	
Formic Acid			0.5-1.5			
Glycolic acid			0.2-0.8			
Lactic acid	0.1-1.3					

Table 3.11 Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of energy crops

Compounds	Barley straw diluite-acid (Almeida et al., 2009)	Pine diluite acid + steam pretreatment (Taherzadeh et al., 1997)	Eucalyptus wood diluite acid pretreatment (Cruz et al., 1999)	Douglas Fir diluite acid steam pretreatment (Robinson et al., 2003)	Rice straw diluite-acid steam pretreatment (Karimi et al., 2005)	Mixed hardwood (50% maple+ beech, birch and poplar) green liquor extraction (Walton et al., 2009)	Olive tree biomass diluite acid pretreatment (Cara et al., 2008)	Rice straw diluite acid pretreatment (Hsu et al., 2010)	Oak diluite acid hydrolysis (Converti et al., 1998)
Sugars									
Glucose	1.1	8.9-40.7	3.6	13.8-14.9	6.0	2.0	12.6-22.2	2.3-8.5	9.0-13.2
Xylose	3.5	0.9-9.5	18.0	3.8-4.9		8.7	0.6-17.2	8.9-14.0	43.5-106.0
Mannose	1.0	4.1-27.3		10.9-15.4			0.2-1.6		3.4-6.8
Galactose	0.5			3.3-3.9			0.4-3.0		3.3-8.6
Arabinose			0.6	1.5-1.8		1.0	0.8-4.8		1.6-2.9
Cellobiose					0.8				
Inhibitors									
Furans									
HMF	0.9	1.7-7.9		1.2-1.6		0.2	0.4-4.8	0.1-0.7	0.3-0.5
Furfural	3.1	0.7-1.7	0.5	0.3-0.4		0.4	0.2-6.6	0.3-3.6	0.9-4.2
Weak acids									
Acetic Acid	5.6	1.8-3.7	2.2		0.0-0.6	25.0	0.8-7.8	1.0-2.0	11.0-27.0
Formic Acid						3.9	1.0-2.2		
Levulinic Acid							0.4-3.0		
Pyruvic acid					0.2-2.8 mg/g				
Lactic acid					7.0-16.7	1.9			
Succinic acid					0.1-1.8 mg/g				

Tab 3.12. Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained after the pretreatment of different types of lignocellulosic biomasses

Table 3.12 describes the concentration of sugars and inhibitors obtained from different types of lignocellulosic biomasses. Pretreatments of pine (Taherzadeh et al., 1997) and oak (Converti et al, 1998) released good concentrations of sugars; in particular pine processing allow to recover a good concentration of glucose compared to oak. Table 3.13 reported below summarizes the concentrations of sugars and inhibitors used to prepare synthetic media with inhibitors cocktails. Regarding furans, the maximum concentrations used were 6.0 g/L for HMF and 5.0 g/L furfural (Larsson et al., 1998). The same author has proposed the highest concentrations for weak acids (30.0 g/L for acetic acid or 23.0 g/L for formic acid).

Compounds	Inhibitor Cocktail (Martin et al., 2003)	Defined medium (Larsson et al., 2000)	Medium Delgenes (Delgenes et al., 1996)	Define Medium + Modified LB (Barber et al., 2000)	Medium Oliva (Oliva et al., 2005)	Spruce medium + inhibitors cocktail (Persson et al., 2002)	Spruce diluite acid + inhibitors (Larsson et al., 1998)
Sugars							
Glucose	25.0	20.0	20.0	5.7	30.0		
Xylose				39.4			
Mannose				3.5			
Galactose				3.1			
Arabinose				1.7			
Total fermentable sugars						35.0	60.0
Inhibitors							
Furans							
HMF	3.8		1.0, 3.0, 5.0	0.2		3.8	0.5, 1.3, 6.0
Furfural	2.9		0.5, 1.0, 2.0	0.7	0.0-2.0	2.9	0.5, 1.2, 2.5, 4.6
Weak acids							
Acetic Acid	4.5		5.0, 10.0, 15.0	10.8	0.0-10.0	4.5	3.0, 4.8, 6.0, 9.0, 12.9, 15.0, 30.0
Formic Acid	3.5					3.4	0.7, 5.0, 4.0, 6.9, 9.9, 11.5, 23.0
Levulinic Acid							0.6, 5.2, 9.3, 10.4, 23.2, 58.0
Phenolic compounds							
Vanillin		0.02, 0.2, 1	0.5, 1.0, 2.0				
Isovanillin		0.02, 0.2, 1					
o-Vanillin		0.02, 0.2, 1					
Syringaldehyde			0.2, 0.7, 1.5				
Cathecol		0.02, 0.2, 1			0.0-1.0		
Hydroquinone		0.02, 0.2, 1					
p-Benzoquinone		0.02, 0.2, 1					
Coniferyl aldehyde	0.2	0.02, 0.2, 1				0.2	
Coniferyl alcohol		0.02, 0.2, 1					
Cinnamic acid	0.1	0.02, 0.2, 1					
4-Methoxy-cinnamic acid		0.02, 0.2, 1					
3-Methoxy-cinnamic acid		0.02, 0.2, 1					
p-Coumaric acid		0.02, 0.2, 1					
Ferulic acid		0.02, 0.2, 1				1.0	
Isoeugenol		0.02, 0.2, 1					
Eugenol		0.02, 0.2, 1					
3-(3,4-Dimethoxy-phenyl)-propanoic acid		0.02, 0.2, 1					
3,4-Dimethoxy-cinnamic acid		0.02, 0.2, 1					
3-Hydroxy-4-methoxy-cinnamic acid		0.02, 0.2, 1					
3,5-Dimethoxy-cinnamic acid		0.02, 0.2, 1					

Tab 3.13. Sugars and inhibitors concentrations (g/L, where not otherwise stated) obtained from papers about the fermentation of nutrient media with the addition of different inhibitors cocktails

According to the tables previously discussed, dilute-acid pretreatment applied to several starting materials ends to the highest amount of glucose and xylose. The situation is completely opposite for the inhibitors released during this process. Alkaline or steam pretreatments released a lower amount of inhibitors (furans, weak acids and phenolic compounds) than dilute acid. This evidence may be due to the fact that harsh conditions involved in dilute acid pretreatment (using strong acids like H_2SO_4) easily yield inhibitory substances, as described by Balat et al. (2008).

On the basis of the above considerations, a new cocktail of inhibitors to be used in the tolerance evaluation was proposed. In general, the concentrations of the inhibitors are the average values calculated from all the data found in literature, increasing by a 1.5 factor. The detailed compositions of the resulting media are reported in Material and Method section. In short, the concentration of the main inhibitors was assessed as following, in g/L: furfural 2.77, HMF 3.75, acetic acid 7.2, formic acid 2.44 and lactic acid 6.79.

3.3 Fermentative abilities of the most promising oenological *S. cerevisiae* strains in the presence of inhibitors.

The same strains, reported in Table 3.1 and 3.2 were tested in MNS (glucose 10% and xylose 5%) supplemented with the cocktail of inhibitors defined above. No strain was able to grow and ferment after 21 days of incubation (data not shown). This result indicates that the cocktail of inhibitors, at the concentrations tested, has a lethal effect on yeast cells. It is not possible to determine whether lethal effect is specifically due to acids or furans or there is a synergistic effect between these substances on the viability of the yeasts. For these reasons a minimum inhibitory concentration for each component of the cocktail had to be determined. However, as an alternative, a new yeast isolation programme based on an artificial selective pressure was considered to be a most promising strategy for the selection of a robust industrial yeast to be used for the second generation bioethanol.

3.4 Isolation of new yeast strains from grape marcs containing high inhibitors concentrations.

To this purpose, the choice of an appropriate environment was strategic. To search for yeasts with interesting fermentative properties and high robustness, grape marcs were chosen as an extreme environment because of limited nutrients (N and/or C sources), exposure to solar radiation, temperature fluctuations, low pH and ethanol. In order to enrich this peculiar ecological niche of yeasts with high inhibitors tolerance, grape marcs, collected immediately after crushing, were sprayed with the inhibitors cocktail named 1X (g/L: furfural 1.85, acetic acid 4.8, formic acid 1.63 and lactic acid 4.53). The inhibitors levels used in this experiment were specifically lower than those tested in the Paragraph 3.3 which resulted to be lethal for all the yeasts screened.

As described in Materials and Methods, five-kg aliquots of grape marcs were transferred into sterile plastic bags, with or without the inhibitors cocktail, and incubated at 30 and 40 °C. For strains isolation, samples of grape marcs were collected at regular intervals, serially diluted and plated on YPD supplemented with each single inhibitor at concentration 1X and another series at concentration 0.5X (g/L: furfural 0.92, acetic acid 2.40, formic acid 0.81 and lactic acid 2.26). YPD plates were also prepared with the addition of the entire inhibitors cocktail, both at concentration 1X and 0.5X. YPD agar without inhibitors was used as reference medium.

A number of 336 yeast isolates, considered representative of all the isolation programme, was stored at -80 °C.

3.5 Genetic characterization of new isolates

In order to proceed to a reliable identification, the yeasts obtained in the new isolation programme were analyzed by ITS amplification and subsequent sequencing, as described in the Paragraph 2.4 of Materials and Methods. The ITS1 and ITS4 primers were used to amplify a regionof the rRNA gene repeat unit, which includes two non coding regions designated as the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. As described in Figure 3.7, representative examples of the 336 tested yeasts, PCR products showed a high length variation in this region.

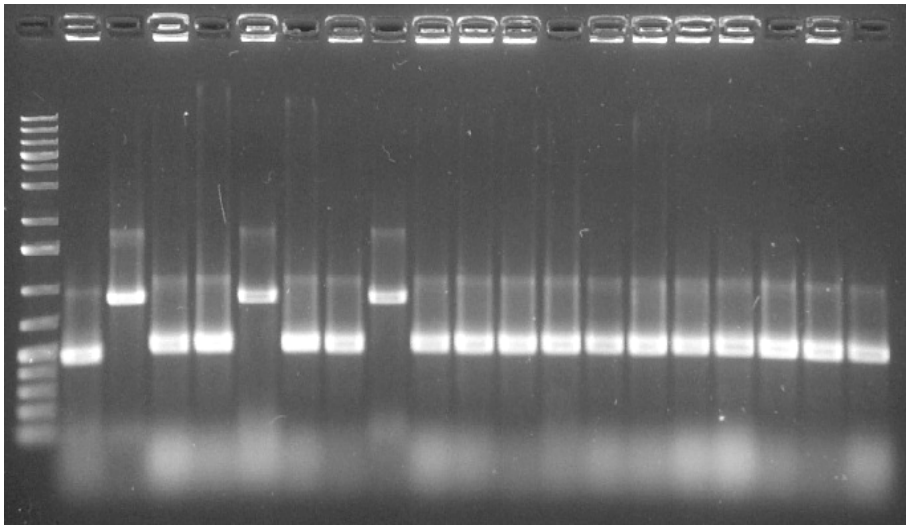

Fig 3.7. Agarose gel with amplification products of ITS region of new isolates. (Marker used 100 bp ladder, Euroclone)

When the rRNA gene region was digested with *Hinf*I, each species exhibited a specific restriction pattern (Figure 3.8), with nine major profiles.

Representative isolates from each *Hinf*I restriction pattern were then subjected to ITS region sequencing (BMR Genomics, University of Padova). Their 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.

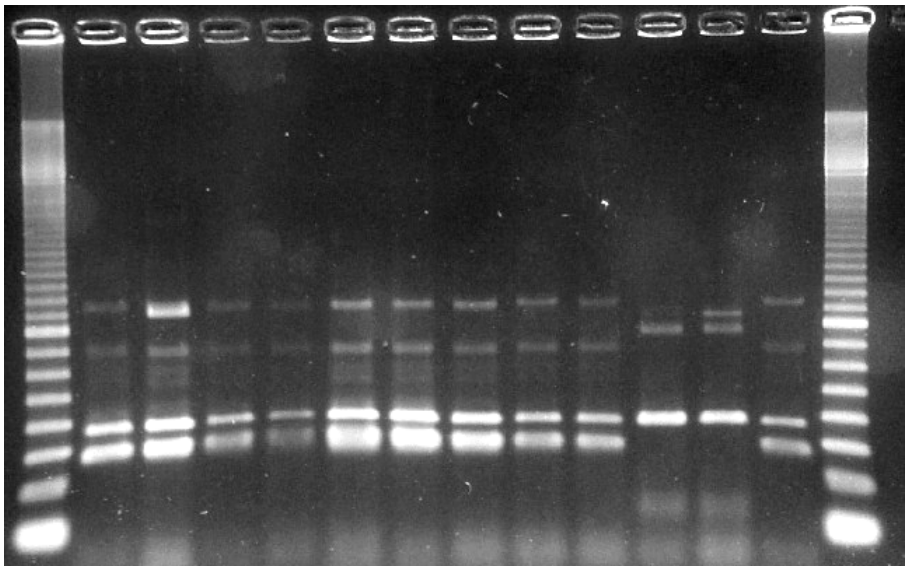

Fig 3.8. Restriction patterns of the amplification products of ITS after digestion with *Hinf*I enzyme. Marker used 100 bp ladder (Euroclone)

As reported in Table 3.14, ITS sequencing indicated that the 336 isolates belong to the following four major species: *Candida glabrata*, *C. zemplinina*, *Issatchenkia orientalis* and *S. cerevisiae*. All the yeasts identified as *I. orientalis* were isolated from marcs incubated at 40 °C, while the other strains have been obtained at 30 °C. These results are consistent with Kwon et al. (2011) reporting that *I. orientalis* has good tolerance to high temperature. Since this ability is one of the most desired traits for the development of an industrial CBP yeast, in the next future, the 155 *I. orientalis* isolates will be screened for their fermentative vigour and inhibitor tolerance, in order to evaluate their potential applicability as candidates for the production of ethanol from lignocellulose.

The high abundance of *C. zemplinina* isolates obtained in this study (Table 3.20) should not be considered surprising since such species has been already reported for good ethanol and acetic acid tolerance (Magyar et al., 2011). The presence of sixteen *C. glabrata* yeasts, among the 336 isolates, can be justified by the fact that this species possess high tolerance to acids, such as sulfate and acetate, as described by Watanabe et al. (2008)

Twenty-one yeasts have been identified as *S. cerevisiae*. All these strains were isolated from grape marcs incubated at 30 °C in the presence of the inhibitors cocktail.

		Temperature of isolation	
Yeast species	%	30°C	40°C
Issatchenkia orientalis	46.1	--	155
Candida zemplinina	42.9	144	--
Saccharomyces cerevisiae	6.2	21	--
Candida glabrata	4.8	16	--
	100	181	155

Table 3.14. Species identified with ITS sequencing and their percentage on a total of 336 selected isolates (temperatures of isolation are also reported)

Such yeasts, able to grow in the presence of high inhibitors concentrations, should be of great impact in the second generation bioethanol. In order to confirm this hypothesis, their fermentative ability and inhibitors tolerance were evaluated as described below. The *S. cerevisiae* were screened for their fermentative vigour in MNS medium with glucose and xylose, and their tolerance to inhibitors commonly present in lignocellulosic hydrolysates was tested.

3.6 Evaluation of the fermentative vigour of the newly isolated *S. cerevisiae* yeasts.

The twenty-one strains genetically identified as *S. cerevisiae* were evaluated for their fermentative abilities according to Delfini (1995) in MNS broth with 20% glucose or 10% glucose and 5% of xylose. *S. cerevisiae* 27P (Favaro et al., 2012a) was used as reference strain.

Figure 3.9 reports the fermentative kinetics of the newly isolated *S. cerevisiae* strains once inoculated at 25°C in MNS medium with 20% glucose. Most of the yeasts consumed about all the glucose available with a fermentative vigour even higher than that showed by the benchmark *S. cerevisiae* 27P. As compared to the oenological strains (Paragraph 3.1), the newly isolated *S. cerevisiae* presented higher fermentative vigour. This is a promising feature towards the selection of a host strain to be engineered for the lignocellulose-to-ethanol route. However, few strains (*S. cerevisiae* T1, T5, T18, T19, T20 and T21) exhibited a reduced glucose consumption. Considering that *S. cerevisiae* is an uppermost fermenting species, this behavior cannot be easily explained, but it will be further investigated in future studies.

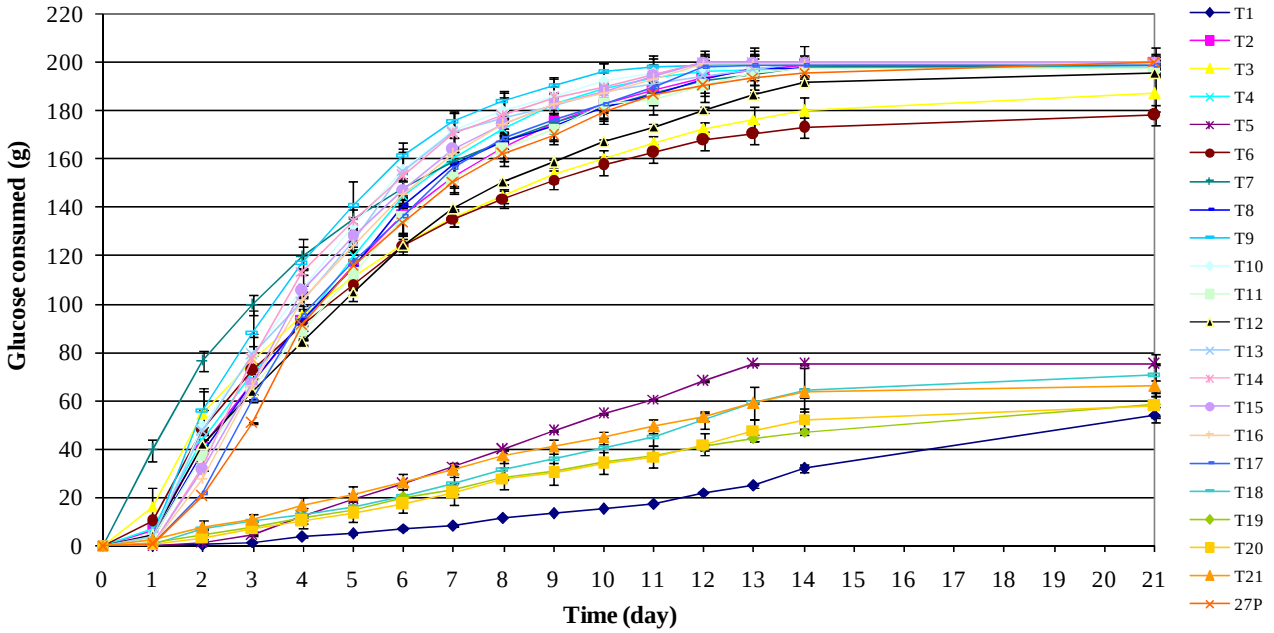

Fig. 3.9. Fermentative performance at 25 °C of 21 *S. cerevisiae* strains in MNS medium with glucose (200 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

Once inoculated at 40 °C, the strains showed much lower fermenting vigour (Figure 3.10). For most of the strains tested, the fermentative kinetics stopped after about 8 days of fermentation with the highest glucose consumption exhibited by *S. cerevisiae* T2 and T3. As compared to the fermentative abilities of oenological yeasts reported in the paragraph 3.1, the newly isolated strains showed lower performances. Nevertheless, their fermentative vigour was comparable to those of the benchmark yeasts.

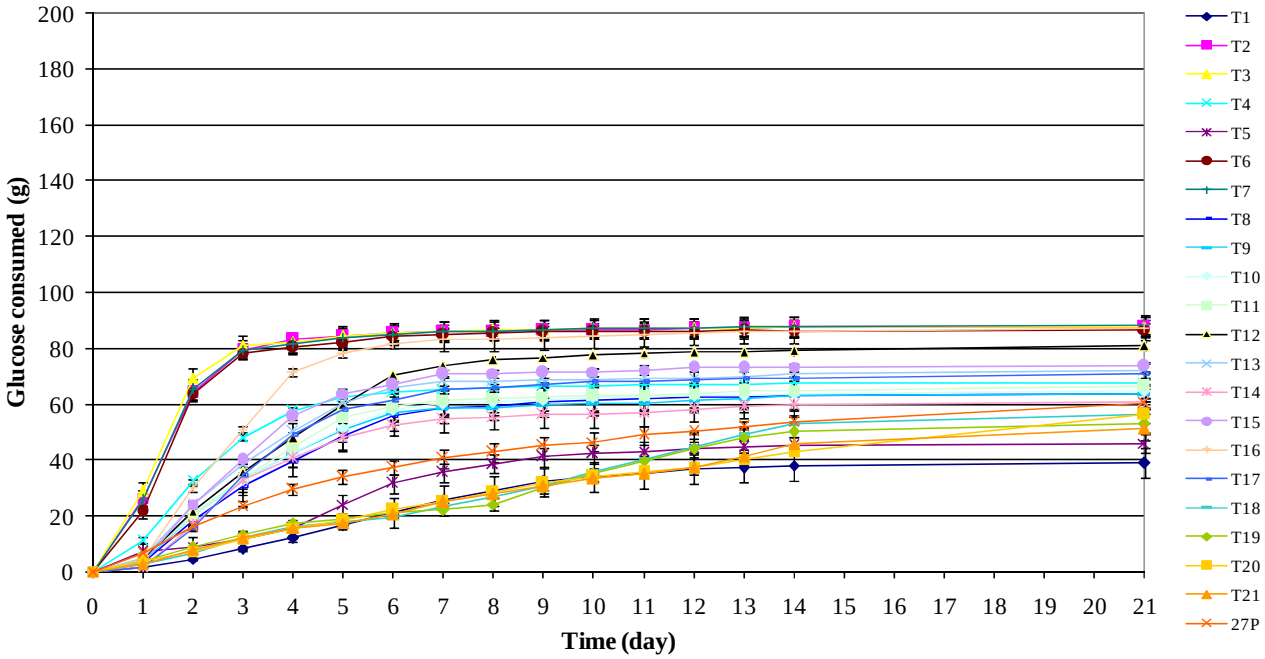

Fig. 3.10. Fermentative performance at 40 °C of 21 *S. cerevisiae* strains in MNS medium with glucose (200 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

Figures 3.11 and 3.12 show the fermentative kinetics of the *S. cerevisiae* strains in MNS medium with 10% glucose and 5% xylose at 25 and 40 °C, respectively. At 25 °C, the majority of the strains rapidly consumed all the glucose available within 7 days (Figure 3.11) while xylose content remained constant through the fermentation (data not shown). *S. cerevisiae* T1, T5, T18, T19, T20 and T21 confirmed their limited fermentative performance also in the presence of xylose.

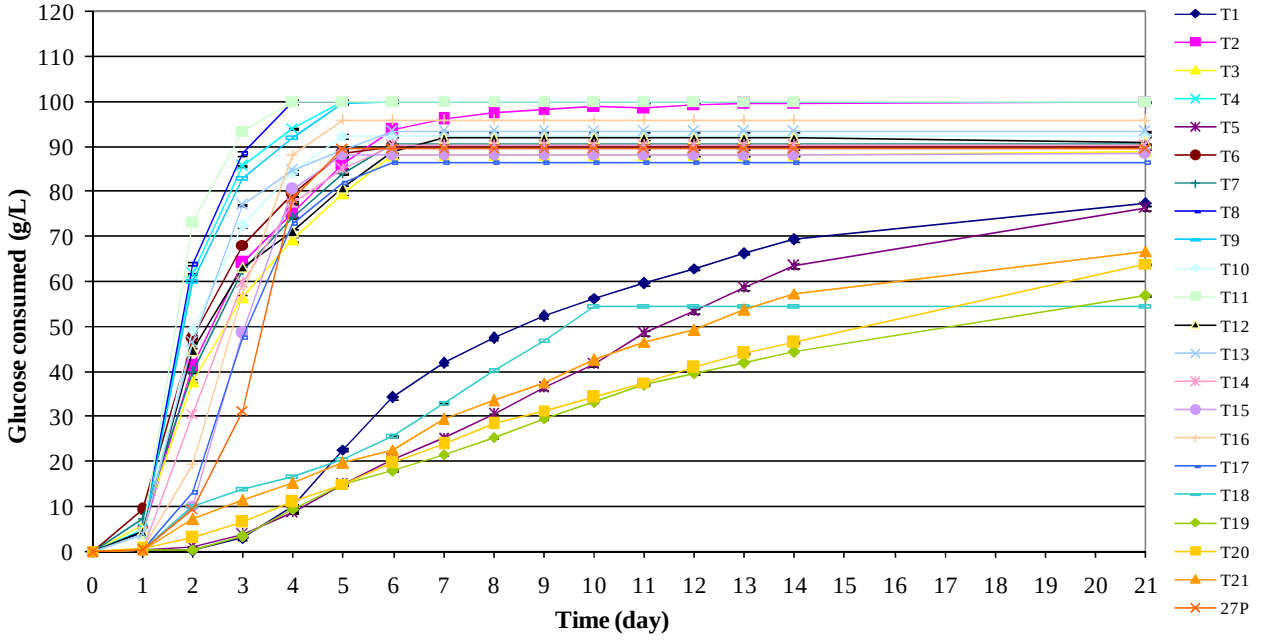

Fig. 3.11. Fermentative performance at 25 °C of 21 *S. cerevisiae* strains in MNS medium with glucose (100 g/L) and xylose (50 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

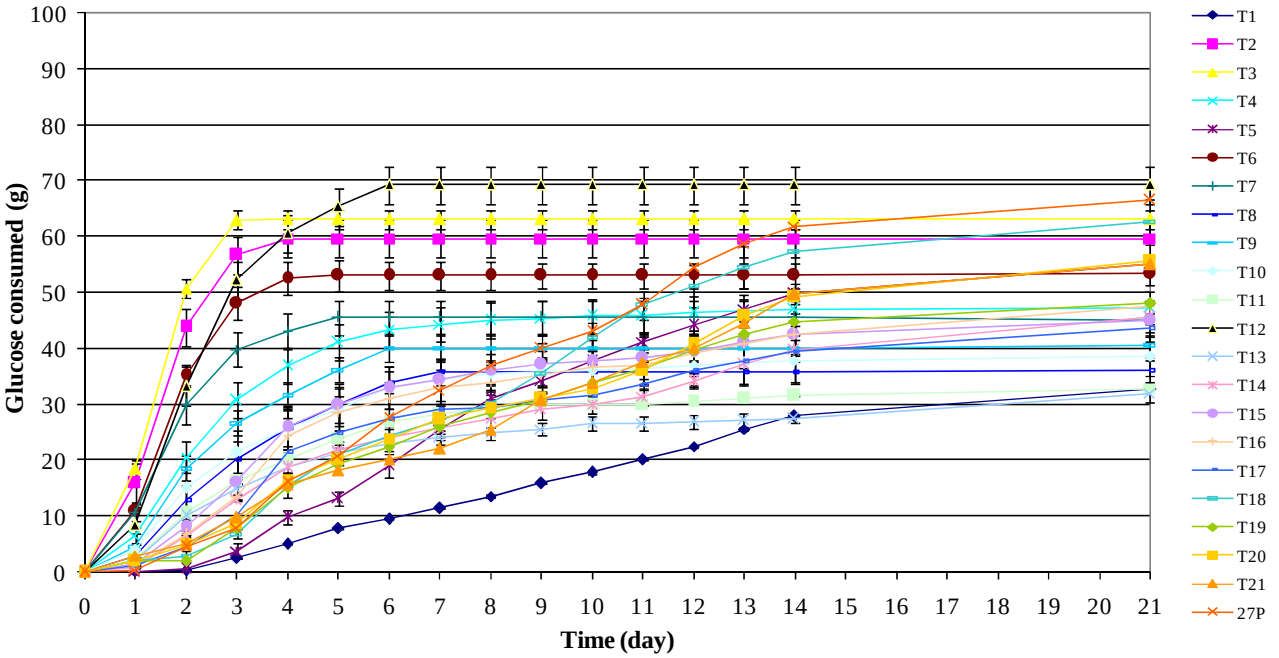

Fig. 3.12. Fermentative performance at 40 °C of 21 *S. cerevisiae* strains in MNS medium with glucose (100 g/L) and xylose (50 g/L) reported as cumulative sugar utilization. The experiment was conducted in triplicate and vertical bars represent SD.

At 40°C, few strains, *S. cerevisiae* T2, T3, T12 and T16, produced the highest fermenting vigour while other yeasts exhibited lower glucose consumption. This finding could be explained

considering that this newly *S. cerevisiae* strains have been isolated from grape marcs incubated at 30 °C.

Table 3.21 shows the grams of glucose consumed by the 21 *S. cerevisiae* strains once grown, for 21 days at 25 and 40 °C, in MNS with glucose 20% and in MNS with glucose 10% and xylose 5%.

At 25 °C, in MNS with 20% glucose, the strains T10, T13, T14, and T15 consumed all the glucose added. At 40°C, *S. cerevisiae* strains T2, T3, T6, T7 and T16 utilised the highest amount of glucose but they were not able to metabolise all the sugar available. In MNS containing both 10% glucose and 5% xylose, *S. cerevisiae* T4, T8, T11 presented the best fermentative vigour at 25°C while the yeasts T3, T12 and T18 exhibited the highest performance at 40 °C (68.8, 63.1 and 62.7 grams of glucose, respectively).

At 25°C, the most promising yeasts in both media resulted to be *S. cerevisiae* T8, T9, T11 and T13, while, at 40° C, *S. cerevisiae* T2 and T3 showed interesting fermenting abilities in both MNS broths.

	Grams of glucose consumed			
	MNS with 20% glucose		**MNS with 10% glucose and 5% xylose**	
Strains	**25 °C**	**40 °C**	**25 °C**	**40 °C**
T1	53.9	38.8	41.6	32.7
T2	195.2	85.5	91.0	59.7
T3	182.1	85.5	89.9	63.1
T4	193.5	66.0	100.0	42.9
T5	73.0	28.1	70.6	55.5
T6	173.4	84.7	89.8	53.3
T7	198.2	85.9	89.6	45.3
T8	197.4	62.1	100.0	36.7
T9	199.0	62.1	99.6	40.7
T10	200.0	63.1	92.3	35.2
T11	197.1	64.6	100.0	29.8
T12	195.2	78.7	90.8	68.8
T13	200.0	70.0	92.5	29.1
T14	200.0	59.4	90.5	45.5
T15	200.0	71.7	88.3	44.9
T16	199.3	84.8	93.8	47.5
T17	198.2	68.9	88.7	43.7
T18	70.6	56.1	56.8	62.7
T19	58.2	52.9	56.9	48.7
T20	57.3	51.1	61.9	5.56
T21	65.9	53.4	66.7	55.2
27P	195.6	58.7	87.7	56.5

Table 3.15: Grams of glucose consumed by *S. cerevisiae* strains in MNS with glucose 20% (left) and MNS with glucose 10% and xylose 5% (right) once grown for 21 days at 25 and 40 °C. The values are expressed as the mean of two replicates

After the analysis of the fermentative kinetics of the twenty-one *S. cerevisiae* isolates and the evaluation of the glucose consumption exhibited by the yeasts at 25 and 40 °C, the strains T2, T9, T11 and T12 were selected as the most talented fermenting yeasts. HPLC analysis of their fermentation broths were performed (Tables 3.16 and 3.17).

S. cerevisiae	**T2**	**T9**	**T11**	**T12**
MNS 20% glucose at 25 °C				
Glucose (g/L)	0.79	1.25	1.52	2.44
Glycerol (g/L)	4.64	5.53	5.37	4.81
Acetic acid (g/L)	0.53	0.73	0.87	0.63
Ethanol (g/L)	95.11	94.74	93.07	87.94
Ethanol yield				
g/g of glucose	0.48	0.48	0.47	0.44
% of the theoretical maximum	94	94	92	86
MNS 20% glucose at 40 °C				
Glucose (g/L)	51.52	58.13	57.95	53.92
Glycerol (g/L)	3.72	3.93	4.15	3.79
Acetic acid (g/L)	0.49	0.79	0.82	0.50
Ethanol (g/L)	37.77	28.64	29.94	36.94
Ethanol yield				
g/g of glucose	0.25	0.20	0.21	0.25
% of the theoretical maximum	49	39	41	49

Tab. 3.16. Product formation by *S. cerevisiae* T2, T9, T11, T12 strains after 21 days fermentation, at 25 and 40 °C, in MNS with glucose (200 g/L) as substrate.

S. cerevisiae	**T2**	**T9**	**T11**	**T12**
MNS 10% glucose 5% xylose at 25 °C				
Glucose (g/L)	0.68	0.69	0.60	0.69
Xylose (g/L)	35.13	34.7	34.35	34.48
Xylitol (g/L)	3.95	3.3	3.15	2.89
Glycerol (g/L)	2.85	3.4	3.38	3.03
Acetic acid (g/L)	0.32	0.56	0.55	0.46
Ethanol (g/L)	47.37	45.66	44.69	45.29
Ethanol yield				
g/g of glucose	0.48	0.46	0.45	0.46
% of the theoretical maximum	94	90	88	90
MNS 10% glucose 5% xylose at 40 °C				
Glucose (g/L)	29.06	37.97	37.32	14.5
Xylose (g/L)	36.8	37.29	36.77	35.05
Xylitol (g/L)	2.5	1.27	1.36	3.32
Glycerol (g/L)	2.52	2.45	2.61	3.5
Acetic acid (g/L)	0.48	0.49	0.57	0.52
Ethanol (g/L)	27.6	14.53	14.51	35.87
Ethanol yield				
g/g of glucose	0.38	0.23	0.23	0.42
% of the theoretical maximum	74	45	45	82

Tab. 3.17. Product formation by *S. cerevisiae* T2, T9, T11, T12 strains after 21 days fermentation, at 25 and 40 °C, in MNS with glucose (100 g/L) with xylose (50 g/L) as substrates.

The HPLC analysis confirmed the good fermentative performances exhibited by the strains in MNS medium with 20% glucose at 25 °C. Their fermenting abilities revealed to be comparable to those of the oenological strains reported in the Paragraph 3.1 (see Table 3.1 and 3.2).

At 40 °C, *S. cerevisiae* T2 and T12 exhibited promising ethanol yields in both media. Their glucose to ethanol conversion efficiency was about 49% of the theoretical maximum in MNS with 20% glucose and 74% and 82% of the theoretical, respectively, in MNS broth supplemented with 10 % glucose and 5% xylose.

3.7 Evaluation of the inhibitors tolerance of the newly isolated *S. cerevisiae* yeasts

The newly isolated *S. cerevisiae* strains were studied also for their inhibitor tolerance once grown in YNB and YPD broths supplemented with several inhibitors cocktails as described in Materials and Methods. The pH of the medium, after the addition of the inhibitors, was set to 4.5. Among a number of different cocktails tested, the following were adopted: *Cocktail B* (g/L: furfural 1.4, acetic acid 3.6; formic acid 1.2; lactic acid 3.4) and *Cocktail C* (g/L: furfural 2.1, acetic acid 5.4; formic acid 1.8; lactic acid 5.2).

For each strain the tolerance was evaluated by comparing the growth in the medium with and without the inhibitors, as OD value (%). The results are reported in Figure 3.13.

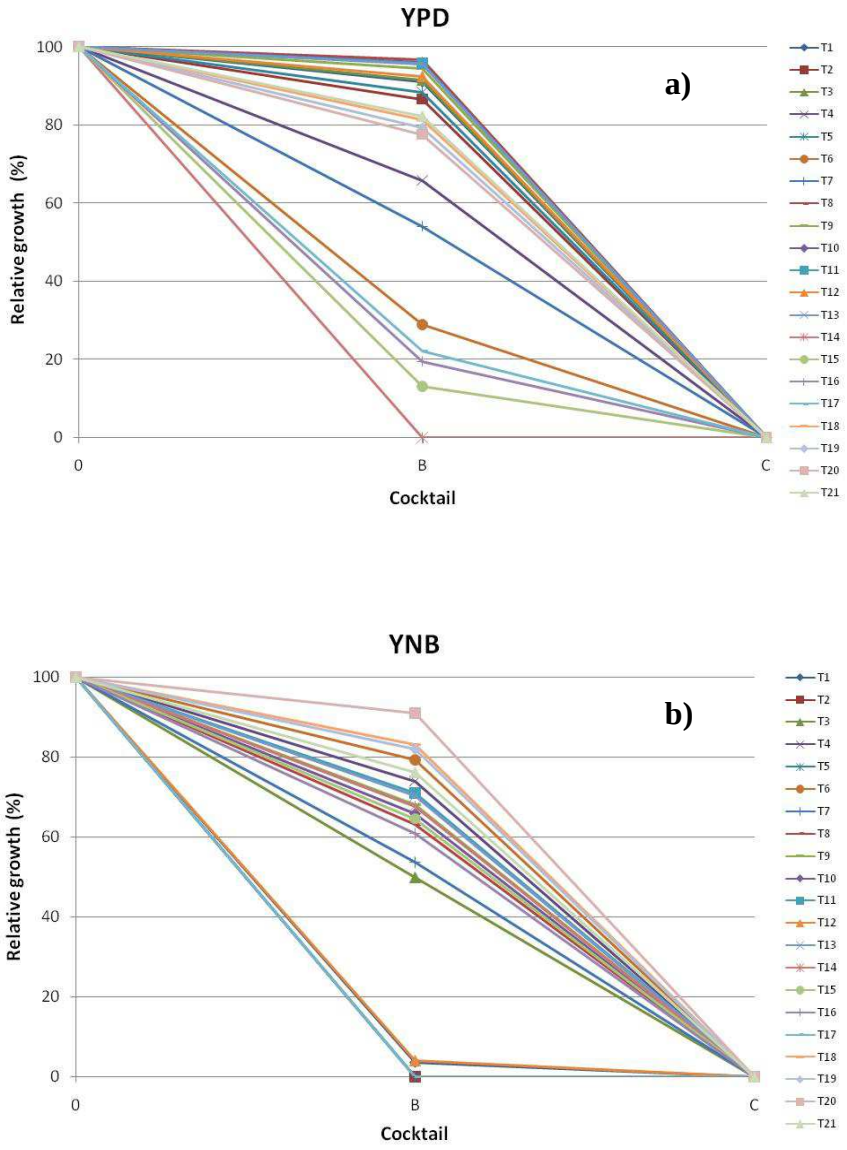

Fig .3.13. Growth of 21 *S. cerevisiae* strains in (a) YPD and (b) YNB with increasing concentration of inhibitors (cocktails B and C).

In both media (YPD and YNB), none of the 21 strains showed any growth in the presence of cocktail C and this indicates that the highest concentration of inhibitors had a lethal effect on the yeasts. In general, YPD medium (Fig 3.13 a) seemed to support cell growth better than YNB (Fig. 3.13 b). This evidence can be explained by the fact that YPD medium provides a greater supply of nutrients to the yeasts than YNB.

In YPD broth, a group of strains (*S. cerevisiae* T2, T8, T9, T10, T11, T12, T13) showed good tolerance to the inhibitors cocktail B. Among these strains, *S. cerevisiae* T11 exhibited also one of the highest inhibitors tolerance in YNB broth (Fig. 3.13b)

Interestingly, *S. cerevisiae* T2, T9, T11 and T12, selected as the most promising fermenting yeasts (Table 3.22), were among the strains having the best inhibitors tolerance in YPD medium (Figure 3.13a). This evidence indicates that such *S. cerevisiae* strains could be very attractive as yeasts to be used in the second generation bioethanol production and/or as host strains for the development of a CBP microbe.

3.8 Evaluation of growth at 40 °C of the selected strains in YNB medium

The four selected *S. cerevisiae* strains T2, T9, T11 and T12 were evaluated for their ability to grow aerobically in YNB medium at 40 °C. The incubation temperature of 30 °C was assessed as control. Yeast growth was evaluated at regular intervals monitoring optical density at 600nm (Figure 3.14).

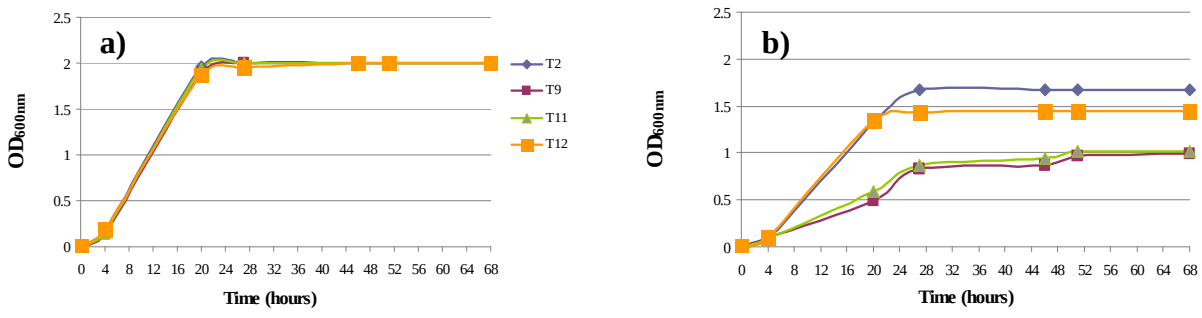

Fig. 3.14. Growth of the four *S. cerevisiae* strains obtained in YNB medium with 20 g/L of glucose at 30 (a) and 40 °C (b).

As expected, all the strains grew very well at 30 °C (Fig. 3.14a). At 40 °C, the yeast growth was affected and the highest OD value was reached by *S. cerevisiae* T2 after 28 hours while the strains T9 and T11 achieved lower OD values after a longer incubation time.

At final sampling time, aliquots of the YNB cultures were collected for the evaluation of dry biomass. Table 3.18 shows, for each strain, the value of dry biomass obtained at 40°C as percentage of that obtained at 30 °C. High values will be considered a good ability of the yeasts to grow at 40 °C.

Strain	% of dry biomass 40/30 °C
T2	56
T9	44
T11	30
T12	39

Table 3.18. Relative dry biomass of *S. cerevisiae* strains T2, T9, T11, T12 expressed as percent value of dry biomass obtained at 40 °C with respect to the value obtained at 30 °C.

S. cerevisiae T2 exhibited the highest relative growth. Moreover, according to the HPLC analysis conducted in the spent YNB samples of each strain, such yeast produced the best ethanol yields at both temperatures (data not shown). As a result, *S. cerevisiae* T2, having also high inhibitors tolerance (Figure 3.13), was selected in order to start a molecular biology programme for the development of an efficient cellulolytic yeast.

3.9 Engeneering *S. cerevisiae* strains for the expression of *bglI* β-glucosidase gene from *Saccharomycopsis fibuligera*.

To this aim, within a bilateral project with Stellenbosch University, the β-glucosidase gene sequence from *S. fibuligera* has been selected to be integrated into *S. cerevisiae* T2. *S. cerevisiae* yeasts 27P, F12, Fp96 and Y130 were also included in this research programme as reference strains. *S. cerevisiae* 27P has promising industrial traits (Favaro et al. 2012a), *S. cerevisiae* F12 is characterized by high thermo-tolerance (Favaro et al. 2012c) while *S. cerevisiae* Fp96 possess high thermo-tolerance and inhibitors tolerance (Favaro et al., 2012c) and YI30 has high furans tolerance (Favaro L., personal communication).

The wild type *S. cerevisiae* strains were transformed with the XhoI digested pBKD1_BGL1

integrative plasmid for chromosomal integration (see paragraph 2.7.1). pBKD1_BGL1 plasmid contains DNA sequences for the resistance to antibiotic geneticin (G418 resistance) and the *bglI* gene from *S. fibuligera* for the expression of the β-glucosidase enzyme (Figure 2.1).

3.9.1 Evaluation of the resistance to geneticin of the selected *S. cerevisiae* strains

Unlike laboratory haploid strains of *S. cerevisiae,* wild type isolates lack selective genetic markers and thus could only be transformed with vectors containing dominant selection markers such as zeocin and geneticin genes. Prior to proceed with the yeast engineering, each *S. cerevisiae* strain was evaluated for resistance to geneticin (Table 3.19).

***S. cerevisiae* strains**	**T2**	**27P**	**F12**	**Fp96**	**YI30**
Geneticin (µg/mL)					
0	++++	++++	++++	++++	++++
10	+	++++	++++	++++	+
20	ng	++	ng	ng	ng
30	ng	ng	ng	ng	ng
40	ng	ng	ng	ng	ng
50	ng	ng	ng	ng	ng
100	ng	ng	ng	ng	ng

Tab 3.19. Geneticin resistance of *S. cerevisiae* strains T2, 27P, F12, Fp96 and YI30 grown on YPD plates supplemented with increasing concentrations of antibiotic (++++ remarkable growth; ng: no growth)

The tested *S. cerevisiae* strains were very sensitive to the antibiotic. *S. cerevisiae* T2 and YI30 were not able to produce a remarkable growth already at 10 µg/mL of geneticin. At 20 µg/mL, only *S. cerevisiae* 27P produced a moderate growth on YPD agar plates. The following concentrations of geneticin were adopted for the experiment, µg/mL: 30 for strains T2, F12, Fp96, 35 for strain 27P and 25 for strain YI30.

3.9.2 Cellobiose-hydrolyzing yeast strain generation.

pBKD1_BGL1 plasmid extraction was performed following the protocol of Sambrook et al. (1989). The concentration and purity of plasmid was evaluated by Nanodrop instrument. The integrative plasmid pBKD1_BGL1 contains a unique XhoI site in the δ-sequence for an efficient homologous recombination into yeast chromosomes. As a result, pBKD1_BGL1 was digested with XhoI and used to transform *S. cerevisiae* T2, 27P, F12, Fp96 and YI30.

At the end of digestion, samples of the reaction were run into an agarose gel (Figure 3.15).

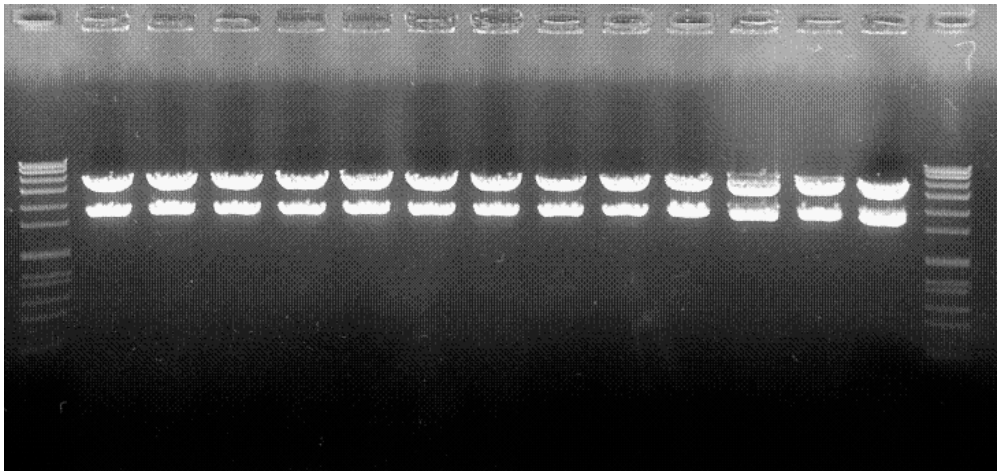

Fig. 3.15. Plasmid pBKD1_BGL1 digested overnight at 37 °C with XhoI enzyme (Marker used 1kb ladder, Euroclone)

To obtain the chromosomal integration of *bglI* gene *S. cerevisiae* strains were subjected to electroporation protocol (Favaro et al., 2012a). Recombinant cells were plated onto YPDS plates supplemented with geneticin and incubated at 30 °C for 3 days in order to select the positive transformed yeasts. Once grown on YPDS plates (Figure 3.16), the yeast colonies of greater size were selected for further tests of β-glucosidase activity. At the end of the integration phase, more than 170 recombinant strains (obtained from the selected five *S. cerevisiae* strains) were available and stored at – 80 °C.

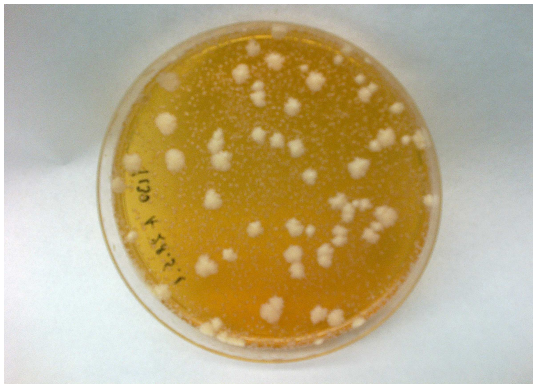

Fig 3.16. Recombinant colonies of *S. cerevisiae* T2 grown for 72 h on YPDS plates at 30 °C.

3.10 Evaluation of β-glucosidase activity by recombinant strains

3.10.1 Detection of enzymatic activity on MUG plates and evaluation of the mitotic stability of engineered yeasts

To evaluate their β-glucosidase, recombinant strains were transferred onto fresh YPD plates formulated with 4-methyl-umbelliferyl-β-D-glucopyranoside (4-MUG) as substrate. This substrate allows to detect a β-glucosidase activity as, once hydrolyzed, it produces fluorescence under the long-wave ultraviolet light.

The plates were incubated at 30 °C and examined after 24 and 48 h under the long-wave ultraviolet light. Many recombinant yeasts showed variable β-glucosidase activity giving a fluorescent halo, while the relative wild type strains did not hydrolyze MUG (data not shown). In particular, two engineered yeasts, T2[pBKD1_BGL1] (Figure 3.17) and 27P[pBKD1_BGL1], obtained respectively from their parental *S. cerevisaie* T2 and 27P, exhibited the largest hydrolysis halos and were maintained for further analysis.

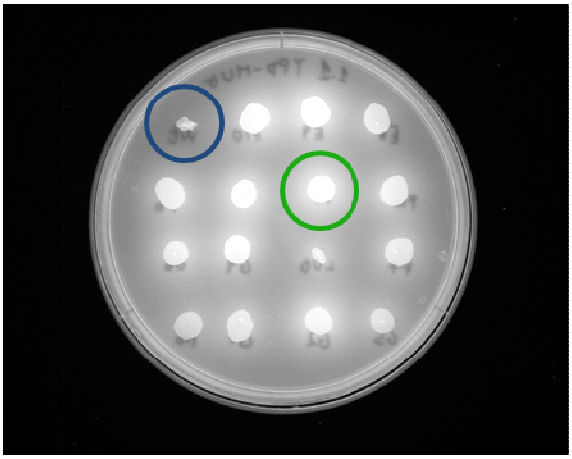

Fig. 3.17. β-glucosidase activity of the recombinant strain *S. cerevisiae* T2[pBKD1_BGL1], highlighted by green round. The parental *S. cerevisiae* T2 was spotted on the plate (blue round) as negative control.

To study their mitotic stability, the recombinants were grown in sequential batch cultures using non-selective YPD broth, as described in Favaro et al. (2012a). After 120 generations, both yeasts were found to be mitotically stable, since they displayed both resistance to geneticin and hydrolytic ability on 4-MUG.

Genomic DNA isolated from the engineered strains served as template for PCR to confirm the presence of the recombinant gene. As shown in Figure 3.18, the presence of the *bglI* gene from *S. fibuligera* was confirmed in both recombinants.

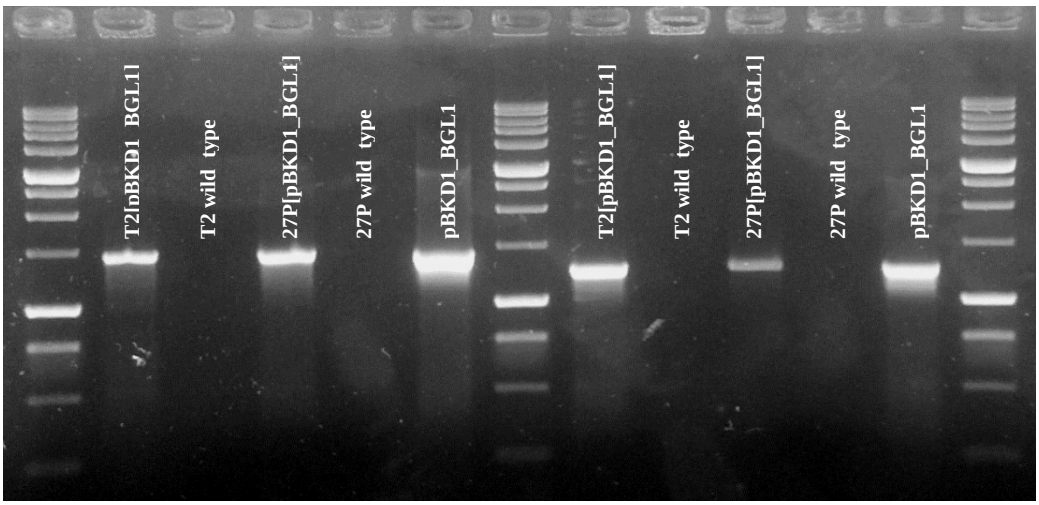

Fig. 3.18. PCR amplification products of the two different primers BGL1-1 (left part) and BGL1-2 (right part) using, as template, genomic DNA isolated from *S. cerevisiae* T2 [pBKD1_BGL1], 27P[pBKD1_BGL1] and their parental yeasts. pBKD1-BGL1 plasmid was used as positive control. 1 kb ladder was used (Euroclone).

3.10.2 Enzymatic assays of the recombinant strains

The enzymatic activity of engineered yeasts was then detected in liquid assays, using p-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate. The enzymatic assays were conducted also with the wild type *S. cerevisiae* T2 and 27P. β-glucosidase enzyme hydrolyzes pNPG and release *p*-nitrophenol which can be detected at 405 nm with spectrophotometric techniques.

In short, yeasts were grown in YPD medium and, at regular intervals, samples of cultures were mixed with the substrate (4mM pNPG in 0.1M citrate-phosphate buffer, pH 5.0) and incubated at 55 °C for 20 minutes, assay conditions previously described as optimal for BglI β-glucosidase of *S. fibuligera* by den Haan et al. (2005). Each sample, once inactivated, was transferred in a 96-well flat transparent microplate for the evaluation of the absorbance at 405 nm.

At final sampling time, aliquots of the YPD cultures were collected for the evaluation of dry biomass. β-glucosidase activity was expressed as units per mg dry cell weight (Meinander et al., 1996) where one unit was defined as the amount of enzyme required to produce 1 µmol of a *p*-nitrophenol or reducing sugar per minute under the assay conditions.

In Figure 3.19, the β-glucosidase activity of the recombinant strains T2[pBKD1_BGL1] and 27P[pBKD1_BGL1] is reported.

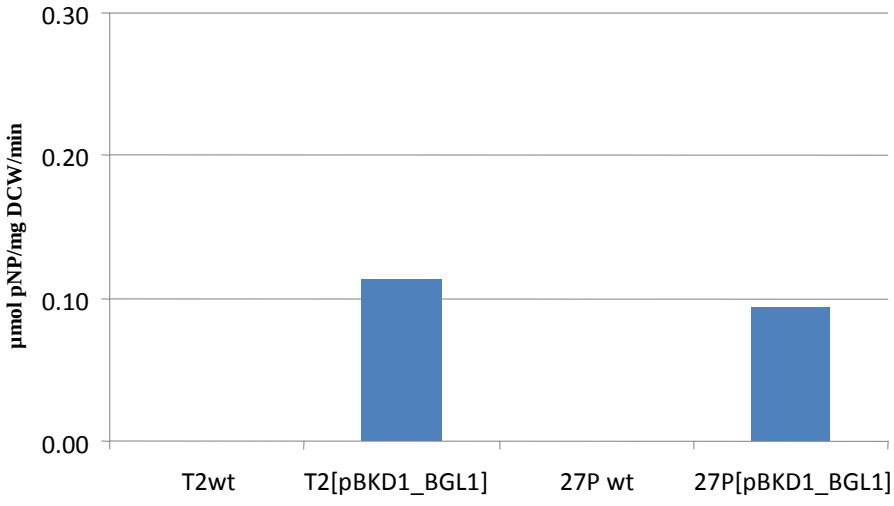

Fig. 3.19: β-glucosidase activity of the recombinant strains T2[pBKD1_BGL1] and 27P[pBKD1_BGL1] expressed as µmol of pNP released per mg of dry cell weight (DCW) per minute of reaction. *S. cerevisiae* T2 and 27P were assayed as negative control.

Their parental yeasts, *S. cerevisiae* T2 and 27P, did not produce any detectable activity while the engineered strains exhibited hydrolytic abilities. However, their enzymatic activity was found to

be lower than that previously described by den Hann et al. (2005) for the laboratory strain *S. cerevisiae* Y294 engineered with episomal plasmid for the production of BglI of *S. fibuligera* (0.7 µmol pNP/mg DCW/min). The higher β-glucosidase activity described by den Hann et al. could be explained considering that the laboratory strain would have produced high copy numbers of the episomal plasmid harboring the *bglI* gene sequence, resulting in higher β- glucosidase expression. Nevertheless, this is the first work reporting such hydrolytic ability in an industrial *S. cerevisiae* strain.

3.10 Growth of the recombinants in medium with cellobiose

To assess if this enzymatic activity produced *in vitro* by the recombinant strains would be enough to ensure a significant cell growth directly on cellobiose, the engineered strains were evaluated for their growth in liquid media supplemented with cellobiose, as only carbon source. Both recombinant strains were tested for their ability to grow in medium with cellobiose using two broths: minimal (YNB) and rich medium (YP Yeast extract, Peptone). For each medium, three different conditions were evaluated: no sugar addition, 10 g/L glucose addition or 10 g/L cellobiose supplementation. Yeast cell growth was monitored measuring the optical density at 600 nm at regular intervals. The behavior of the recombinant strains was similar in minimal and rich medium (data not shown). In YNB, the integration does not affect the ability of the strains to metabolize glucose and the two recombinants grew rapidly on glucose as their parental yeasts (Figure 3.20a). In YNB supplemented with cellobiose, the engineered strains were able to grow while the relative wild type strains did not exhibit any growth (Figure 3.20 b). However, their OD values in the YNB with cellobiose were much lower than those obtained in the glucose medium. This result can be explained by the fact that recombinant strains are able to hydrolyze only a little amount of cellobiose.

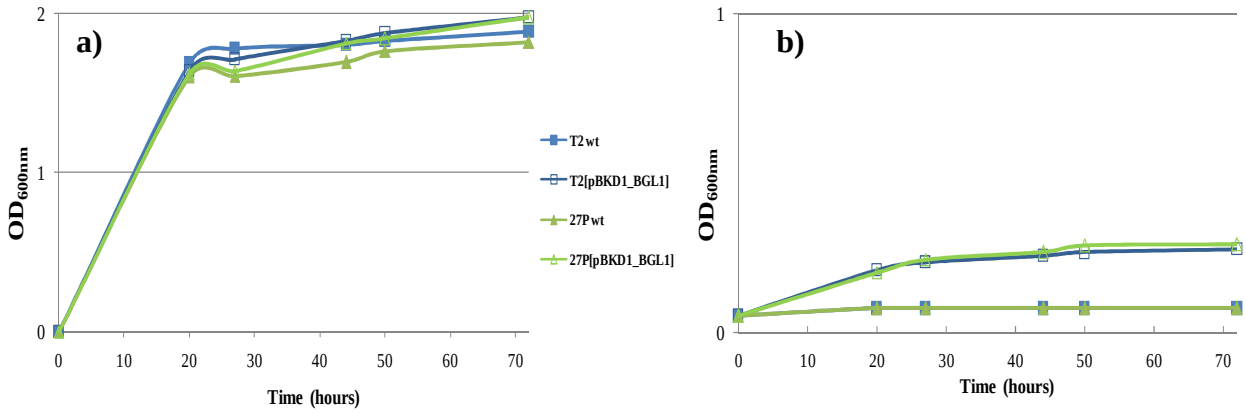

Fig. 3.20. Growth curves of the recombinant strains T2[pBKD1_BGL1] and 27P[pBKD1_BGL1] and the relative wild type strains in YNB medium with 10 g/L glucose (a) and cellobiose (b)

As a result, surprisingly, recombinant strains selected in this study showed reduced ability to hydrolyze the dimer and to utilize the released glucose. Considering that both strains were found to be mitotically stable, this finding may be due to a low number of the *bglI* gene integrations occurred throughout the dispersed delta sequences into the *S. cerevisiae* 27P and T2 genomes. Alternatively, the integration of *bglI* gene could have been in a region of the genome with low transcription level. However, further genetic studies are required to confirm both hypothesis.

The low number of integration events could be ascribed to different reasons. For example electroporation parameters would have been not optimal. Alternatively, the starting concentration of plasmid DNA should be higher or the concentration of dominant marker used in the plates to select for the positive recombinant strains should be increased to ensure the selection of transformed yeasts with higher *bglI* copy numbers. In the next future, the optimization of all these parameters will be useful to obtain a higher efficiency of the *bglI* gene integration into the selected strains.

4. CONCLUSIONS

Recently increasing attention has been devoted to bioethanol, considered the cleanest liquid fuel alternative to fossil fuels. It can be produced using different conversion technologies and from several biomass feedstocks. Between these, lignocellulosic biomasses like wood from forestry activities, agro-industrial residues and energy crops represent interesting resources for second generation bioethanol production.

The main obstacle hampering the utilization 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. In this context the development of a *Saccharomyces cerevisiae* yeast strain, able both to hydrolyze and ferment the cellulose present in this type of biomasses, could be a promising strategy to obtain a sustainable process for the production of second generation bioethanol. The full enzymatic hydrolysis of cellulose requires three major types of enzymatic activity: (1) endoglucanases, (2) exoglucanases and (3) β-glucosidases. In this work, peculiar attention has been focused on β-glucosidase, since cellobiose (the main substrate of β-glucosidase) is the major soluble by-products of cellulose hydrolysis and its efficient utilization is of primary importance for the high efficiency of the overall lignocellulose-to-ethanol process. As a result, a CBP *S. cerevisiae* yeast producing satisfactory levels of β-glucosidase enzyme would be a promising starting point for the engineering of a CBP microbe that completely hydrolyzes and efficiently ferments cellulose.

In this study, the development of such microorganism was started with a strategy that comprises two big phases: (1) selection of a host strain with promising fermenting vigour and resistance to inhibitors commonly presents in lignocellulosic substrates, (2) the construction of an engineered yeast strain able to convert cellobiose into ethanol.

Fundamental prerequisite for the production of CBP microbe is that the selected microorganism possesses industrial traits such as high fermentative vigour and tolerance to inhibitors present in lignocellulosic hydrolysates. In the first phase of this study, a new method for screening fermentative abilities, at 25 and 40 °C, and inhibitor tolerance in yeasts strains has been developed. The *S. cerevisiae* strains tested came from a collection available in the DAFNAE department while a new isolation programme of yeast strain was conducted in order to obtain yeasts that present interesting tolerance to inhibitors. This new method allowed to select a cluster of promising yeast strains for the development of CBP microbe to be used for second generation bioethanol. *S.*

cerevisiae strain T2 was selected as the most promising strain for the construction of a cellobiose hydrolyzing engineered yeast.

In the second phase of this study, the selected strain T2 was transformed with a DNA sequence encoding β-glucosidase *bglI* from *Saccharomycopsis fibuligera*. The *bglI* sequence was successfully integrated into *S. cerevisiae* and the recombinant T2[pBKD_BGL1] strain, found to be mitotically stable, was selected as the most interesting cellobiose hydrolyzing engineered strain. The recombinant strain T2[pBKD_BGL1] showed low but improvable abilities to both hydrolyze and grow on cellobiose.

In conclusion, in this work a new method for the selection of microorganisms suitable for the production of second generation bioethanol has been developed. The method involved the screening of the fermentative abilities, at 25 and 40 °C, and inhibitor tolerance for the yeast strains candidates for the development of a CBP microbe. In particular, for the first time, the fermenting vigour of yeast strains at 40 °C has been evaluated, and this procedure provided interesting results. Moreover, a new industrial recombinant yeast strain able to produce β-glucosidase enzyme has been obtained.

Further studies are required to obtain a full cellulolytic CBP microorganism for a single-step process for the production of second generation bioethanol from lignocellulose. However, this multidisciplinary work seems to be a promising platform to achieve the one step bioconversion of lignocellulosic biomass to ethanol.

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