UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Università degli Studi di Padova

Dipartimento di Agronomia Animali Alimenti risorse Naturali e Ambiente
(DAFNAE)

CORSO DI DOTTORATO DI RICERCA IN
SCIENZE DELLE PRODUZIONI VEGETALI

XXIX CICLO

Engineering robust *Saccharomyces cerevisiae* yeast for consolidated bioprocessing of lignocellulose into bioethanol

Coordinatore: Prof. Antonio Berti
Supervisore: Prof. Sergio Casella
Prof. Marina Basaglia
Co-supervisiore: Dott. Lorenzo Favaro

Dottorando: Lorenzo Cagnin

Summary

LIST OF ABBREVIATIONS

AFEX	Ammonia Fiber EXplosion
CBP	Consolidated BioProcessing
DCW	Dry Cell Weight
E10	10% ethanol blend with gasoline
E95	95% ethanol blend with gasoline
EJ	Exajoule
EU	European Union
GHG	GreenHouse Gas
HMF	5-hydroxymethyl-2-furaldehyde
HPLC	High Performance Liquid Chromatography
LHW	Liquid Hot Water
MUG	methyl-umbelliferyl-ß-D-glucoside
nKat	nanoKatals
OD_{600}	Optical Density measured as absorbance at 600 nm
*p*NPG	*p*-nitrophenyl-ß-D-gluco-pyranoside
RC	Relative Concentration
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and CoFermentation
SSF	Simultaneous Saccharification and Fermentation
YEp	Yeast Epismal plasmid
YIp	Yeast Integrative plasmid

Abstract

Second generation bioethanol, making use of the polysaccharides included in the lignocellulosic biomass, represents a promising alternative approach to overcome the limitations revealed by first generation bioethanol. The main issue hindering the effective industrial scale utilization of biomass is the lack of low-cost technology. In fact, lignocellulose hydrolysis requires expensive pre-treatments and large dosages of commercial enzymes. Moreover, feedstock pre-treatment results in the formation of inhibitors, mainly weak acids (acetic and formic), furans (furfural and 5-hydroxymethyl-2-furaldehyde) and phenolics, which affect the fermentation phase.

Consolidated BioProcessing (CBP) of lignocellulosic biomass is gaining increasing attention as a potential strategy to reduce production costs both by integrating different production steps and by lowering the need for supplying of commercial cellulases. As no naturally occurring fermenting microorganism suitable for CBP has been described yet, genetic engineering of highly fermentative microorganisms, particularly yeast, will be required. To further improve the economic feasibility of the process in industrial scenarios, the search of robust yeast with high inhibitors tolerance as platform for genetic engineering would be desired.

In this study, a collection of wild type *Saccharomyces cerevisiae* yeast, previously selected for their robustness and high ethanol yield, was characterized for inhibitors tolerance on synthetic mixtures of the inhibitors typical of lignocellulosic pre-hydrolysates and on real pre-hydrolysates, rich in these toxic compounds.

The best performing strain was chosen as a robust candidate for the expression of three fungal ß-glucosidases by δ-integration, together with the benchmark strain Ethanol Red®, currently utilized in industrial bioethanol production. Similarly, two wild-type yeast that were previously successfully used as parental to develop CBP strains, were used for the same purpose. Among the

cellulases required for cellulose degradation, ß-glucosidase was selected as it plays a key role in the process, representing the rate limiting enzyme.

A large amount of recombinant clones, secreting ß-glucosidases from the fungal species *Saccharomycopsis fibuligera* and *Phanerochaete chrysosporium*, were obtained. The engineered clones were firstly screened for high enzyme activity in a quantitative assay, using esculin as substrate. The ß-glucosidase activity of the best performing strains was then quantified on *p*NPG. One recombinant able to produce high amounts of ß-glucosidase demonstrated to be mitotically-stable and capable of sustaining the growth in presence of cellobiose as sole carbon source. The enzymatic activity of the recombinant was characterized *in vitro* in terms of enzyme localization, optimal pH and temperature, and stability. The fermentative abilities were assessed in defined medium containing cellobiose.

The obtained recombinant showed comparable performances to the parental strain on glucose, indicating that ß-glucosidase secretion does not cause any severe metabolic burden to the host. Further, the engineered strain could display high ethanol yield when fermenting cellobiose, comparable to those of a laboratory yeast strain expressing the same ß-glucosidase via multicopy episomal plasmid, despite the remarkable disadvantage of lower gene copy number guaranteed by gene integration.

This study reports the successful construction of *S. cerevisiae* strains capable of tolerating high inhibitors concentrations and expressing fungal ß-glucosidases. To our knowledge, this work represents the first attempt to produce a CBP microorganism for lignocellulosic bioethanol via integration of ß-glucosidases into tolerant yeast selected for thermotolerance and resistance to the inhibitors typically present in lignocellulosic pre-hydrolysates.

The fermentation performances of the engineered strain will next be studied on sugarcane bagasse hydrolysate, with the aim to confirm the inhibitors tolerance traits.

1. Introduction

1.1 Bioethanol: an alternative to depleting fossil fuels

Worldwide energy demand has been increasing since the rise of the industrial revolution and it has been growing exponentially during last decades, due to the increment in world population and in the number of developing countries (Demırbas, 2016). In 2012, 579 EJ (exajoule) were consumed and the number is expected to increase at a faster rate, reaching 663 EJ in 2020 and 859 EJ in 2040, thus marking a +48% difference between 2012 and 2040 (EIA, 2016). Fossil fuels, including oil, coal and natural gas, represent the most widely used source of energy. They cover over 80% of energy demand today (Table 1.1) and are expected to maintain their primary role for the next decades (Ak and Demirbas, 2016).

Main resources of energy consumption	%
Oil	37
Coal	25
Natural gas	23
Nuclear power	6
Biomass	4
Other	5

Table 1.1 - World's energy consumption by resource type (modified from Ak and Demirbas (2016))

Fossil fuels are non-renewable sources of energy whose availability is diminishing over time towards its depletion. International Energy Agency (IEA, 2013) predicts that current reserves of oil will only last until 2050 at the current consumption rate, despite technological advances that now allow to extract petrol from difficult substrates like bituminous and shale oil. Similarly, natural gas and coal are expected to exhaust by 60 and 120 years, respectively (Guo et al., 2015; IEA, 2013).

Uncertainties about fossil fuels availability, especially in the case of oil, increasing political instability and economic contrasts between producing countries cause wide fluctuations in fuels price, which in turn results in decreased production and consumption of goods (Ebrahim et al., 2014). During recent years, for example, the cost of oil dramatically peaked 150$ per barrel in 2008, and fell to just 40$ per barrel within a few months.

Fossil fuels originate from decomposition of organic material that was removed from the carbon cycle over millions of years. During last two centuries, massive and steady utilization resulted in the release of immense amounts of greenhouse gases (GHGs) into the atmosphere, mainly CO_2. Photosynthetic organisms cannot keep pace with such increased input of GHGs. As a consequence, CO_2 increased in the atmosphere from the pre-industrial level 280 ppm (parts per million) to the actual 400 ppm (Guo et al., 2015). Increment of CO_2 and other GHGs in the atmosphere due to anthropic activity is causing climate changes that led to increased global temperature of around 0.8 °C over the last hundred years and 0.6 °C during last three decades (Hansen et al., 2006; Panwar et al., 2011). Negative effects such as increase of sea levels, retreat of glaciers and sea ice, extinction of biological species are to be attributed to climate change.

The need for energy security and the growing concerns posed by environmental issues and oil price volatility directed the attention to new forms of cleaner and inexhaustible energy that will not be subjected to depletion. Renewable energy is the alternative to finite fossil sources. Main renewable forms of energy are photovoltaic solar, thermal solar, wind power, geothermal, hydroelectric, biomass (Ak and Demirbas, 2016). Biomass actually represents the most relevant source of renewable energy, as it can be used for generating heat and electricity or converted into biofuels. Wind energy and photovoltaic showed the highest development rate among renewable energies during recent years, the latter being expected to reach 25% of global power generation by 2040 (Demırbas, 2016). Nevertheless, also the amount of CO_2 and other greenhouse gases generated during ethanol production must be taken into account.

In 2009, the European Union's (EU) Renewable energy directive set a goal of 20% energy consumption from renewable sources by 2020, including at least 10% of transportation fuels. As for 2030, the EU aims to reach 27% of internal energy consumption from sustainable resources (EU, 2009). With the Climate Action Plan, the American Environmental Protection Agency set the target of 32% reduction (compared to year 2005) in GHGs emissions by 2030 (EPA, 2015).

All renewables can be implemented as sources of electricity and heat; liquid fuels for transportation, however, can be obtained only from biomass (Bisaria and Kondo, 2013). Despite great achievements in the development of full electric vehicles (IEA, 2016), transportation will rely on liquid fuels for many years. At the time, around 27% of global energy is used for transportation, in a growing trend. Introduction of renewable transportation fuels thus represents a promising target for reduction of greenhouse gases (Antoni et al., 2007). Although biomass-derived energy already supplies 10% global annual energy, it accounts to only 2% of transportation fuels (Srirangan et al., 2012). Biofuels lead to lower carbon emissions as, differently than fossil fuels, their combustion returns to the atmosphere only as much CO_2 as the vegetal fixed into organic carbon during its growth (Gomez et al., 2008).

The most common biomass-derived liquid biofuels are: bioethanol, biodiesel and biobuthanol, the first one being far the most abundantly produced, representing over the 90% of the market (Antoni et al., 2007; Srirangan et al., 2012). All these can be processed via thermochemical routes. Bioethanol, instead, is the only one obtainable also through biological conversion mediated by microorganisms. This is considered a much sustainable and environmental friendly approach, as it operates at much lower temperatures and results in less byproducts and pollutants (Srirangan et al., 2012). As for bioethanol production, the biochemical route is also economically more feasible than the thermochemical (Gomez et al., 2008).

Bioethanol and biodiesel are the sole fuels that are already being applied for transportation purposes, thanks to the possibility to share the existing

infrastructure that serves common fossil fuels. Biodiesel, which is currently made from soybean, rapeseed and palm oils, is already blended with petrodiesel up to 20% and used in common vehicles without requiring any engine modification (Demirbas, 2011; Schroder et al., 2013). Similarly, bioethanol is considered a gasoline replacement since it can be used in a mixture up to 10% in all vehicles (E10 fuel) and up to 95% (E95 fuel) in specifically designed engines (Bajpai, 2016). USA are world's major suppliers of bioethanol, providing 40 million tons in 2015. Together, USA and Brazil supply 87% of globally produced bioethanol, while only 5% is from Europe (Ajanovic, 2011; RFA, 2015).

Bioethanol will play a major role in replacing fossil fuels and decreasing greenhouse gases. In particular, lignocellulosic bioethanol is expected to result in 93% reduction in petrol consumption and 88% reduction in emissions in respect to gasoline (Farrell et al., 2006).

1.2 First and second generation bioethanol

Ethanol can be produced from a variety of different organic materials. First generation bioethanol is obtained from sugar crops such as sugar cane and beet as well as from starchy substrates like wheat, corn, sorghum. Second generation bioethanol derives from lignocellulosic material, such as corn stover, wheat and rice straw, sugar cane waste (Naik et al., 2010). The wide availability of lignocellulosic substrates as inexpensive byproducts of agricultural and forestry activities makes second generation bioethanol particularly appealing in comparison to first generation technologies, for which the raw material represents the highest cost (Demirbas, 2011). Moreover, the effective sustainability of bioethanol from sugar and starch arose many concerns (Alvira et al., 2010). First generation bioethanol is considered to have a negative impact on biodiversity, water resources, soil quality as well as poor net energy balance, in terms of ratio between energy outputs as biofuels and inputs required by production (Groom et al., 2008; Simpson et al., 2008). Common substrates for first

generation bioethanol are commodities harvested from cultivations dedicated to fuel production, which could serve also as food and feed. This establishes a direct competition between energy and food market, that may result in food shortage, especially in developing countries (Srirangan et al., 2012), even though there is no full agreement within the scientific community. Moreover, reduction in GHGs emissions is not as high as expected, as the decomposition of the non-starchy fraction of the biomass further releases GHGs (Farrell et al., 2006; Kim and Dale, 2005). Consequently, biofuel industry needs to address these aspects before claiming sustainability of its product as a key benefit over fossil fuels.

Production of first generation bioethanol, despite the strong substrate cost, is currently a much cheaper process than from lignocellulose, as the latter requires pre-treatments to alter its complex structure and be exploitable for ethanol production. Pre-treatment costs need to be lowered in order to make lignocellulosic ethanol competitive from an economic standpoint.

Bioethanol is produced by microorganisms that convert simple sugars into the final product by fermentation. The most employed organism in bioethanol industry is the yeast *Saccharomyces cerevisiae*, that can ferment several hexose sugars (glucose, fructose, mannose, galactose). Several strains of *S. cerevisiae* display high ethanol yields, as well as tolerance to high concentrations of ethanol. This yeast, however, cannot ferment pentose sugars present in the hemicellulose fraction of lignocellulosic biomass. For this reason, attention has been devoted to studying the fermentation abilities of other microbial species, as more thoroughly discussed in *1.9*.

1.3 Ethanol from fermentable sugars

Fermentation of sugars from sucrose-rich substrates, mainly sugar cane and sugar beet, is the simplest and earliest technology for bioethanol production. It requires no biomass pre-treatment, except for size reduction and pressing, to extract sugar juice which is then fermented by yeast and finally distilled to the

desired concentration (Wilkie et al., 2000). Since sugars has a high market value as food, ethanol is also often produced from byproducts of sugar preparation, like beet molasses and cane molasses. The vast majority of first generation bioethanol from sugars originates from sugar cane in Brazil, where this market for ethanol fuel flourished starting from 1970s, as a consequence of the oil crisis. Brazil currently produces 20 million tons of bioethanol, which is consumed in flex fuel cars typically in a 25% mixture with gasoline, and higher concentrations are also available (Amorim et al., 2011). Sugar cane is also the most implemented feedstock in India, where the utilization of sweet sorghum as source of fermentable sugars is gaining increasing attention (Prasad et al., 2007).

1.4 Ethanol from starch

Starch represents the most relevant source of storage energy in plants, for periods of dormancy, germination, and growth. It can be deposited in seeds, fruits or tubers. First generation bioethanol can be produced from the starchy content of several crops, including corn, wheat, barley, oats, sorghum and tubers of potato and cassava. Starch content varies from an average of 33% in mature cassava roots (Van Zyl et al., 2012) to 70% in corn kernel and triticale.

Industrial production of bioethanol from starch is a well-established technology, most widely developed in the USA, corn being the major source of raw starch (Gray et al., 2006).

Starch is made up of individual units of glucose, linked in two types of polysaccharides: amylose and amylopectin. Amylose is composed by chains of up to 1000 α-1,4-linked glucose monomers, resulting in a flexible linear molecule. Amylopectin has a more pronged structure consisting of similar α-1,4-linked chains with α-1,6 linkages serving as branching points, every 10-12 glucose monomers. Linear amylose and branched amylopectin chains are packed together in semi-crystalline regions within each starch granule. Semi-crystalline regions are interspaced by amorphous regions, consisting of sole amylopectin. The latter

are less ordered than crystalline regions, resulting in increased surface areas. As a consequence, they are more susceptible to the attack of hydrolytic enzymes (Viktor et al., 2013). As starch is not readily convertible into ethanol by fermenting yeast such as *S. cerevisiae*, it needs to be hydrolyzed to sugar monomers. For the complete conversion, enzymes acting on both α-1,4 and α-1,6 linkages are required. In industrial processes, α-amylases are used to randomly cleave α-1,4 linkages within amylose and amylopectin, while glucoamylases break the α-1,6 ones.

Fuel ethanol from corn starch is typically produced by either wet mill or dry grind processes. Wet mill strategy yields less ethanol than the counterpart but, along with bioethanol, it also generates higher-value byproducts, such as corn gluten feed and corn gluten meal to be used as feed. Wet mill requires to separate corn grains into their components (starch, fiber, gluten and germ). For this reason, more capitals and energy are required (Bothast and Schlicher, 2005).

Dry grind process instead aims to ferment as much as corn kernels as possible. It yields higher ethanol while being a cheaper technology. For this reason, ethanol production by dry grinding account to about 67% of the total. In this process, no starch is separated from the kernel, which is entirely ground and slurred with water into a mash. A thermostable α-amylase is added for randomly cleaving α-1,4 linkages while the temperature is increased to above 100°C by a jet cooker to liquefy the starch. After several minutes, additional α-amylase is supplemented, at a slightly lower temperature, to continue the hydrolysis. After cooling the liquefied starch, at the beginning of the fermentation phase, glucoamylase is added to cleave α-1,6 linkages, so that saccharification continues while the yeast consume the glucose released, in a typical Simultaneous Saccharification and Fermentation (SSF) (for a description of SSF, please refer to *1.11.2*). Consequently, glucose concentration is contained, thus not affecting the fermentation phase. Fermentation lasts up to 72 hours, producing a final ethanol concentration of 10-12%. Ethanol is finally distilled to 95% pure ethanol by heat separation or to anhydrous ethanol (100%). Solid and liquid fractions remaining

after distillation are processed into dried distillers grains with solubles (DDGS) and used as feed. Their economic valorization is crucial to ensure the feasibility of bioethanol production (Bothast and Schlicher, 2005; Cinelli et al., 2015).

1.5 Ethanol from lignocellulosic biomass

Current bioethanol production utilizes starch and sugars as feedstock. This could limit the availability of raw material for the biofuels industry and increase volatility of its price, while posing ethical concerns on the exploitation of these substrates for biofuel production (Brown, 2006; Hahn-Hägerdal, Galbe, Gorwa-Grauslund, Lidén, and Zacchi, 2006).

Conversely, to have available a technology that allows the conversion of cheap, non-edible materials would be desirable. Lignocellulosic biomass represents an interesting alternative, as it is already widely available as a waste product, such as forest and agricultural residues and food processing wastes. Cultivation of dedicated crops could instead lead to the valorization of marginal rural areas without competing with other markets (Alvira et al., 2010).

Lignocellulosic biomass is composed of cellulose (40-50%), hemicellulose (25-35%) and lignin (15-20%), strongly associated in a hetero-matrix (Gray et al., 2006). Relative composition can change among plants species, as reported in more detail in Table 1.2.

Cellulose is the main constituent of biomass cell wall, conferring structural support to the plant. It is mostly constituted by chains of linear polymers of ß-D-glucopyranose moieties linked by ß-(1,4) glycosidic bonds. The degree of polymerization ranges from 10000 to 15000 units. Repeating units of the disaccharide cellobiose constitute cellulose chains, which are grouped together (20-300) by van der Waals and hydrogen bonds to form microfibrils. Groups of microfibrils in turn constitute cellulose fibers. Hydrogen bonds are responsible for conferring straightness to the microfibrils structure. At the same time, bonds between microfibrils result in more organized (crystalline) or less ordered

(amorphous) cellulose structure (Bajpai, 2016; Laureano-Perez et al., 2005). Amorphous cellulose is more susceptible to the attack of cellulolytic enzymes required for conversion into fermentable sugars.

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40-50	24-40	18-25
Softwood stems	45-50	25-35	25-35
Bagasse	44	23	20
Corn cobs	45	35	15
Corn stover	40	25	18
Wheat straw	33-40	20-25	15-20
Wheat bran	10-12	25-35	2-6
Rice straw	40	18	5-7
Switchgrass	30-50	10-40	5-20
Paper	85-99	0	0-15
Waste paper from chemical pulps	60-70	10-20	5-10

Table 1.2 - The contents of cellulose, hemicellulose and lignin in potential bioethanol feedstocks (modified from Duenas et al. (1995), Sun and Cheng (2002), Kim et al. (2003))

Differently than cellulose, hemicellulose is not a chemically homogeneous polymer, as it presents as a branched, heterogeneous sequence of pentose (xylose, arabinose) and hexose (mannose, glucose, galactose) sugars. Hemicellulose has lower molecular weight and lower degree of polymerization than cellulose, with shorter lateral chains and it varies in composition among plants. Softwood for example contains mostly galactomannan, a polymer of mannose and glucose, while agricultural biomass and hardwood contains mostly xylan, a polysaccharide made from units of xylose (Agbor et al., 2011; Fengel and Wegener, 1984).

Hemicellulose connects lignin and cellulose fibers and gives the whole network more rigidity (Hendriks and Zeeman, 2009).

Lignin confers rigidity and impermeability to the structure, offering resistance to microbial attack and oxidative stress. It is an amorphous heteropolymer composed by a variety of phenolic monomers. Lignin binds cellulose and hemicellulose, in conjunction with less abundant compounds, in the final structure of lignocellulose. Herbaceous grasses are typically low in lignin content, while softwoods and hardwoods present higher amounts (Agbor et al., 2011; Hendriks and Zeeman, 2009).

Fermentable sugars can be obtained from cellulose and hemicellulose. However, techno-economic challenges have to be solved in order to ensure the feasibility of the conversion process. In particular, efficient depolymerization of these polymers, by effective pre-treatment and hydrolysis, and proficient fermentation of both hexose and pentose sugars must be achieved to increase the overall ethanol yield. Advanced process integration and valorization of lignin as a byproduct for the production of resins, adhesives and coatings, currently derived from petroleum refining, will be required to lower the production costs (Hahn-Hägerdal et al., 2006).

1.6 Pre-treatment of lignocellulosic material

Saccharification of polymers from lignocellulose into simple sugars is typically obtained by using specific hydrolytic enzymes, in a similar fashion to starch hydrolysis. Lignocellulosic biomass, however, is extremely recalcitrant to enzymatic digestion. For this reason, a number of pre-treatment methods have been developed to improve substrate digestibility (Gray et al., 2006), which is achieved by acting on multiple factors (Alvira et al., 2010), including:

- Reduction of cellulose crystallinity
- Lowering the cellulose degree of polymerization (i.e. number of monomers per cellulose chain)
- Increase of the surface area available for the enzymatic attack
- Removal of lignin, which both acts as a barrier and aspecifically binds hydrolytic enzymes
- Separation or removal of hemicellulose, to favor access to cellulose fibers

Lignocellulosic materials can differ widely in their physico-chemical characteristics. Similarly, different pre-treatment approaches can be more suitable for some substrates than others, resulting in higher digestibility, formation of less inhibitory chemical compounds or in lower energy demand of downstream processes (Galbe and Zacchi, 2007). Since pre-treatment also represents one of the most relevant costs in the bioethanol production process, choice of the more appropriate technology must be wisely considered (Mosier et al., 2005b).

The ideal pre-treatment process presents several key properties (Yang and Wyman, 2008):

- Low need for chemicals and their following neutralization
- Use of chemicals that do not require costly disposal challenges
- Little or no loss of cellulose and hemicellulose content
- Adaptability to a wide range of crops
- Minimum amount of toxic compounds produced
- No need for expensive thorough biomass size reduction
- Small working size, to lower the production costs of the pre-treatment plant
- Recovery and valorization of byproducts (lignin)
- Low heat and power demand

Pre-treatment methods can be divided into four categories, according to different approaches: physical, chemical, physico-chemical and biological.

1.6.1 Physical pre-treatments

Physical pre-treatment aims to reduce particle size of the substrate, yielding to a decrease in cellulose crystallinity and degree of polymerization, and to an increase in surface area available for the enzymatic hydrolysis. Chipping, grinding and milling are all used to this purpose. Grinding and milling are the most effective, but at the same time the more energy demanding ones (Behera et al., 2014). High power demand render these pre-treatment methods generally not economically feasible (Hendriks and Zeeman, 2009).

1.6.2 Chemical pre-treatments

Chemicals like acids, alkali, organic solvents, and ionic liquids have been reported to have significant effect on altering the structure of lignocellulosic biomass. Chemical pre-treatments are divided according to the nature of the chemical compounds used.

1.6.2.1 Alkali pre-treatments

Exposition of lignocellulosic biomass to bases, like sodium, potassium, calcium, ammonium hydroxides, yield lignin solubilization and increas cellulose digestibility by causing swelling of the structure and decrease in degree of polymerization, while resulting in low cellulose and hemicellulose solubilization (Carvalheiro et al., 2008). Alkali pre-treatment can be performed at room temperature, thus lowering energy requirements. However, efficiency of the process strongly depends on lignin content of the biomass. As a consequence, alkali pre-treatment is more effective on agricultural residues than lignin-richer biomass like softwoods and hardwoods (Kumar and Wyman, 2009).

1.6.2.2 Acid pre-treatments

Acid treatment of lignocellulosic biomass is an effective technology to modify lignocellulose structure and make it suitable for the following enzymatic hydrolysis. It results in the complete solubilization of hemicellulose fraction, making cellulose readily available for saccharification. Concentrated and diluted acids can be utilized. Latter ones are considered more attractive, as the corrosive effect on treating plant equipment are reduced, and the process results in the formation of less inhibitors from hemicellulose hydrolysis (Wyman, 1996), at the cost of a diminished sugar yield.

Diluted acid pre-treatment is performed with inorganic acids (mostly sulfuric acid, but also nitric, hydrochloric, phosphoric acids) at high temperature (180°C) for few minutes or at lower temperatures (120°C) for longer periods of time (30 to 90 min) (Mosier et al., 2005a). Treatment with diluted sulfuric acid was shown to yield about 75% of fermentable sugars from corn stover and olive tree (Cara et al., 2008; Saha et al., 2005).

1.6.2.3 Ionic liquids pre-treatment

Ionic liquids are solvents with high polarities presenting in liquid form at low temperatures. These salts, typically composed of large organic cations and small inorganic anions, alter the hydrogen bonds that ensure the complex interconnection between lignin, cellulose and hemicellulose. As a result, the lignocellulosic structure is disrupted, and low inhibitory degradation products are formed, thanks to the low processing temperature. Ionic liquids received great attention also due to other interesting properties, including thermal and chemical stability and non-flammability (Hayes, 2009; Zavrel et al., 2009). Further studies are required to decrease the operational costs, that currently impede the implementation of this pre-treatment at industrial level (Alvira et al., 2010). Salts toxicity on enzymes and microorganisms must be assessed, as well as the possibility to recycle ionic liquids after treatment (Yang and Wyman, 2008).

However, Li and colleagues (2009) showed that ionic liquid pre-treatment did not affect *S. cerevisiae* metabolism.

1.6.3 Physico-chemical pre-treatments

Chemical and physical processes can be combined to maximize cellulose and hemicellulose degradability and minimize costs and byproduct formation. This category includes the vast majority of available pre-treatment methods, such as steam explosion, liquid hot water, ammonia fiber explosion (Behera et al., 2014).

1.6.3.1 Steam explosion

Steam pre-treatment, also referred to as steam explosion, is the most employed physico-chemical method for altering lignocellulosic structures. Physically treated biomass is exposed to pressurized steam (0.7 – 4.8 MPa) at temperatures ranging from 160 to 240 °C for a variable period of time, up to several minutes. The pre-treatment reactor is suddenly depressurized (Alvira et al., 2010). Lignin structure is disrupted due to high temperature and pressure. Hemicellulose is mostly solubilized and hydrolyzed by the acetic acid produced from acetyl groups associated with hemicellulose and other acids released during the pre-treatment. Acids act as catalysts and result in the production of sugar monomers from part of the hemicellulose, in a process named autohydrolysis (Mosier et al., 2005b). The abrupt pressure decrement finally results in separation of cellulose fibers and swollen biomass, increasing the surface available to enzymatic attack. Higher temperatures (270°C for 1 min) can be implemented to improve hemicellulose removal and increase cellulose digestibility. At the same time, exposition to extremely high temperatures can excessively degrade hemicellulose and eventually cellulose, resulting in release of monomeric sugars and their thermochemical conversion into fermentation inhibitors (Alvira et al., 2010).

Steam pre-treatment represents a valid technology, since it requires modest amounts of chemical and energy input, with no recycling or environmental costs and relatively low economic investment (Avellar and Glasser, 1998) to yield high sugar recovery. It also does not outcome in undesired dilution of the resulting sugars, that can affect the fermentation phase. Conversely, lignin is not completely removed from cellulose, thus limiting cellulose digestibility, and hemicellulose is partly loss or transformed into inhibitors (Agbor et al., 2011).

Agricultural residues and hardwood are particularly susceptible to steam explosion (Sun and Cheng, 2002), which instead performs poorly on softwood, due to low content of acetyl groups in the hemicellulose portion. Yield from this substrate can be increased by addition of external acid, typically sulfuric acid as catalyst to improve hemicellulose solubilization and reduce inhibitor formation (Tengborg et al., 1998). In this case, however, additional costs are added as washing the pre-treated biomass is necessary to remove excess of acid that can impair the following processes.

1.6.3.2 Liquid hot water

Like steam explosion, liquid hot water (LHW) pre-treatment aims to remove lignin and to hydrolyze hemicellulose, while improving the digestibility of the cellulose fraction. LHW uses water in liquid state at high temperatures (160-240°C) and requires no rapid decompression or addition of chemicals, as steam explosion does (Yang and Wyman, 2004). Hot water breaks hemiacetal links and liberates acids that mediate hemicellulose hydrolysis in oligosaccharides (Agbor et al., 2011). Further degradation into monosaccharides and, as a consequence, to inhibitory compounds as 5-hydrohymethyl-2-furaldehyde (HMF) and furfural, can be minimized by maintaining the pH of the slurry between 4 and 7 (Mosier et al., 2005a).

Use of water without supplementation of chemicals make LHW particularly attractive, as no washing is required and the solvent does not pose any risk of equipment corrosion. For this reason, plant construction results less expensive, as well. When compared with steam pre-treatment, LHW has the advantage of yielding less inhibitors formation and higher amount of solubilized product (Agbor et al., 2011). However, it also results in lower sugars concentration in the final slurry, which increase the energy demand, as higher volumes of liquid need to be processed.

1.6.3.3 Ammonia Fiber EXplosion (AFEX)

AFEX pre-treatment uses liquid ammonia to pre-treat biomass. In a similar fashion to steam explosion, substrate is exposed to high pressure at lower temperature (60°C to 100°C) in presence of ammonia for a variable period of time (10-60 min). When the environment is depressurized, ammonia gas causes swelling and disruption of biomass structure, affecting cellulose crystallinity.

During the pre-treatment, lignin is strongly altered but only little amount of solids solubilizes. Other chemical and physico-chemical pre-treatments result in separation of cellulose, which remains in solid form, and hemicellulose, partly degraded into shorter oligosaccharides. According to the process configuration, hemicellulose can be discarded or be separately converted into bioethanol. Instead, ammonia fiber explosion produces only solid material. Choosing AFEX pre-treatment thus implies utilization of both cellulases and hemicellulases in next enzymatic hydrolysis followed by conversion of pentose sugars into ethanol, as relevant amounts of hemicellulose will be retained in the solid fraction. (Agbor et al., 2011; Mosier et al., 2005a). AFEX is widely more effective on agricultural crops than on woody biomass. Despite little removal of non-cellulosic material, this method can achieve over 90% conversion of useful polysaccharides (Wyman et al., 2005). Compared with other pre-treatment methods, AFEX gives low inhibitors formation, mostly due to lower temperatures and no hemicellulose saccharification. Together with modest cost of the ammonia, low working

temperatures also result in better chances of economic feasibility at the industrial level (Agbor et al., 2011). Furthermore, spent ammonia can be collected and recycled.

1.6.4 Biological pre-treatment

Biological pre-treatment relates to the utilization of fungal species capable of degrading lignocellulosic material. White-rot fungi are the most suitable for this application, as they primarily attack lignin and hemicellulose, while leaving cellulose almost unaltered. Several white-rot fungi, including *Phanerochaete chrysosporium*, were found to be particularly selective in terms of substrate of action (Kumar and Wyman, 2009; Sun and Cheng, 2002).

However, while this pre-treatment offers relevant advantages, such as low capital and energy and no chemicals requirement, the process results too slow for an effective industrial applicability, due to a residence time of 10-14 days. Large scale implementation would then require large space and important efforts for careful growth control (Behera et al., 2014).

1.7 Inhibitors formation and effects

Pre-treatments remove the physical barrier that make biomass recalcitrant to enzymatic hydrolysis of the cellulosic and hemicellulosic fractions, to allow high recovery of sugars in the following phases of bioethanol production process. As a side effect, harsh conditions required for efficient pre-treatments result in the formation of derivative byproducts that are inhibitory to microbial metabolism or to the activity of hydrolytic enzymes used before fermentation (Jönsson and Martín, 2016). Type and amount of inhibitors released during pre-treatment depend on the intrinsic characteristics of each different substrate and to the specific pre-treatment conditions applied. Inhibitors accumulation becomes more relevant in case of pre-treatment methods that involve recycling of process water, due to accumulation over time.

In the vast majority of pre-treatments, cellulose structure is altered, but not degraded. Hemicellulose is instead typically solubilized and partly degraded to oligomers of various length. Lignin is normally modified, still remaining for the most part in solid form. Inhibitors are mostly formed by degradation of lignin and of sugars released from hemicellulose (Figure 1.1). These molecules can be grouped in three major groups: furans, weak acids, phenolic compounds (Palmqvist and Hahn-Hägerdal, 2000a). In addition, other compounds can exert a negative influence on the activity of enzymes involved in the following hydrolysis step.

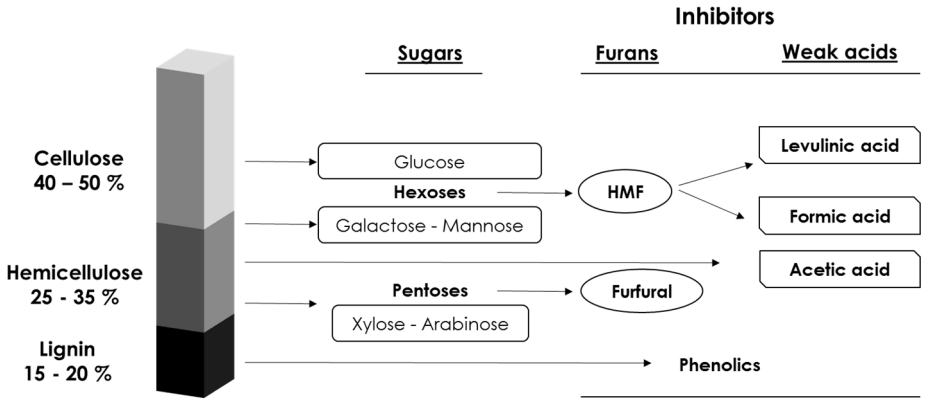

Figure 1.1 - Average composition of lignocellulosic biomass and main derived hydrolysis products (modified from Almeida et al. (2007)).

1.7.1 Inhibitors of microbial metabolism

1.7.1.1 Furans

Furan, specifically furfural and HMF, are formed by dehydration of pentose and hexoses sugars, respectively (Jönsson et al., 2013), in particular under acidic conditions. Their formation at the expense of fermentable sugars reduces the final product yield. In addition, furfural and HMF can directly affect microbial metabolism. Under fermentative condition, *S. cerevisiae* yeast can cope with the presence of these inhibitory compounds, by converting furfural to less

toxic furfuryl alcohol (Horváth et al., 2003) and HMF to 2,5-bis-hydroxymethylfuran (Taherzadeh et al., 2000), even though this occurs at lower rate than furfural conversion. However, at concentration as low as 0.1 mM, furan aldehydes can already show inhibitory effects on the fermenting yeast (Larsson et al., 2000).

1.7.1.2 Weak acids

Lignocellulosic hydrolysates contain a broad spectrum of weak acids, in particular acetic, levulinic and formic acid.

Under severe pre-treatment conditions, levulinic acid originates from degradation of HMF, which can also be transformed, as well as furfural, into formic acid. Acetic acid, instead, is not a degradation product, as it is directly released from the hydrolysis of acetyl groups of hemicellulose (Jönsson and Martín, 2016; Ulbricht et al., 1984). As previously discussed in case of furan aldehydes, formation of degradation compounds, produced at the expenses of fermentable sugars, has a strong negative effect on the overall conversion process. For these reasons, pre-treatment should be adapted in order to minimize weak acids formation.

Weak acids inhibit cell growth, as a result of the diffusion of undissociated forms across the plasma membrane, that lower cytosolic pH and can lead to cell death. However, weak acids concentration lower than 100 mM was found to promote rather to inhibit yeast fermentation (Larsson et al., 1999; Pampulha and Loureiro-Dias, 1989). At these concentrations, yeast cells can cope with pH decrease by pumping protons outside the cell. This requires utilization of ATP molecules, that are produced at the expenses of biomass formation (Palmqvist and Hahn-Hägerdal, 2000a).

1.7.1.3 Phenolics

Pre-treatment of lignin, in particular under acidic conditions, originates a multiplicity of phenolic compounds. Due to the high diversity among chemical

species, identification and quantification of each individual compound is particularly challenging (Jönsson et al., 2013). The mechanism of inhibition still remains unclear. Phenolics however are responsible for the loss of membrane integrity and the consequent permeabilization and change in protein-to-lipid ratio (Palmqvist and Hahn-Hägerdal, 2000a). *S. cerevisiae* can cope with low concentration of phenolics, by converting them into less harmful compounds (Larsson et al., 2000).

Despite these compounds are found in lignocellulosic hydrolysates in lower concentrations than other inhibitors, their negative effects are higher than other inhibitors like weak acids. Consequently, presence of phenolics should be minimized by carefully tuning pre-treatment processes according to the intrinsic characteristics of the biomass in use.

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1.7.2 Inhibitors of hydrolytic enzymes

Hydrolytic enzymes, responsible for the saccharification of pre-treated cellulose prior to the fermentation phase, can be inhibited by products and byproducts of pre-treatments. Lignin and residual hemicellulose, for example, can aspecifically bind or absorb enzymes, resulting in the need for addition of costly cellulases (Jönsson et al., 2013).

As discussed in paragraphs 1.7.1.1 and 1.7.1.2, harsh pre-treatments can result in the formation of weak acids and furans, originating from sugar monomers released by hemicellulose and cellulose degradation. These monosaccharides, including glucose and xylan, together with few disaccharides, like cellobiose, exhibit undesired inhibitory effects on the enzymes utilized for polysaccharides hydrolysis (Kumar and Wyman, 2014; Teugjas and Väljamäe, 2013). This requires, once again, to reach a compromise between desired substrate digestibility and resulting inhibitory effects of the obtained pre-treated material. Finally, phenolic compounds can, as well, affect enzymatic activity, especially on cellulases and particularly on ß-glucosidases (Ximenes et al., 2011).

1.8 Detoxification of pre-treated lignocellulose

One possibility to avoid formation of relevant amounts of inhibitors would be the selection of less recalcitrant feedstock coupled with the application of mild pre-treatments. However, since the main purpose of producing second generation bioethanol is represented by the exploitation of waste material, the possibility to use different lignocellulosic feedstocks is more than desirable. Moreover, aiming for poor sugar yield in change of low inhibitors loading, is not reasonable in an industrial production scenario (Jönsson et al., 2013).

Washing the pre-treated material is the simplest and most economic countermeasure to eliminate soluble inhibitory compounds. However, this would result in loss of huge amount of sugars, as well as requiring processing large amounts of wastewater. A number of detoxification processes has been developed to increase hydrolysate digestibility while minimizing the intrinsic costs caused by introducing one more processing step (Moreno et al., 2014).

Detoxification methods, also referred as conditioning, can be divided into three categories: chemical, physical and biological.

1.8.1 Chemical conditioning

Many pre-treatments involve addition of acids to maximize hemicellulose solubilization and cellulose digestibility. This results in a strong decrease in pH, which must be raised to a level that fermenting microorganisms can tolerate (Pienkos and Zhang, 2009). Although the mechanism is not fully elucidated, pH increase is known to result in less inhibiting material.

One of the most common and effective methods is referred as “overliming”. Addition of calcium hydroxide results in the formation of an insoluble precipitate of calcium phosphate, that can be removed by centrifugation (Alriksson et al., 2005; Nevoigt, 2008). pH is increased to high values, up to pH 10, and subsequently lowered to values that can be tolerated by fermenting yeast. The detoxification effect, initially thought to be caused by salts precipitation (Van Zyl

et al., 1988), is instead due to chemical conversion of the inhibitors (Persson et al., 2002a).

Utilization of other bases like sodium, calcium, potassium or ammonium hydroxides was found to be effective in conversion of HMF, furfural and phenolics to less toxic compounds (Persson et al., 2002a), giving levels of fermentability that are comparable to those obtained with overliming (Alriksson et al., 2005).

Despite alkaline conditioning reaches high levels of detoxification, thus high final ethanol yields, a disadvantage of this method is represented by sugar loss by degradation, especially when harsh conditions are applied (Jönsson et al., 2013).

1.8.2 Physical conditioning

Differently from chemical conditioning, physical detoxification aims to remove inhibitors from the hydrolysate, rather than converting them into less toxic substrates. In general, physical methods are less effective than chemical ones. Supercritical fluid extraction, for example, is known to remove a number of toxic compounds, like over 90% of furfural and phenolics from softwoods, while being almost no effective on HMF and acetic acids (Persson et al., 2002b).

Other methods include electrodialysis, for acids removal, and liquid extraction with diethyl ether, with a broader effect on a wider range of inhibitors. Activated carbon can be used for solid phase extraction and reduce weak acids without significantly affecting sugars load (Berson et al., 2006; Pienkos and Zhang, 2009). Different types of ion exchange resins can be used to remove higher portions of HMF and furfural from agricultural residues (De Mancilha and Karim, 2003).

1.8.3 Biological conditioning

1.8.3.1 Enzymatic detoxification

Enzymatic treatment is one of the main biotechnological methods for diminishing inhibitor load in pre-treated material. Most efficient enzymes are laccases and peroxidases produced by white rot fungi, like *Trametes versicolor, P. chrysosporium, Coriolopsis rigida,* among others (Pienkos and Zhang, 2009). Enzymatic conditioning is selectively effective on inhibitors of phenolic origin. Laccases and peroxidases, in particular, catalyze the oxidation of monoaromatic phenolics from pre-treated lignin into less toxic aromatic compounds (Alvira et al., 2013).

Enzymatic detoxification is characterized by lower reaction times than other methods of biological conditioning, while high costs of the enzymes and strict selectivity for phenolic compounds represent strong disadvantages (Pienkos and Zhang, 2009). Elimination of the sole phenolic inhibitors fraction, however, demonstrated to be still effective in reducing the toxicity of pre-treated material. This approach, in addition, does not suffer from the downsides typical of physical and chemical detoxification, like loss in fermentable sugars (Palmqvist and Hahn-Hägerdal, 2000b).

1.8.3.2 Microbial detoxification

Microorganisms can be used as cell factories for the production of detoxification enzymes, which are later applied in conditioning industrial plants. At the same time, fungi, bacteria and yeast can be directly implemented in order to mitigate the inhibitory effect of phenols, furans and weak acids. *Trichoderma reesei* and *Coniochaeta ligniaria* were thoroughly studied for this purpose, resulting in the ability to remove furfural, HMF and phenols without altering weak acids concentrations or consuming relevant amounts of fermentable sugars

(Moreno et al., 2014). A *S. cerevisiae* strain was described for metabolizing acetic acid but not sugars (Schneider, 1996).

A different approach focuses on the selection of fermentative microorganisms suitable for *in-situ* detoxification, showing intrinsic ability to tolerate high amounts of pre-treatment inhibitors. Harsh environments posing high stress levels to microorganisms, as for example grape marc from wineries, proved to be a promising source for wild type yeast with remarkable tolerance levels to a wide range of inhibitors (acetic acid, formic acid, furfural, HMF). At the same time, these yeast strains could exhibit high fermentation performances required for industrial applicability for bioethanol production (Favaro et al., 2013a). Inhibitors resistance traits of fermenting microorganisms can be improved by means of adaptive evolution. As for the selection of robust fermentative microbes, discussed above, this technique mostly applies to yeast with high fermentation abilities. The constant exposition to sublethal concentrations of inhibitors is used to isolate adapted yeast strains with improved tolerance to these undesired compounds (Wallace-Salinas and Gorwa-Grauslund, 2013).

Selection of tolerant and adapted microorganisms leads to a diminished need for reduction of total inhibitors concentration, since these microbes, generally yeast, can convert higher amount of deleterious compounds into less harmful molecules (Favaro et al., 2013a; Favaro et al., 2014; Mukherjee et al., 2014). This, in turn, results in lower or no expenses for conditioning and, consequently, in improving the economic feasibility of the overall production process.

To the same aim, genetic engineering of highly fermentative yeast can be used to confer new characteristics not present in the wild type. Selected strains can be modified for the secretion of fungal laccases and peroxidases, to cope with presence of phenolic compounds (Larsson et al., 2001). Other studies have shown the possibility to confer increased furfural and HMF resistance (Petersson et al., 2006).

1.9 Hydrolysis of cellulose

After a successful pre-treatment, hemicellulose is removed for the most part, leaving the altered cellulosic structure readily available for hydrolysis. As the most commonly used fermentative microorganisms can only utilize sugar monomers for ethanol production, cellulose needs to be hydrolyzed (Olofsson et al., 2008).

Cellulose hydrolysis was historically obtained in acid-catalyzed processes. Pre-treated cellulose can be saccharified by addition of acids, typically chloridric acid or diluted sulfuric acid, in a process similar to acid pre-treatments. Reaction is carried out in a range from 150°C to 190°C, in the case of sulfuric acid, while lower temperatures are required for chloridric acid (Rinaldi and Schüth, 2009). Fermentable sugar yield, initially lower than 50% of the theoretical, was improved by introducing a two-stage system (Harris et al., 1985). Highly pure glucose can be obtained after initially hydrolyzing hemicellulose, which requires lower temperatures than cellulose, in a first processing step (170°C, 0.4 wt% H_2SO_4). Cellulose is later degraded at higher temperature (190°C) with double concentrated sulfuric acid. As hemicellulose is not subjected to as harsh conditions as cellulose is, formation of inhibitors is limited.

A different method for degrading cellulose into simple sugars involves utilization of cellulolytic enzymes. This approach offers several advantages, compared to acid hydrolysis. While the latter requires high temperature and low pH, leading to corrosion of mechanical components in industrial plants, enzymatic hydrolysis operates at milder conditions. Further, fermentable sugars reach much closer concentrations to the theoretical yield than in acid hydrolysis, without resulting in degradation of the hemicellulose fraction into inhibitory compounds. Several disadvantages, however, cannot be omitted. Process retention time is longer (days, compared to minutes in acid hydrolysis) and released sugars can cause inhibition of cellulase activity (Olofsson et al., 2008; Taherzadeh and Karimi, 2007). Finally, despite enzymes can be recycled with the

purpose of costs reduction, presence of solid lignin residuals hinders enzyme recovery as lignin absorbs part of the enzymes introduced. Solubilization of the cellulases in the liquid hydrolysate obstructs enzyme recovery.

Enzymatic hydrolysis is currently the most promising technology for industrial applications. Consequently, description of next steps of ethanol production will refer to enzymes-mediated saccharification of cellulose.

Three types of enzymes, collectively referred as "cellulases", characterized by highly specific activity on ß-1,4-glycosidic bonds within cellulose structure, are needed to complete the hydrolysis: endoglucanases, exoglucanases (or cellobiohydrolases) and ß-glucosidases. Endoglucanases attack amorphous and low-crystallinity regions of cellulose, which increased as a result of pre-treatments. Role of endoglucanases is to reduce the degree of polymerization by randomly cleaving ß-1,4-glycosidic linkages within cellulose chains, generating shorter oligomers with reducing ends. Cellobiohydrolases target the reducing ends and release cellobiose units. Cellobiose units are finally cleaved into glucose by ß-glucosidases (Lynd et al., 2002; Taherzadeh and Karimi, 2007). Cellobiohydrolases can also exert activity on microcrystalline cellulose. For this reason, two enzymes of cellobiohydrolases are used in industrial applications, namely CBHI and CBHII, having different preferences for oligomers reducing ends or microcrystalline chains (Teeri, 1997).

Many fungal species were investigated for the ability to sustain production of cellulases, including *T. reesei, Aspergillus niger, P. chrysosporium, Humicola insolens*. Industrial scenario is currently dominated by *T. reesei*, which produces endoglucanases, cellobiohydrolases and ß-glucosidases of industrial grade. In addition, ß-glucosidases from *A. niger* are also used, as they are more tolerant to high concentrations of glucose in the medium (Lynd et al., 2002).

1.10 Hydrolysis of hemicellulose

Saccharification of hemicellulose poses a bigger challenge than cellulose hydrolysis, due to the complexity of its structure. Expression of a larger number of enzymes will be required. ß-xylanases and ß-xylosidases cleave the hemicellulose backbone in xylan-rich hardwood, while other enzymes are necessary for debranching the remaining structure, including α-D-glucuronidases, α-L-arabinofuranosidases and acetylesterases. Softwoods instead are richer in mannans, requiring secretion of different mannanases and α-galactosidases (van Zyl et al., 2007). Complete hydrolysis of hemicellulose results in the release of pentose sugars, mainly xylan and arabinose, as well as several hexose sugars.

Ability to ferment pentoses is not widespread among microbial species. Most of the research has been devoted to xylose fermentation, as this sugar is present in much higher concentration than arabinose in lignocellulosic substrates (Kuhad et al., 2011).

Many obligate anaerobic bacteria are capable of converting pentoses into ethanol. Thermophiles, in particular members of genus *Clostridium* and *Thermoanaerobacter*, could offer the advantage of low cooling requirements and limited risk of contamination. However, low ethanol tolerance and formation of a range of byproducts make these bacteria industrially unviable (Hahn-Hägerdal et al., 2007). Scarcity of defined protocols for genetic engineering of these strains represents one additional disadvantage. Among facultative anaerobes, possible utilization of *Escherichia coli* was investigated, as this bacterium can metabolize a variety of pentose sugars. The mixed fermentation pattern exhibited by *E. coli* required metabolic engineering approaches to improve final ethanol yield (Olofsson et al., 2008). Nevertheless, the high susceptibility to inhibitory compounds hinders the industrial applicability of this bacterium. *Zymomonas mobilis,* instead, shows outstanding ethanol yield and productivity. This species is not able of fermenting pentose sugars, though. Pentose utilization pathway

could be expressed by genetic engineering (Mohagheghi et al., 2002). However, like other bacterial species, *Z. mobilis* suffers from lacking desired robustness.

Aerobic filamentous fungi, including *T. reesei* and *Fusarium oxysporum*, can ferment pentose sugars, albeit at extremely poor rates. This results in prolonged processing time and extended area requirements, which represent the major disadvantages of fungal industrial implementation, together with low ethanol tolerance and necessity for fine tuning of oxygen levels (Kuhad et al., 2011).

Many yeast species are described for xylose utilization, including *Pichia stipitis* and *Candida shehatae* (Olofsson et al., 2008). However, the inability to produce ethanol as major end-product represents the biggest disadvantage. In addition, such yeast shows scarcer tolerance to low pH, high ethanol and inhibitors concentration than bioethanol *S. cerevisiae* strains.

Due to the unavailability of suitable industrial pentose-fermenting microorganisms, attention has been dedicated to modification of laboratory and industrial *S. cerevisiae* strains by means of genetic engineering. Effective pentose fermentation was obtained by expressing fungal xylose reductase and xylitol dehydrogenase, as well as by the overexpression of endogenous xylulose kinase and expression of membrane proteins for facilitating pentose diffusion (Hahn-Hägerdal et al., 2007; Hong and Nielsen, 2012; Laluce et al., 2012; Sànchez Nogué and Karhumaa, 2015).

1.11 Fermentation of biomass hydrolysates and process configurations

Industrial scale bioethanol production from pre-treated biomass requires four biologically mediated events: i) cellulase production, ii) hydrolysis of cellulose and, if present, hemicellulose (, according to the applied pre-treatment and industrial configuration), iii) fermentation of soluble sugars of cellulosic origin and iv) fermentation of soluble sugars from hemicellulose (Lynd et al., 2002).

These events can be consolidated to several degrees of integration, leading to four different process configurations: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP). The different level of integration is schematically represented in Figure 1.2.

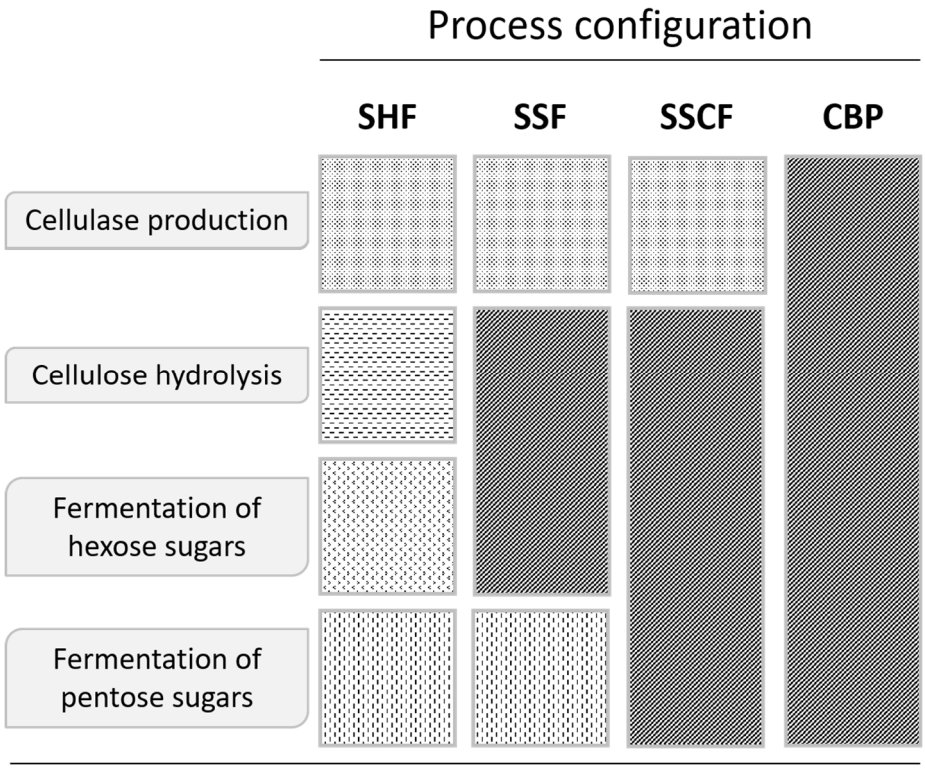

Figure 1.2 Consolidation of biologically mediated events in cellulosic ethanol production (modified from Lynd (1996)).

1.11.1 Separate hydrolysis and fermentation

This process requires the utilization of four steps to complete the conversion of pre-treated cellulose into bioethanol. Hydrolytic enzymes, produced in aerobic conditions by fungal species, as discussed *1.9* and *1.10*, are supplied in a second bioreactor for cellulose and hemicellulose hydrolysis. Released sugars, hexoses and pentoses, are finally fermented by yeast in separate environments under anaerobic conditions (Lynd et al., 2002).

Main advantage of this configuration is the possibility to complete hydrolysis and fermentation under their optimal operational conditions. Enzymes can exhibit cellulase activity at temperatures that also allow microbial fermentation (i.e. 25 to 30°C for yeast, 37°C for bacteria). However, in this scenario the hydrolytic performances are dramatically decreased in comparison to the optimal temperature and pH, which ranges from 50 to 60°C, at pH close to 5 (Paulova et al., 2015).

Conversely, high sugars concentration reached in the last phase of enzymatic hydrolysis has a negative impact on cellulase activity. Cellobiose can reduce performances of endoglucanases and cellobiohydrolases by more than 50% at concentration as low as 6 g/L, while glucose, released by ß-glucosidases, strongly inhibits the same enzyme already at half that concentration (Taherzadeh and Karimi, 2007). As a consequence, the major disadvantage of SHF is the risk of incomplete hydrolysis of the substrate, caused by the inhibitory effect the end products exhibit on cellulases. SHF can suffer from contamination problems, as well. Even though hydrolysis is conducted at high temperature, sterilization of hydrolytic enzymes is difficult to achieve, since autoclaving is not permitted as it would result in enzyme deactivation. Finally, the cost of building and managing four different vessels has a strong impact on production costs (Ask et al., 2012).

1.11.2 Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation (SSF) is a modification of the original SHF process. Saccharification of pre-treated material by cellulases is integrated with fermentation of released sugars in a single step (Paulova et al., 2015). The more immediate advantage of this process implementation is the reduction of capital costs required, as hydrolysis and fermentation take place in the same bioreactor.

SSF offers a stronger advantage over SHF because continuous conversion of fermentable sugar into ethanol by fermenting organisms occurs during the hydrolysis, minimizing any sugar inhibition on cellulase activity (Cardona and Sánchez, 2007). Benefits on the enzymatic activity of cellulases results in turn in a reduction of conversion time. During the early stages, however, lower ethanol productivity is shown by SSF when compared to SHF, as glucose is still present in relatively small amounts (Paulová et al., 2014).

As a consequence of consolidating hydrolysis and fermentation in a single step, it is necessary to identify a trade-off between ideal working parameters of each process. As mentioned in *1.11.1*, optimal temperature for saccharification is much higher than that of fermentation. For example, to perform SSF at a temperature close to 50°C would result in the complete inhibition of yeast fermenting abilities. Thus, SSF is normally conducted at a temperature that favors microbial over enzymatic activity. For this reason, identification of highly processive cellulases displaying optimal performances at low temperatures, is crucial (Olofsson et al., 2008; Taherzadeh and Karimi, 2007).

Ethanol accumulation in the bioreactor makes the selection of fermentative microorganisms capable of tolerating high ethanol concentration mandatory. At the same time, potential risk of contamination by non-fermentative microbes, which could lead to relevant product losses, is reduced by the presence of the alcohol (Ojeda et al., 2011). Ethanol, however, can also negatively affect the performance of cellulases (Holtzapple et al., 1990), but to a lesser extent than exhibited by cellobiose or glucose.

In SHF, saccharified cellulose must be separated from the solid part, rich in lignin, and transferred to a distinct vessel to proceed with the fermentation phase. Part of the sugars remains associated with the solid fraction, thus causing sugar losses that decrease final product yield. In SSF, this separation is not necessary, marking one more reason to prefer this processing method over SHF (Olofsson et al., 2008).

Recovery of enzymes and yeast is hampered in SSF by the presence of high amounts of solids in the hydrolysate. As a consequence, to find a balance between enzyme and yeast concentration is of fundamental importance for reducing production cost and final ethanol yield (Olofsson et al., 2008).

As earlier discussed, the final step of SSF, as well as of SHF, is represented by conversion of pentose sugars eventually present in the hydrolysate into bioethanol by pentose-fermenting microorganisms.

Simultaneous saccharification and fermentation step can be combined with fermentation of pentoses, in a process called simultaneous saccharification and co-fermentation (SSCF), where five-carbon and six-carbon sugars are converted into ethanol in a single reactor, while pre-treated polymers are being degraded by cellulases and hemicellulases. SSCF is considered an improvement of SSF, as it aims to further reduce production costs by further limiting the number of vessels required for converting the pre-treated material (Mcmillan et al., 1999).

1.12 Consolidated bioprocessing

Integration of three out of the four biological events that mediate the conversion of pre-treated lignocellulosic material into bioethanol to reduce production costs and complexity lead to the development of SSCF technology. In this configuration, however, production of hydrolytic enzymes remains a separate process, carried out in a distinct aerobic bioreactor. The ultimate process simplification is represented by the definition of a single step consolidate bioprocessing (CBP), where a consortium or, preferably, a single microorganism, would be able to mediate all the reactions necessary to convert the substrate into ethanol, in a single bioreactor (van Zyl et al., 2007). Such CBP microbe is required to both hydrolyze pre-treated biomass and convert it to the final product at high yield and titer under stressful industrial conditions (Olson et al., 2012).

This configuration offers strong advantages in terms of capital and managing costs reduction when compared to SSF, as it significantly lowers the efforts required for enzyme production.

Although many fungal and bacterial species possess some of the characteristics required, no single microorganism is eligible for consolidated bioprocessing. Two main approaches, both involving genetic engineering of selected microbes, have been identified. Native cellulolytic strategy involves improvement of naturally occurring cellulolytic and/or hemicellulolytic organisms by conferring high fermentative traits by means of genetic manipulation. Recombinant cellulolytic strategy, instead, relates to engineering organisms that exhibit high ethanol yields but are incapable of hydrolyzing cellulose or hemicellulose (Lynd et al., 2005).

One of the main challenges for the native approach is represented by the limited options available for genetic modification, since gene transferring to non-model cellulolytic and hemicellulolytic organisms is only rarely a standardized process. These difficulties affect in particular the possibility to obtain a CBP organism of fungal origin (Olson et al., 2012), while anaerobic cellulolytic bacteria belonging to *Clostridium* and *Thermoanaerobacterium* genus were successfully engineered for ethanol production (Lynd et al., 2005).

However, fungi show slow hydrolysis activity at low ethanol yield (Panagiotou et al., 2005). Anaerobic bacteria produce a broad number of fermentation products in addition to ethanol, requiring additional efforts for pathway modification by metabolic engineering in order to re-route energy conversion to a single end-product, ethanol. Further, native cellulolytic species generally lack in robustness towards other stressful industrial process conditions, including inhibitor tolerance and low performances at high substrate concentration (Olson et al., 2012).

Non-cellulolytic microorganisms with high fermentation performances represent platforms for developing CBP organisms, according to the recombinant cellulolytic strategy. The primary challenge is the expression of cellulases and

hemicellulases in sufficient quantities to allow sufficient conversion of pre-treated material. To this purpose, a number of bacterial (*Z. mobilis*, *E. coli*, *Klebsiella oxytoca*) and yeast (*S. cerevisiae*, *P. stipitis*) have been subjected to heterologous protein production, resulting in many cases in secretion-related issues (den Haan et al., 2015).

Among those species, *S. cerevisiae* is currently considered one of the most promising platforms for CBP development, thanks to high levels of inhibitors tolerance and adaptability to industrial conditions (van Zyl et al., 2007).

Construction of a fully operational cellulolytic CBP organism requires the efficient co-expression of all cellulases necessary for cellulose hydrolysis: endoglucanases, exoglucanases and ß-glucosidases (as discussed in *1.5*).

Despite complete conversion of pre-treated cellulosic material by engineered yeast has not been achieved yet, significant advances have been made, regarding the expression levels of cellulases. Strains expressing two cellobiohydrolases at titers sufficient for industrial applications and co-expression of endoglucanases and cellobiohydrolases are some of the examples (Ilmén et al., 2011). In general, sufficient expression of cellobiohydrolases represents the main challenge, as endoglucanases and ß-glucosidases showing higher activities on their substrates, require lower expression levels (W. H. van Zyl et al., 2007).

Characteristic of consolidated bioprocessing is the conversion of the hemicellulose along with cellulose. Hemicellulases have been already singly expressed in *S. cerevisiae*, but the consolidated bioprocessing of hemicellulose is far from being obtained by a single microorganism (van Zyl et al., 2007).

Successful expression of cellulases and hemicellulases in *S. cerevisiae* strains supports the potential of this yeast as CBP host. However, the challenges posed by the expression of multiple genes should not be underestimated. The need for high-level expression is likely to result in strong stress responses. Factors that

may cause unwanted cell stress include: i) effect of unfavorable codon bias, ii) improper protein folding, resulting in protein degradation and iii) accumulation of proteins within cytoplasm or cell wall due to low permeability (van Zyl et al., 2007).

Heterologous genes can be expressed via two main different strategies: utilization of episomal Yeast Episomal plasmid (YEp) vectors and chromosomal integration. YEp vectors are present in high copy-number within the host cell and are replicated during cell cycle, so that vector copy number is maintained during cell growth. This approach offers the advantage of ensuring high enzymatic activity due to conspicuous gene transcription. However, stability of recombinant strains requires specific selection markers in defined mediums, which hampers the application of these strains in complex industrial configurations. Cellulase and hemicellulase genes can instead be integrated into yeast chromosomes by using Yeast Integrative plasmids (YIp). Gene integration results in improved expression stability, irrespective of the growth medium. As a downside, the number of integrated copies is normally low, thus affecting expression levels (Romanos et al., 1992; Da Silva and Srikrishnan, 2012; Yamada et al., 2010b).

A suitable method for industrial-grade heterologous gene expression would benefit from the advantages of each technique: i) high copy-number integration and ii) high mitotic stability of the gene of interest. These characteristics can be merged by integrating multiple copies of the target genes within chromosomal rRNA coding sequence and repetitive δ-sequences (Lopes et al., 1996). Integration within non-transcribed sequences of rDNA locus offers the possibility to produce clones that express multiple gene copies, since up to 300 sites are available in the haploid genome. The use of selection markers based on lactose assimilation avoids conferring antibiotic-resistance to industrial yeast. This facilitates the accomplishment of bio-safety requirements necessary for large-scale production (Leite et al., 2013). In addition, this approach favors the multiple integration of different genes. However, expression of long sequences significantly compromises mitotic stability of the construct, which length cannot exceed 9.1 kb. In addition,

localization of rRNA in the nucleolus could affect the accessibility to RNA polymerase transcription (van Zyl et al., 2007).

δ-sequences, instead, are long terminal repeats of the *Ty* retrotransposon of *S. cerevisiae*, present in higher copy number within the genome. More than 400 copies exist in the haploid yeast genome, thus offering the opportunity for multiple highly stable gene integration (Dujon, 1996; Parekh et al., 1996). Despite the high number of δ-sequences, integration after yeast transformation often occurs within a single location (Da Silva and Srikrishnan, 2012). Acquired resistance against antibiotic, such as geneticin, is typically used as dominant selection marker. Laboratory strains expressing heterologous cellulases were previously successfully developed by δ-integration (Cho et al., 1999).

Consolidated bioprocessing approach does not solely apply to the conversion of lignocellulosic biomass into ethanol. Recently, CBP wild-type *S. cerevisiae* strains for the direct fermentation of raw starch were developed by secreting fungal amylases (Favaro et al., 2015). This result indicates the viability of CBP as a valid technique for merging strong process integration and implementation of robust yeast, isolated from environmentally harsh condition, to stressful industrial applications.

1.13 Role of ß-glucosidase in CBP yeast

Among cellulases, ß-glucosidases represent the key enzyme for cellulose hydrolysis. Endoglucanases and cellobiohydrolases are inhibited by cellobiose. ß-glucosidases, cleaving cellobiose into glucose monomers, represent the rate limiting enzyme in the overall saccharification, as their activity avoids decreasing rates in cellulose hydrolysis over time (Sørensen et al., 2013), in addition to finalizing the cellulose degratation process. ß-glucosidases also suffer from similar inhibition by high glucose concentrations. Such downside is particularly relevant in SHF fermentations, while almost insignificant in SSF and CBP, as glucose is continuously consumed by fermenting microorganisms.

ß-glucosidases are a heterogeneous group of hydrolytic enzymes. They can be found in cellulolytic microorganisms, as well as in plants, where they serve roles in cell wall development, fruit ripening pigment metabolism, and in mammals, associated with hydrolysis of glucosyl ceramides (Singhania et al., 2013). ß-glucosidases hydrolyze the O-glycosyl linkage of terminal, non-reducing ß-D-glucosyl residues, with variable substrate specificity. In this regard, the enzymes can be divided into i) cellobiases, with high degree of specificity towards cellobiose, ii) arlyl-ß-glucosidases, with high specificity towards aryl-glucosides and iii) broad substrate specificity enzymes, which act on a wide spectrum of substrates (Sørensen et al., 2013). Most of the fungal ß-glucosidases described so far belongs to the last group. However, the more suitable enzymes for industrial strains for bioethanol production are cellobiases (Njokweni et al., 2012).

Most of the cellulases employed in large scale cellulose hydrolysis originates from *T. reesei*, as discussed in *1.9.* The scarce ability of this fungus to secrete ß-glucosidases required investigation of more suitable sources. Highly processive ß-glucosidases have been identified in *A. niger, Aspergillus oryzae, Thermoascus aurantiacus, Saccharomycopsis fibuligera, P. chrysosporium*, among others (Hong et al., 2014; Tang et al., 2013; Tsukada et al., 2006). Typical approaches for enzymes identification and isolation require culturing of the producing organism. Only about 1% of the species can be grown in culture media. Thus, the vast majority of microorganisms, including ß-glucosidase producing ones, cannot be studied with classic methods. However, metagenomic approaches, which can be used for studying population genomes directly from environmental samples, proved to be successful for discovering novel promising enzymes, paving the way for further improving hydrolysis performances. Several ß-glucosidases have already been obtained by means of metagenomics analysis (Bao et al., 2012).

With specific attention towards development of CBP yeast, conferring cellobiose hydrolytic activity can be achieved via two main routes. Host strains can be engineered for expressing either an intracellular ß-glucosidase or cellobiose phosphorylase, together with a cellodextrin importer. Recombinant

yeast is thus capable of importing cellobiose within the cytoplasm, where it is hydrolyzed. This approach limits the possibility of contamination in an industrial bioreactor, due to the scarcity of glucose in the medium (Ha et al., 2011; Sadie et al., 2011). Differently, yeasts can be engineered to express extracellular ß-glucosidases, to be released in the medium. Cellobiose hydrolysis occurs extracellularly and released glucose is later assimilated and metabolized (Eriksen et al., 2013). Despite the advantage of consuming cellobiose within the cell, successful expression of cellobiose importers is challenging, yielding to poor conversion performances (Njokweni et al., 2012).

Extracellular ß-glucosidase activity can be assayed through a variety of different methods, involving the consumption of substrates like cellobiose, salicin or esculin, or the hydrolysis of artificial compounds that release chromogenic or fluorescent substrates (Wood and Bhat, 1988). Hydrolysis of cellobiose, salicin and esculin can be quantified by measuring the amount of released sugars, via dinitrosalicylic acid or Nelson-Somogyi assays. However, the accuracy of these detection methods is no longer satisfactory. In addition, activity on salicin or esculin is not always representative of the ability to hydrolyze cellobiose (Schwald et al., 1988). Saccharification of this substrate can instead yield to extremely precise quantification of the enzymatic activity when coupled with detection of underutilized cellobiose and released glucose by High Performance Liquid Chromatography (HPLC) (Dashtban et al., 2010; Schwald et al., 1988). HPLC analysis, despite being extremely accurate, is a high time and resource consuming technology. More immediate enzymatic assays for ß-glucosidases involves use of the chromogenic compound *p*-nitrophenyl-ß-D-gluco-pyranoside (*p*NPG) or the fluorogenic methyl-umbelliferyl-ß-D-glucoside (MUG) (Singhania et al., 2013). *p*NPG is cleaved by ß-glucosidases and *p*-nitrophenol is released and quantified using a spectrophotometer in order to detect the hydrolysis rate (Dashtban et al., 2010; Kubicek, 1982). Similarly, MUG is cleaved into methylumbelliferone, which is then quantified using a fluorometer (Setlow et al., 2004). *p*NPG and MUG

assays offer strong advantages over other techniques, including immediate results and ease in quantification of large number of samples. However, these methods suffer from a number of false positive effects. Samples exhibiting ß-glucosidase activity, basing on *p*NPG and MUG assays, were reported not to yield any detectable activity on cellobiose (Singhania et al., 2013). In fact, successful hydrolysis of cellobiose requires a conformational change of ß-glucosidase, which is not necessary on *p*NPG and MUG, despite all three molecules display the same O-glycosyl linkage. Thus, some ß-glucosidases may not be able to display any activity on cellobiose, while expressing high activity on artificial molecules (McCarthy et al., 2004).

2. Materials and methods

2.1 Cultivation media

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

Medium	Reference or supplier
Luria-Bertani (LB)	Oxoid – Thermo Fisher Scientific (Waltham, MA, USA)
Nutrient Broth (NB)	Oxoid – Thermo Fisher Scientific (Waltham, MA, USA)
Yeast Peptone Dextrose (YPD)	(Atlas, 2010)
Yeast Nitrogen Base Without Amino Acids (YNB)	Sigma-Aldrich (Sant Louis, MO, USA)
Yeast Peptone Dextrose Sorbitol (YPDS)	(Nickoloff, 1995)

Table 2.1 - Summary of the media used in this study.

2.2 Strains and plasmids

Genotypes, phenotypes and sources of bacterial and yeast strains used in this work are summarized in Table 2.2.

Strain	Relevant genotype / phenotype	Reference
E. coli JM109	*end*A1, *rec*A1, *gyr*A96, *thi, hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15]	Promega (Fitchburg, MI, USA)
S. cerevisiae Ethanol Red®	Industrial yeast strain for bioethanol production	Lesaffre (Marcq-en-Barœul, France)
S. cerevisiae Fm17	Industrial strain with high fermentative vigour and inhibitors tolerance	(Favaro et al., 2013a)
S. cerevisiae Fm89	Newly isolated industrial strain with high inhibitors tolerance	(Favaro et al., 2014)
S. cerevisiae Fm90	Newly isolated industrial strain with high inhibitors tolerance	(Favaro et al., 2014)
S. cerevisiae Fm96	Newly isolated industrial strain with high inhibitors tolerance	(Favaro et al., 2014)
S. cerevisiae M2n	Industrial distillery strain	(Viktor et al., 2013)
S. cerevisiae M2n[pBDK1-BGL3]-C1	*BGL3* multiple copy δ-integration into M2n strain	This work
S. cerevisiae MEL2	Industrial strain with high fermentative vigour	(Favaro et al., 2013b)
S. cerevisiae Y130	Wild type strain with high inhibitor tolerance	Stellenbosch University (ZA)
S. cerevisiae Y294[*Pccbgl1*]	*URA3 ENO1$_P$-XYNSEC-BGL3-ENO1$_T$*	(Njokweni et al., 2012)

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

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

Genotypes and sources of plasmids used in this work are summarized in Table 2.3.

Plasmid	Relevant genotype	Reference
pBKD1	*amp* δ-sites-*PGK1*$_P$-*PGK1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	(Mcbride et al., 2008)
pBKD2	*amp* δ-sites-*ENO1*$_P$-*ENO1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	(Mcbride et al., 2008)
pBKD1-*BGL1*	*amp* δ-sites-*PGK1*$_P$-*BGL1-PGK1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	Stellenbosch University (ZA)
pBKD1-*BGL2*	*amp* δ-sites-*PGK1*$_P$-*BGL2-PGK1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	Stellenbosch University (ZA)
pBKD1-*BGL3*	*amp* δ-sites-*PGK1*$_P$-*BGL3-PGK1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	Stellenbosch University (ZA)
pBKD2-*BGL1*	*amp* δ-sites-*ENO1*$_P$-*BGL1-ENO1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	This work
pBKD2-*BGL2*	*amp* δ-sites-*ENO1*$_P$-*BGL2-ENO1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	This work
pBKD2-*BGL3*	*amp* δ-sites-*ENO1*$_P$-*BGL3-ENO1*$_T$ *TEF*$_P$^{a}-*KanMX-TEF*$_T$^{a}-δ-sites	This work

Table 2.3 - Summary of plasmids used in this study. [a] *TEF1* promoter and terminator from *Ashbya gossypii*

Recombinant plasmids were constructed and amplified in *E. coli* JM109. The bacterial strains were cultured at 37°C on a on a rotary shaker in LB medium or on LB agar (Sambrook and Russell, 2001). Ampicillin was added to a final concentration of 100 μg/mL for the selection of resistant bacteria.

2.3 Evaluation of inhibitor tolerance of selected wild type and industrial yeast

Eight yeast strains (Ethanol Red®, Fm17, Fm89, Fm90, Fm96, M2n, MEL2, Y130), were screened for their industrial fitness. In particular, inhibitors tolerance in presence of four synthetic inhibitors mixtures and seven inhibitors-rich lignocellulosic pre-hydrolysates, was evaluated. *S. cerevisiae* Ethanol Red® was used as reference industrial strain.

2.3.1 Inhibitors tolerance in synthetic inhibitor mixtures

The selected strains were firstly evaluated for their inhibitor tolerance in defined YNB medium supplemented either with 20 g/L or 100 g/L of glucose and containing increasing concentrations of weak acids (acetic, formic acids) and furans (furfural, HMF). Medium was filter sterilized using a 0.22 μm sterile filter. Inhibitors were formulated into four mixtures, namely RC_{25}, RC_{50}, RC_{100}, RC_{200} (RC: Relative Concentration), obtained by adding increasing doses of each toxic compound. Detailed composition of each mixture is reported in Table 2.4. RC_{25}, RC_{50} were respectively obtained as 4-fold and 2-fold dilutions of RC_{100}, which was formulated using the highest concentration of the tested inhibitors present in common lignocellulosic pre-hydrolysates. RC_{200} is a 2-fold concentration of RC_{100} (Favaro et al., 2016). pH was adjusted to 5.0, using 5M NaOH.

This particular pH value was chosen since it is widely used in the bioethanol production process (Kádár et al., 2007; Lin and Tanaka, 2006).

Inhibitor	Concentration (g/L)			
	RC_{25}	RC_{50}	RC_{100}	RC_{200}
Acetic acid	1.80	3.60	7.20	14.40
Formic acid	0.60	1.20	2.40	4.80
Furfural	0.68	1.35	2.70	5.40
HMF	0.95	1.89	3.78	7.56

Table 2.4 – Inhibitors composition of four quaternary mixtures for assessing yeast inhibitors tolerance. pH values of inhibitor mixtures RC_{25}, RC_{50}, RC_{100}, RC_{200} were 2.60, 2.50, 2.40, 2.20, respectively.

Overnight cultures of each yeast strain, cultured at 30°C in YNB medium containing 20 g/L of glucose, were transferred, in triplicate, at an inoculum concentration of 1 x 10^6 cells/mL in 2 mL Eppendorf tubes containing 0.9 mL of medium. After 40 h of aerobic growth, the optical density at 600 nm (OD_{600}) was measured. For each strain, the tolerance was evaluated as relative growth, calculated as ratio between measured OD_{600} values of the medium with inhibitors and the control medium, devoid of any inhibitor mixture. For each strain, the tolerance was evaluated as relative growth (OD_{600} value, %) by comparing the growth in the medium with and in the medium without the inhibitors (Favaro et al., 2013a).

2.3.2 Inhibitors tolerance in lignocellulosic pre-hydrolysates

Inhibitors tolerance of the selected strains was assayed also on eight lignocellulosic pre-hydrolysates, obtained by steam explosion of *Phragmites australis*, *Cynara cardunculus* and *Saccharum officinarum* (sugarcane) bagasse, using different pre-treatment conditions. Pre-treatment parameters, pH and composition of the pre-hydrolysates are reported in Table 2.5.

	Substrate	$LogR_0$	pH	Glucose	Formic acid	Acetic acid	Furfural	HMF	Reference
PG1	*P. australis*	3.60	3.75	0.145	0.324	0.996	0.241	0.051	(Cotana et al., 2015b)
PG2	*P. australis*	4.00	3.29	0.289	0.779	2.184	0.973	0.130	(Cotana et al., 2015b)
PG3	*P. australis*	4.40	3.23	0.427	1.083	3.504	1.432	0.482	(Cotana et al., 2015b)
PG4	*C. cardunculus*	3.85	3.86	0.303	2.731	3.153	0.459	0.298	(Cotana et al., 2015a)
PG5	*C. cardunculus*	4.28	3.79	0.132	4.281	5.799	0.640	0.386	(Cotana et al., 2015a)
PG6	*C. cardunculus*	4.02	3.93	0.201	2.180	2.762	0.439	0.205	(Cotana et al., 2015a)
PG7	*C. cardunculus*	4.28	4.10	0.014	0.498	0.715	0.086	0.049	(Cotana et al., 2015a)
SH	*S. officinarum*	n.a.	3.28	0.500	3.000	11.200	1.700	0.500	(Favaro et al., 2013a)

Table 2.5 – Pre-treatment parameters, pH and composition of the pre-hydrolysates used in this study. Severity factor $LogR_0$ correlates with the harshness of the pre-treatment (Cotana et al., 2015b). Glucose, formic and acetic acid, furfural and HMF are reported as concentration (g/L) in the pre-treated biomass. n.a.: not available

Overnight cultures of each yeast strain were used to inoculate, as described in *2.3.1*, a volume of 200 µL of eight different YNB media, each formulated with one of eight lignocellulosic hydrolysates. Each medium was supplemented with 20 g/L of glucose. pH of the medium was not modified. Medium was filter sterilized using a 0.22 µm sterile filter. The experiment was carried out in quintuplicate for each condition in 96-well plates. Increase in turbidity indicated the ability of the strain to sustain growth in presence of the specific pre-hydrolysate.

Similarly, yeast strains were evaluated in 0.9 mL of YNB medium formulated with pre-hydrolysates PG3, PG5, PG6 and containing 20 g/L glucose, as described in *2.3.1*. pH was either not modified, or adjusted at values of 4.5 and 5.0 by adding 5 M NaOH. The experiment was carried out in triplicate for each condition. Cell cultures preparation, analytical methods and evaluation of inhibitors tolerance in terms of relative growth were performed as described in *2.3.1*.

2.3.3 Fermentation performances on lignocellulosic pre-hydrolysates

Fermentation performances of Fm17 and Ethanol Red® yeast strains were evaluated in YNB medium formulated with PG6 pre-hydrolysate and supplemented 20 g/L glucose. pH was adjusted to 5.0 by adding 5M NaOH. Medium was filter sterilized using a 0.22 µm sterile filter.

Precultures of yeast strains grown to stationary phase in YNB medium containing 20 g/L of glucose were used as inoculum. Cells were collected and used to inoculate 50 mL medium to an initial OD_{600} of 1.0 in triplicate experiments using 55 mL glass serum bottles. The small-scale fermentations were carried out under oxygen-limited conditions. Bottles were sealed with rubber stoppers, incubated at 30°C and mixed on a magnetic stirrer.

Samples were taken through a capped syringe needle pierced through the bottle stopper. Anaerobic growth was measured as absorbance at 600 nm. Samples taken before and during fermentation were analysed for glucose, ethanol, glycerol, acetic acid, formic acid, furfural and HMF. Samples were filtered through a 0.22 µm pore filter and diluted prior to HPLC analysis, performed as described in *2.14.*

2.4 DNA manipulation

Restriction enzyme digestion, electrophoresis, DNA ligation, *E. coli* DNA isolation and transformation were performed using the standard methods according to Sambrook and Russell (2001). DNA fragments were purified from agarose gels by using the Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, MI, USA). Restriction enzymes were supplied by New England Biolabs (Ipswich, MA, USA) and Fermentas - Thermo Fisher Scientific (Waltham, MA, USA). T4 DNA ligase and RNAse was provided by New England Biolabs (Ipswich, MA, USA) and Sigma-Aldrich (Sant Louis, MO, USA), respectively.

2.5 Construction of integrative plasmids for ß-glucosidase secretion

Three fungal genes, *BGL!* from *Saccharomycopsis fibuligera*, *BGL2* and *BGL3* from *P. chrysosporium* were selected for the construction of new integrative vectors targeted to the δ-sequences of the yeast retrotransposon Ty1. These genes, encoding ß-glucosidases, were previously described as highly active on cellobiose (Njokweni et al., 2012). The genes were initially hosted in pBKD1 plasmids and later subcloned into pBKD2 plasmids. pBKD1 and pBKD2 are integrative plasmids differing in the *S. cerevisiae* promoter and terminator sequences, respectively *PGK1* (Phosphoglycerate Kinase) and *ENO1* (EnolaseI).

2.6 Yeast dominant marker resistance

To establish the innate dominant marker resistance, wild type *S. cerevisiae* strains Fm17, MEL2, M2n and Ethanol Red® were grown in YPD broth at 30°C for 24 h. Yeast cells were serially diluted in NaCl (0.9%) and plated onto YPD agar supplemented with increasing amounts of geneticin (0, 50, 100, 150, 200 µg/mL). After 48 h incubation at 30°C, each strain was then evaluated for sensibility to the antibiotic.

2.7 Electrotransformation of yeast strains with integrative δ-vectors

Wild type *S. cerevisiae* strains Fm17, MEL2, M2n and Ethanol Red® were transformed with restricted pBKD1-*BGL1*, pBKD1-*BGL2*, pBKD1-*BGL3*, pBKD2-*BGL1,* pBKD2-*BGL2* and pBKD2-*BGL3* integrative plasmids for multi-copy chromosomal integration. A new protocol was developed from those described in Ausubel (2003), Delorme (1989), and Gysler et al. (1990).

An overnight culture of each host strain was used to inoculate fresh YPD broth at OD_{600} equal to 0.15 and incubated at 30°C on a rotary shaker for 3 h. 10

mL of the culture were harvested in Falcon tubes by centrifugation at 5400 × *g* for 3 min, washed twice in distilled deionized water and resuspended in 800 µL of 0.1 M Lithium Acetate solution into Eppendorf tubes. After 45 min incubation on a rotator wheel at 30°C, 20 µL of 1M Dithiothreitol (Sigma-Aldrich, Sant Louis, MO, USA) were added to the tubes and incubated for additional 15 min. The cells were washed again in distilled deionized water and finally resuspended in 1 mL electroporation buffer containing 1 M sorbitol and 20 mM HEPES (Sigma-Aldrich, Sant Louis, MO, USA). After centrifugation at 3000 × *g* for 2 min, the pellet was resuspended in 250 µL of electroporation buffer. 50 µL of resuspended cells were transferred into electroporation cuvettes (0.2 cm electrode, Bio-Rad, Hercules, CA, USA). After adding 1 µg of linearized plasmid, an electric pulse of 1.4 kV, 200 Ω, 25 µF was applied by using a Gene-Pulser electroporation system (Bio-Rad, Hercules, CA, USA). After delivering the pulse each cuvette was added 1 mL of YPD supplemented with 1 M sorbitol and incubated at 30°C for 3 h.

Optimal cell density and pulse voltage were defined to maximize the number of recombinants.

Electroporated cells were then plated in YPD plates containing 1 M sorbitol supplemented with 200 µg/mL geneticin for selective pressure and incubated at 30°C for 48 h.

2.8 Screening of recombinant clones

Isolated colonies were patched onto new YPDS plates supplemented with 200 µg/mL geneticin with sterile pipette tips as a first screening for identifying stable transformants. After 24 h at 30°C, clones capable of displaying appropriate antibiotic resistance were further evaluated in different screening methods, in order to detect the production of ß-glucosidase.

2.8.1 Enzymatic activity on *p*NPG in 96-well plates

Antibiotic-resistant clones were used to inoculate 180 µL YPD medium in 96-well plates and incubated at 30°C for 48 h. 10 µL of each yeast culture were used to detect the production of ß-glucosidase via enzymatic assay. Each sample was added to a 90 µL mixture containing 88 µL 50 mM citrate buffer pH 5.0 (Colowick and Kaplan, 1956) and 2 µL 250 mM 4-Nitrophenyl ß-D-glucopyranoside (*p*NPG) (Sigma-Aldrich, Sant Louis, MI, USA), in 96-well plates. After 15 min incubation at 60°C, 100 µL 1 M Na_2CO_3 was added to stop the enzymatic reaction. Parental wild-type strains were used as negative control. A significant increase in absorbance (400 nm) over the value displayed by the negative control, indicated presence of ß-glucosidase activity. Positive clones also resulted in the production of a dark yellow solution.

2.8.2 Enzymatic activity on YPD plates containing MUG

Clones displaying resistance to geneticin were point-inoculated with sterile pipette tips on YPD plates supplemented with MUG. 50 µL of a 37 mM solution of MUG (Sigma-Aldrich, Sant Louis, MO, USA) in dimethylformamide was previously spread on the agar surface, using a L-shape spreader. The plates were incubated at 30°C for 48 h and examined under the long-wave ultraviolet light of a transilluminator. Strains with ß-glucosidase activity hydrolyze the substrate, resulting in a fluorescent halo (Fia et al., 2005). Parental wild-type strains were used as negative control.

2.8.3 Enzymatic activity on agar plates containing esculin

Recombinant clones were point-inoculated with sterile pipette tips on YNB agar plates supplemented with 1 g/L esculin (Sigma-Aldrich, Sant Louis, MO, USA) and 0.5 g/L ferric citrate. After incubating at 30°C for 48 h, agar plates were evaluated for the presence of clones producing extracellular ß-glucosidase. The enzymatic activity results in the release of esculetin, produced by cleaving the

glucoside group from esculin. Esculetin reacts with ferric citrate, producing dark areas around the positive clones (Njokweni et al., 2012; Qadri et al., 1980). Parental wild-type strains were used as negative control.

2.8.4 Growth in liquid medium containing cellobiose

Single colonies of antibiotic-resistant clones were resuspended in 500 µL NaCl 0.9% solution, used to inoculate 20 mL of YNB medium containing 10 g/L of cellobiose (Sigma-Aldrich, Sant Louis, MO, USA) to an initial OD_{600} of 0.2, in 50 mL Erlenmeyer flasks. Cell cultures were incubated at 30°C on a rotary shaker. Cell growth was monitored every 24 h by measuring the optical density. ß-glucosidase producing clones exhibited a significant increase in the optical density (OD_{600}) after 48 h, when compared to those of the parental strains, used as negative control.

2.9 Evaluation of mitotic stability

Yeast clones showing ß-glucosidase activity were studied for the mitotic stability of the integrated construct according to Favaro et al. (2012). The recombinants were cultured in sequential batch cultures in non-selective YPD broth (5 mL) on a rotating wheel and transferred (0.1% v/v) to fresh YPD after glucose depletion. After 120 generations, recombinant strains were serially diluted in NaCl (0.9%) and plated onto five YPD plates supplemented with 0 or 200 µg/mL geneticin. After 48 h incubation at 30°C, stable recombinants showed a comparable number of colonies both in presence and in absence of selective pressure.

2.10 Growth kinetics

Aerobic growth performances of recombinant and parental yeast, along with the laboratory Y294[*Pccbgl1*], were studied in buffered (citrate buffer 0.05 M pH 5.0) and unbuffered YNB medium supplemented with 10 g/L cellobiose or

the equivalent amount of glucose (10.53 g/L) (Rodrigues et al., 2015). Y294[*Pccbg1*] required supplementation of amminoacids tryptophan (76 mg/L), hystidine (76 mg/L) and leucine (360 mg/L) to ensure auxotrophic growth. Precultures grown to stationary phase in unbuffered medium containing glucose served as inoculum. Cells were centrifuged at 5400 × *g* for 3 min, washed twice with a saline solution (0.9% NaCl) and used to inoculate 120 mL medium to an initial OD_{600} of 0.2 in triplicate experiments using 500 mL Erlenmeyer flasks. The flasks were incubated up to 100 h at 30°C on a rotary shaker. Samples (2 mL) were periodically taken to measure OD_{600} and to detect cellobiose, glucose and ethanol concentration via HPLC, as described in *2.14*.

2.11 Enzymatic assays

The ability of stable clones to produce ß-glucosidase was evaluated with the *p*NPG method (Kubicek, 1982). Yeast cells were anaerobically grown at 30°C for 72 h in 60 mL YPD medium in 250 mL Erlenmeyer flasks. 15 mL samples were taken at 24 h intervals and centrifuged at 5400 × *g* for 3 min. Dry biomass was determined as described in *2.14.*

ß-glucosidase activity was measured in three different systems: i) supernatant of the cell culture, ii) yeast cells and iii) the whole cell culture. Supernatant was obtained by centrifuging 1 mL of the cell culture at 3000 × *g* for 2 min. In order to compare enzymatic activities displayed in the different systems, the initial volume (1 mL) was restored by adding an appropriate amount of sterile deionized water to supernatant and pellet cells. 10 µL samples were added to 90 µL of substrate containing 88 µL of 50 mM buffer (Colowick and Kaplan, 1956) and 2 µL 250 mM *p*NPG, in 1.5 mL Eppendorf tubes. The tubes were incubated in water bath for 5 or 10 min, according to the assay temperature. The specific incubation time for each temperature (10 min at 30°C, 5 min at 40 to 70°C) was experimentally determined. The addition of 100 µL 1M Na_2CO_3 increased the pH

and quenched the reaction. *p*-nitrophenol released during the enzymatic reaction was detected by measuring the absorbance at 400 nm.

Optimal pH was determined at 60°C by conducting the experiment in 50 mM citrate buffer with the following pH values: 4.0, 4.5, 5.0, 5.5 and 6.0. ß-glucosidase activity was evaluated also at lower pH values (2.5, 3.0, 3.5) using 50 mM citrate-phosphate buffer (Colowick and Kaplan, 1956). Optimal temperature was determined at 30, 40, 50, 60 and 70°C in citrate buffer with the optimal pH, previously determined.

Enzymatic activities were expressed as nanokatals per mL (nKat/mL), which is defined as the enzyme activity needed to release 1 nmol of product per second per mL of culture. Enzymatic activities were also reported as nanokatals per milligram dry cell weight (nKat/(mg DCW)), which is defined as the enzyme activity needed to release 1 nmol of product per second per milligram dry cell weight. The experiments were carried out in triplicate.

2.12 ß-glucosidase thermostability

The effect of temperature on the activity of ß-glucosidase enzyme was determined by exposing supernatant of yeast cultures grown in YPD at 30°C for 48 h in water bath at different temperatures: 30, 40, 50, 60 and 70 °C for increasing amounts of time. At specific intervals, ranging from 1 to 20 min, samples of the supernatant were taken to perform enzymatic assays, as described in *2.11*). Enzymatic activity, expressed in nKat/mL, was correlated with exposure duration.

2.13 Fermentation studies

Fermentation performances of *S. cerevisiae* M2n[pBDK1-*BGL3*]-C1 and the parental M2n were studied together with laboratory Y294[*Pccbgl1*] strain in buffered and unbuffered YNB medium supplemented with cellobiose or glucose as described in *2.10.*

Precultures of yeast strains grown to stationary phase in unbuffered broth containing glucose were used as inoculum. Cells were collected, washed as described in *2.10* and used to inoculate 100 mL medium to an initial OD_{600} of 1.0 in triplicate experiments using 120 mL glass serum bottles (Figure 2.1), as described in *2.3.4*. Sampling, quantification of cell growth and HPLC analysis for the detection of glucose, cellobiose, ethanol and glycerol were performed as described in *2.3.4*.

Figure 2.1 – Experimental setup for yeast fermentation in 120 mL serum bottles on magnetic stirrer.

2.14 Analytical methods

A calibration curve was prepared to correlate dry cell weight (DCW) with optical densities (OD_{600}). Dry cell weights were determined from 15 mL culture

samples. Cells were collected after centrifugation at 5400 × *g* for 3 min. The pellet was washed twice in deionized water and finally resuspended in 10 mL deionized water. The sample was dried in and oven at 80°C to constant weight.

Monosaccharides, glycerol and ethanol were detected with high-performance anion- exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Samples were filtered using 0.22 μm pore-size membranes and separated with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) The mobile phase used was H_2SO_4 0.05 M at a flow rate of 0.6 mL/min at room temperature.

3. Results and discussion

3.1 Screening of *S. cerevisiae* yeast strains for inhibitors tolerance

Seven *S. cerevisiae* strains, namely Fm17, Fm89, Fm90, Fm96, M2n, MEL2 and Y130 were previously described for their industrial fitness (Favaro et al., 2013a; Favaro et al., 2013b; Viktor et al., 2013). These strains were selected for further studies on their inhibitors tolerance with the aim to identify the most suitable yeast platforms for the development of robust CBP organism for lignocellulose conversion into bioethanol. *S. cerevisiae* Ethanol Red® was used as reference industrial strain. Inhibitors tolerance was first evaluated in the presence of four synthetic mixtures of inhibitors typically found in lignocellulosic pre-hydrolysates. Yeast growth was then studied in eight inhibitors-rich lignocellulosic pre-hydrolysates obtained from steam-explosion of: *S. officinarum* (sugarcane) bagasse, *P. australis* (common reed) and *C. cardunculus* (cardoon). Three different pre-hydrolysates were used to further assess the effect of pH on toxicity and yeast growth inhibition. The strain displaying the highest inhibitors tolerance was then chosen for characterizing its fermentation performances on a selected pre-hydrolysate.

3.1.1 Inhibitors tolerance in synthetic inhibitor mixtures

Inhibitors tolerance of the selected *S. cerevisiae* strains together with Ethanol Red® used as benchmark was evaluated in YNB medium containing 20 g/L of glucose and increasing concentrations of synthetic inhibitors, weak acids (acetic, formic acid) and furans (furfural, HMF). Each tested concentration was reported as relative concentration (RC) of the third assessed level considered as the highest concentration of the studied inhibitors found in lignocellulosic pre-hydrolysates. Inhibitors were formulated in four mixtures (RC_{25}, RC_{50}, RC_{100}, RC_{200}), as described in *2.3.1.* pH was corrected to 5.0 with 5 M NaOH.

For each strain, the tolerance was evaluated as relative growth (optical density (OD) value, %) by comparing the yeast growth in the medium containing inhibitory compounds with that in medium lacking these compounds, after 40 h incubation at 30°C (Table 3.1).

	Fm17	Fm89	Fm90	Fm96	M2n	MEL2	Y130	Ethanol Red®
RC_{25}	94	81	87	79	50	82	71	65
RC_{50}	71	62	59	53	21	60	63	44
RC_{100}	60	45	42	39	14	28	59	11
RC_{200}	0	0	0	0	0	0	0	0

Table 3.1 - Influence of increasing concentrations of mixtures of weak acids (acetic and formic acid) and furans (furfural and HMF) on yeast growth in defined YNB medium supplemented with 20 g/L of glucose. pH was adjusted to 5.0 with 5M NaOH. Inhibitors tolerance is expressed as relative growth (%) of the optical density measured for each strain after 40 hours of growth in YNB without inhibitors, and are the means of three replicates. Standard error was always less than 7% (not shown).

Inhibitors mixtures hindered cell growth with different degrees of severity. RC_{25} and RC_{50} showed milder inhibitory effects than RC_{100} and RC_{200}. M2n and benchmark Ethanol Red® strains showed high mortality already in the presence of the most diluted mixture (RC_{25}), with a relative growth of 65 and 50%, respectively. These strains displayed the lowest relative growth among tested strains in all inhibitors mixtures. Conversely, Fm17 exhibited the highest degree of tolerance in all the conditions tested, with a relative growth value of 94, 71 and 60% in RC_{25}, RC_{50} and RC_{100}, respectively. By contrast, RC_{200} did not allow any growth of any of the strains tested.

3.1.2 Inhibitors tolerance in lignocellulosic pre-hydrolysates

Inhibitors tolerance of the yeast strains was also evaluated in presence of eight pre-hydrolysates, obtained by pre-treating three lignocellulosic substrates via steam explosion. Several pre-treatment severity factors were applied to the lignocellulosic material, resulting in the release of different inhibitors

concentrations. The detailed composition of each tested pre-hydrolysate is summarized in Table 2.5.

The ability of the yeast strains tested in *3.1.1* to grow in medium formulated with each of the eight pre-hydrolysates was firstly evaluated in a qualitative high-throughput assay in YNB medium containing 20 g/L of glucose, as described in *2.3.2*. Yeast growth was determined by detecting increased turbidity of the medium and is schematically reported in Table 3.2. Each experimental condition was replicated 5 times.

All yeast strains showed the ability to grow in pre-hydrolysates PG1 and PG2 from *P. australis* and in PG4, PG6 and PG7 from *C. cardunculus*, with the exception of Fm89 strain in PG2 and PG6. Pre-hydrolysates PG3 from *P. australis*, PG5 from *C. cardunculus*, and SH from *S. officinarum* bagasse did not support the growth of any yeast indicating that the concentration of toxic chemical species in these pre-hydrolysates was higher than yeast could tolerate. This hypothesis is confirmed by the elevate inhibitors concentrations present in each of these pre-hydrolysates (Table 2.5). In fact, PG3 contains the highest amount of inhibitors among the pre-hydrolysates originating from *P. australis* and the strongest concentrations of furans among all the pre-hydrolysates. Similarly, PG5, which appears as the harshest pre-hydrolysate from *C. cardunculus*, contains the highest concentrations of weak acids.

Pre-hydrolysates PG3 from *P. australis* and PG5 and PG6 from *C. cardunculus* were chosen for additional experimental activities to select highly tolerant yeast. In particular, PG3 and PG5 raised particular interest as they offered the possibility to evaluate whether pH adjustment would improve the yeast ability to grow in their presence.

Pre-hydrolysate	*S. cerevisiae* strains							
	Fm17	Fm89	Fm90	Fm96	M2n	MEL2	Y130	Ethanol Red®
PG1	+	+	+	+	+	+	+	+
PG2	+	-	+	+	+	+	+	+
PG3	-	-	-	-	-	-	-	-
PG4	+	+	+	+	+	+	+	+
PG5	-	-	-	-	-	-	-	-
PG6	+	-	+	+	+	+	+	+
PG7	+	+	+	+	+	+	+	+
SH	-	-	-	-	-	-	-	-

Table 3.2 – Influence of different lignocellulosic pre-hydrolysates on yeast growth. Yeast strains were cultured in YNB medium containing 20 g/L of glucose and formulated with eight different lignocellulosic pre-hydrolysates. + and - indicate yeast ability or inability to grow in the specific medium.

Instead, PG6 was chosen since it contained the higher amounts of inhibitors tolerated by the yeast, among the tested pre-hydrolysates (Table 2.5).

Relative inhibitors tolerance of the eight strains was quantified in YNB medium containing 20 g/L of glucose and formulated with pre-hydrolysates PG3, PG5 and PG6, without altering the pH of the media, as described in *2.3.2*.

In these conditions, yeast growth was completely inhibited in PG3 and PG5, while all strains could grow in presence of the pre-hydrolysate PG6 (Table 3.3). Y130 and Fm17 exhibited the highest relative growth, 70 and 62%, respectively. Reference strain Ethanol Red® showed lower inhibitors tolerance. Higher toxicity of pre-hydrolysates PG3 and PG5 is likely caused by the higher amounts of acetic acid, furfural and HMF (Table 2.5), compared to the less toxic PG6.

The experiment was replicated after adjusting medium acidity to pH 5.0. Relative growth of the tested yeast is reported in Table 3.3.

		PG3		PG5		PG6	
Strain	pH	Unaltered (pH 3.23)	Adjusted (pH 5.00)	Unaltered (pH 3.79)	Adjusted (pH 5.00)	Unaltered (pH 3.93)	Adjusted (pH 5.00)
Fm17		0	63	0	59	62	88
Fm89		0	68	0	63	48	11
Fm90		0	61	0	60	61	80
Fm96		0	3	0	54	50	79
M2n		0	16	0	60	53	57
MEL2		0	2	0	56	30	61
Y130		0	67	0	60	70	76
Ethanol Red®		0	7	0	63	50	78

Table 3.3 - Influence of different lignocellulosic pre-hydrolysates on yeast growth in defined YNB medium supplemented with 20 g/L of glucose with or without pH adjustment to pH 5.0 with 5M NaOH. Inhibitors tolerance is expressed as relative growth (%) of the optical density measured for each strain after 40 hours of growth in YNB, and are the means of three replicates. Standard error was always less than 7% (not shown).

After pH adjustment, all yeast strains could grow in presence of pre-hydrolysates PG3 and PG5, as well as in PG6. While all strains showed similar tolerance to PG5, amounting to about 40% mortality compared to that in the control YNB medium, strong differences could be identified in the case of pre-hydrolysates PG3 and PG6. The reference strain *S. cerevisiae* Ethanol Red® proved to be extremely inhibited when cultured in presence of PG3, while showing high tolerance in PG5 and PG6. pH adjustment resulted in an overall improvement of relative growth, with the exception of M2n and Fm89 strains. Fm17, in particular, exhibited higher relative growth in PG6, as well as high performances in PG3 and PG5.

Benefits generated by pH adjustment can be ascribed to the acidity-related dissociation of weak acids. As extracellular dissociated acids are liposoluble, they can permeate through the cell membrane and lower the cytosolic pH, thus inducing stress levels to the cell that can cause the inhibition of metabolic activities. The amount of dissociate acid is a function of pH and the pK_a of each specific acid, and increases with decrease in pH. The concentration of

undissociated and dissociated acids in lignocellulosic pre-hydrolysates is then very sensible to the medium acidity (Palmqvist and Hahn-Hägerdal, 2000). Increase of medium pH to value closer to or higher than the pK_a of weak acids thus reduces the concentration of harmful dissociate acids, resulting in less stressful conditions for the yeast.

Basing on the high inhibitors tolerance showed in different lignocellulosic pre-hydrolysates, Fm17 strain confirmed the remarkable industrial fitness highlighted in previous studies (Favaro et al., 2013a; Favaro et al., 2016). The performances of this strain were then further characterized, together with the reference Ethanol Red® in terms of fermentation abilities in the lignocellulosic pre-hydrolysate PG6 *C. cardunculus*, chosen for its high inhibitors concentrations (Table 2.5) and on the basis of the higher cell viability displayed by the tested yeast strains in this pre-hydrolysate, compared to PG3 and PG5 (Table 3.3).

3.1.3 Fermentation performances on lignocellulosic pre-hydrolysate

S. cerevisiae Fm17 and Ethanol Red® were evaluated for their fermentation performances in small scale fermentation under oxygen-limited conditions in 50 mL YNB medium containing 20 g/L of glucose, formulated with pre-hydrolysate PG6. Acidity of the medium was adjusted to pH 5.0 with 5M NaOH. Fermentation medium formulated without PG6 was used as control (Figure 3.1).

The strains utilized all glucose available by 20 h of fermentation in both tested media (Figure 3.1a, 3.1b, 3.1c, 3.1d). Ethanol Red® produced higher biomass than Fm17 in both media: final OD_{600} was 4.8 in the control medium and 4.0 in presence of PG6, amounting to 17% and 38% higher than Fm17, respectively.

However, Fm17 displayed better fermentation performances in terms of ethanol yield in presence of the pre-hydrolysate. Fm17 and Ethanol Red®

produced 9.0 g/L and 8.4 g/L of ethanol in the medium formulated with PG6, respectively, corresponding to 88% and 82% of the theoretical yield (Figure 3.1a,

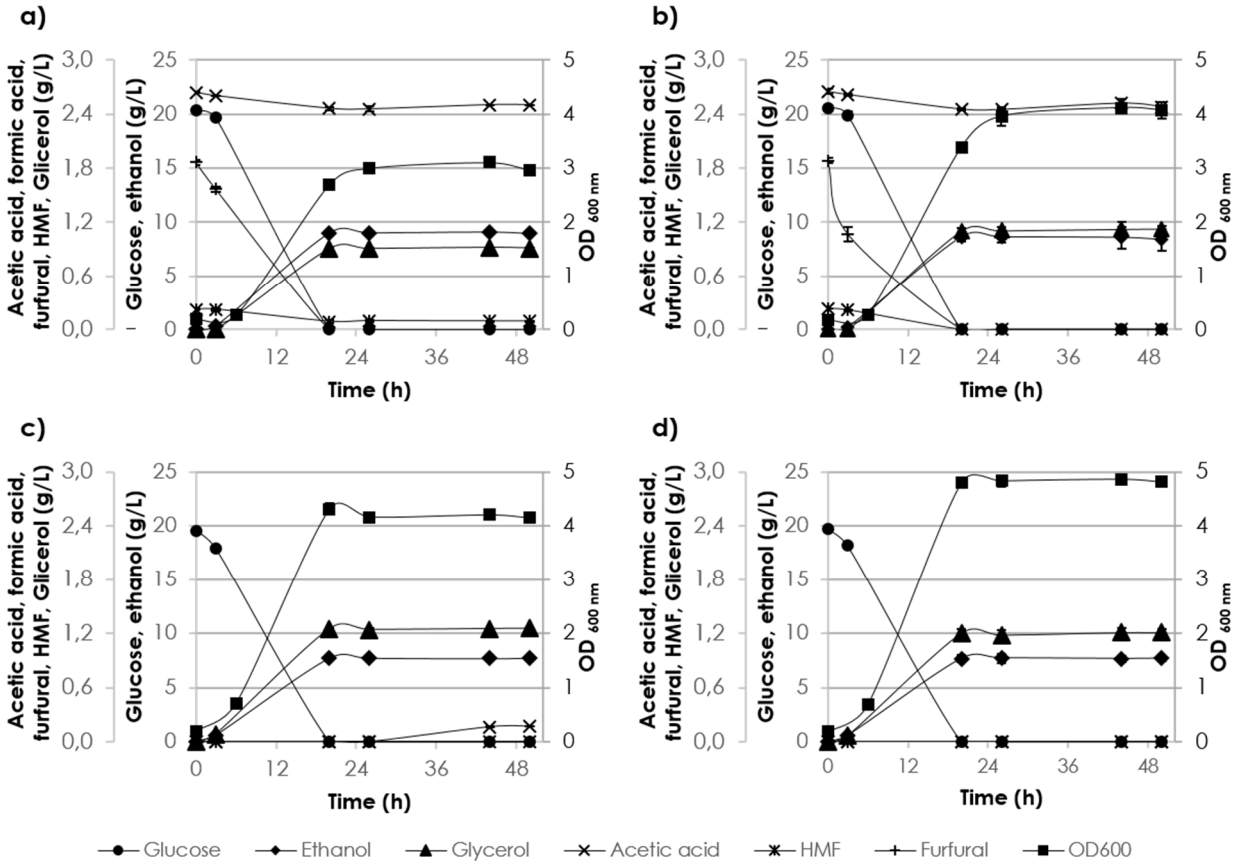

Figure 3.1 - Comparison of fermentation performances of *S. cerevisiae* strains in YNB broth containing 20 g/L of glucose with or without addition of pre-hydrolysate PG6 from *C. cardunculus*: Fm17 (a: supplemented with PG6, c: not supplemented) and Ethanol Red® (b: supplemented, d: not supplemented). Acidity of the medium was adjusted to pH 5.0 with NaOH. The experiment was conducted in triplicate. Error bars correspond to the standard deviation of the means.

3.1b). Both strains yielded 7.7 g/L of ethanol, corresponding to 75% of the theoretical in the control medium (Figure 3.1c, 3.1d).

Since pre-hydrolysates are characterized by a complex chemical composition, presence of additional fermentable sugars in the medium containing PG6 is possible, resulting in higher ethanol production.

However, a higher amount of ethanol produced in presence of inhibitors rich pre-hydrolysate can also be ascribed to the presence of furfural and HMF.

Although these chemical compounds exhibit a negative impact on yeast metabolism, their reduction to less toxic compounds can act as a redox sink, thus preventing redox imbalances and increasing final ethanol yield (Ask et al., 2013; Favaro et al., 2013a; Wahlbom and Hahn-Hägerdal, 2002). Furfural and HMF were completely metabolized by Ethanol Red® (Figure 3.1b), while Fm17 was able to entirely reduce furfural to furfuryl alcohol and over 50% of the available HMF to the less toxic 5-hydroxymethylfurfuryl alcohol (Figure 3.1a). Lower glycerol production observed in presence of PG6 when compared to the control medium further supports this hypothesis, as glycerol production as redox sink is less favored than furans conversion (Martín and Jönsson, 2003; Palmqvist et al., 1999).

Overall, *S. cerevisiae* strain Fm17, previously selected for its outstanding tolerance to high inhibitors levels typical of lignocellulosic pre-hydrolysates, showed higher ethanol yield than reference Ethanol Red® strain currently used in industrial bioethanol production, also in the presence of a pre-hydrolysate from *C. cardunculus*. Therefore, high inhibitors tolerance and promising fermentation performances make Fm17 a strong candidate platform for the development of CBP yeast for lignocellulosic conversion into bioethanol. In addition, this strain previously demonstrated promising phenotypic traits such as thermotolerance ad high ethanol yield (Favaro et al., 2013a).

Fm17 was selected, together with the industrial Ethanol Red® for the expression of heterologous fungal ß-glucosidases as a first step for the development of a recombinant strain suitable for CBP purposes.

In addition to Fm17 and Ethanol Red ®, *S. cerevisiae* MEL2 was chosen as it previously indicated outstanding ethanol yield from wheat bran hydrolysate (Favaro et al., 2013b), together with the industrial distillery strain M2n. Both MEL2 and M2n were previously successfully engineered by δ-integration for the expression of exogenous amylases (Favaro et al., 2015)

3.2 Integrative plasmids construction

Development of CBP yeast for conversion of lignocellulosic substrates into bioethanol requires engineering innately robust yeast platforms, such as the inhibitor resistant and industrial strains selected in this work. Expression of exogenous genes can be achieved via two main strategies, namely episomal plasmids and chromosomal integration, as more thoroughly discussed in *1.12*.

In this regard, integration of exogenous sequences represents the preferred route for developing industrial-grad yeast. In particular, integration at δ-sequences level allows the expression of multiple gene copies. δ-sequences are long terminal repeats of *S. cerevisiae* retrotransposon *Ty*, present in high copy number within the yeast genome. Further, yeast strains with integrated exogenous genes do not require the use of selective medium in order to maintain the new phenotypic traits. Despite the higher enzymatic activity exhibited by yeast with episomal multicopy plasmids, the stability guaranteed by genomic integration favors this approach in industrial application scenarios.

For this reason, two δ-integrative plasmids named pBKD1 and pBKD2 were chosen for the transformation of yeast strains. In pBKD1, a multiple cloning site is located, between the *S. cerevisiae PGK1* (Phosphoglycerate kinase 1) promoter and terminator sequences (Figures 3.2a). An identical multiple cloning site is located between the *S. cerevisiae ENO1* (Enolase1) promoter and terminator sequences in pBKD2 (Figures 3.2b). These particular regulatory sequences allow the exogenous sequence to be constitutively expressed, once transformed into the recipient yeast strain. The suitability of both *PGK1* and *ENO1* for the constitutive expression of exogenous genes, including β-glucosidases, was previously demonstrated in naturally isolated and laboratory *S. cerevisiae* strains (Favaro et al., 2015; Njokweni et al., 2012). Together with the gene of interest, the plasmids contain also *KanMX,* a geneticin (G418) resistance sequence, under the control of the promoter and terminator sequences of the constitutively expressed *TEF* gene from *Ashbya gossypii* (Steiner and

Philippsen, 1994; Wach et al., 1994) (Figure 3.2a, 3.2c). The integrative region, which includes the multiple cloning site, the antibiotic resistance gene, as well as the promoter and terminator sequences, is flanked by repetitive δ-sequences (Figure 3.2, 3.4, 3.5) for the integration within the yeast chromosomes.

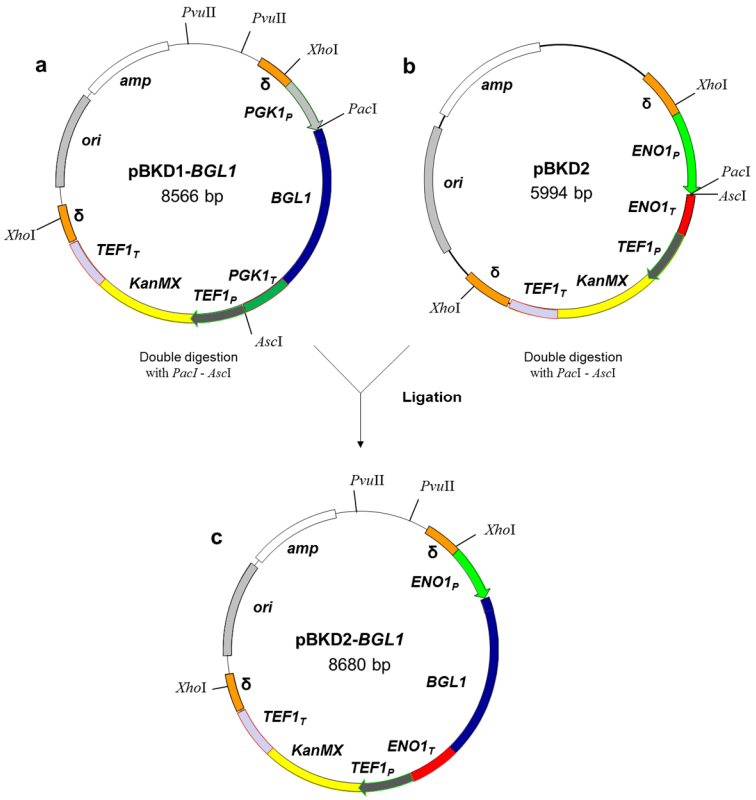

Figure 3.2 - Construction of the δ-integrative vector pBKD2-*BGL1* for *BGL1* constitutive expression in wild type *S. cerevisiae* strains.

pBKD1 and pBKD2 also contain bacterial *ori* and *amp* genes, for plasmid replication and for the expression of ampicillin resistance in *E. coli* strains.

Three β-glucosidase codifying genes *BGL1* from *S. fibuligera*, *BGL2* and *BGL3* from *Phanerochaete chrysosporium* had previously indicated promising hydrolytic activities on cellobiose when expressed in laboratory strains via multicopy episomal plasmids (Njokweni et al., 2012; Van Rooyen et al., 2005). For this reason, they were chosen for engineering wild type yeasts by δ-integration.

BGL1, *BGL2* and *BGL3* were initially singly hosted in pBKD1 plasmids, named pBKD1-*BGL1*, pBKD1-*BGL2*, pBKD1-*BGL3* (Figure 3.2a, 3.4a, 3.5a), that were obtained from Stellenbosch University (ZA).

For the construction of the novel plasmid pBKD2-*BGL1*, *BGL1* was excised from pBKD1-*BGL1* using the restriction enzymes *Pac*I and *Asc*I, each cleaving the plasmid in a unique position within the multiple cloning site. The two resulting fragments were separated via agarose gel electrophoresis.

The fragment containing *BGL1* was recovered from the gel and ligated to pBKD2, previously digested with the same restriction enzymes in order to create cohesive ends necessary to ligate *BGL1*. Plasmid was then extracted and confirmed to be pBKD2-*BGL1* by enzymatic digestion. *Cla*I was used as it yields two different restriction patterns from pBKD2 and pBKD2-*BGL1*: 3211, 1723, 1060 and 4409, 3211, 1060 bp, respectively. The resulting enzymatic digestion gave the expected restriction fragments for both the plasmids (Figure 3.3), indicating that the integrative plasmid pBKD2-*BGL1* was successfully obtained.

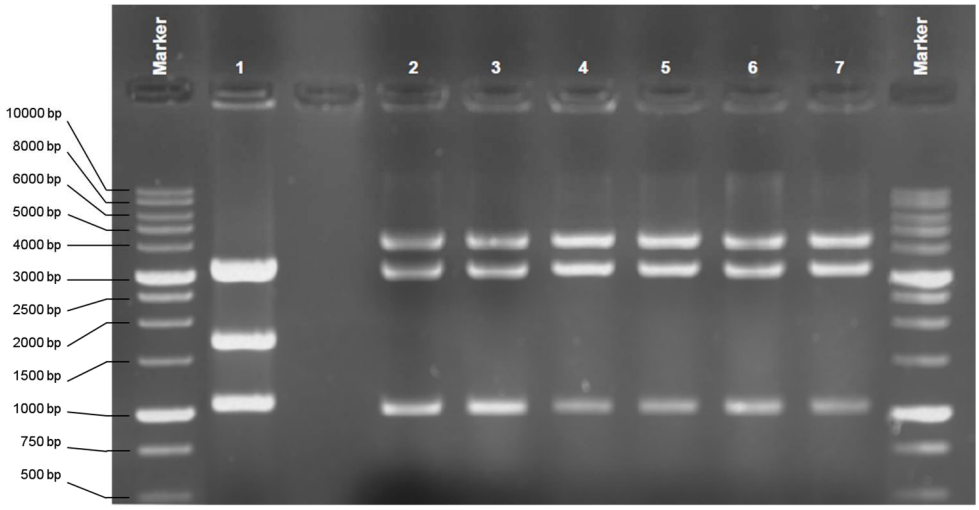

Figure 3.3 - Gel electrophoresis of pBKD2 (lane 1) and pBKD2-*BGL1* (lanes 2-7) digested with *Cla*I; Marker: molecular weight marker 'Sharpmass 1 DNA Ladder' (Euroclone, Milano, IT)

Similarly, pBKD2-*BGL2* and pBKD2-*BGL3* were produced from pBKD1-*BGL2* and pBKD1-*BGL3* (Figures 3.4 and 3.5).

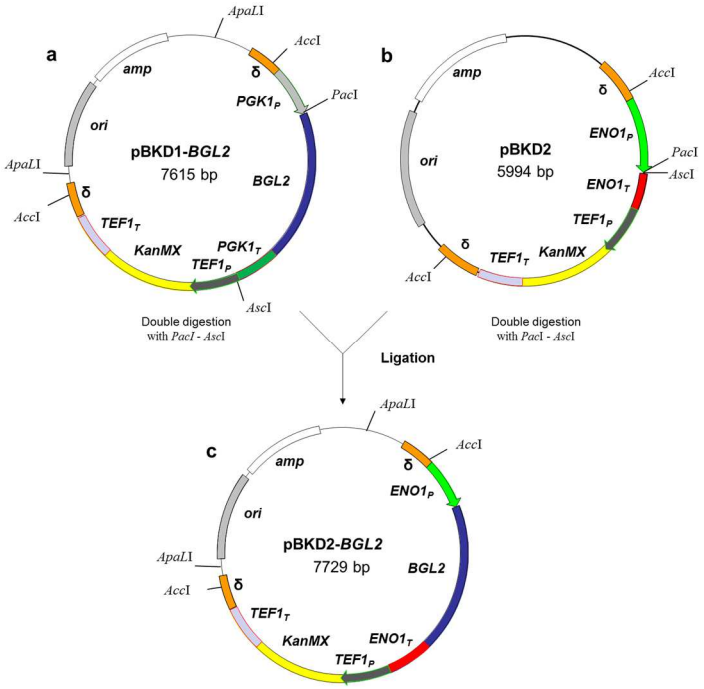

Figure 3.4 - Construction of the δ-integrative vector pBKD2-*BGL2* for *BGL2* constitutive expression in wild type *S. cerevisiae* strains.

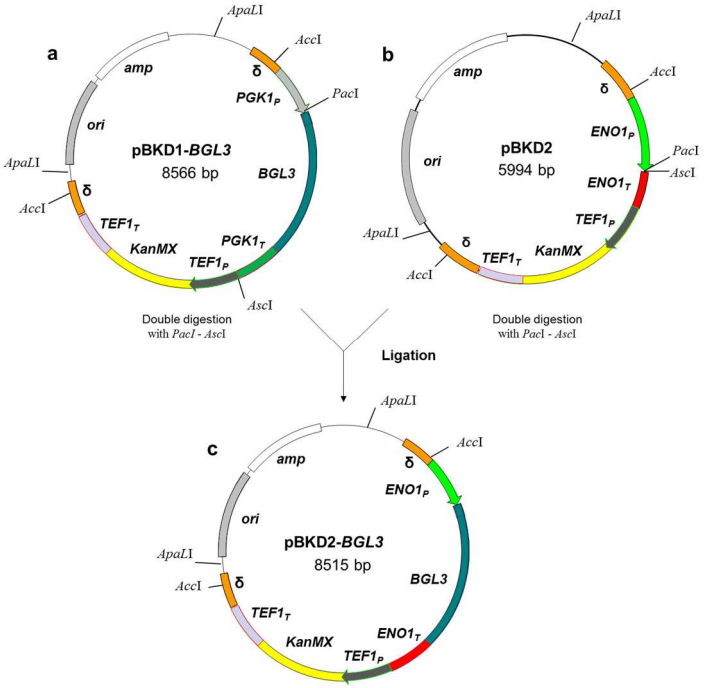

Figure 3.5 - Construction of the δ-integrative vector pBKD2-*BGL3* for *BGL3* constitutive expression in wild type *S. cerevisiae* strains.

3.3 Yeast strains transformation

The integrative plasmids constructed from pBKD1 and pBKD2 were used to insert *BGL1*, *BGL2* and *BGL3* genes into the selected *S. cerevisiae* Fm17, MEL2, M2n and Ethanol Red strains. Exogenous genes were inserted into the yeast platforms by electrotransformation followed by homologous recombination within target δ repetitive sequences.

The integrative plasmids constructed contain unique *Xho*I and *Acc*I sites within the δ-sequence regions. pBKD1-*BGL1* and pBKD2-*BGL1* were linearized by double digestion with *Xho*I in order to create linear vectors flanked by δ-sequences, for an efficient homologous recombination into yeast chromosomes, and *Pvu*II, to cleave the plasmid backbone, for preventing the possibility of integrating unwanted sequences (Figures 3.2a, 3.2c). As *BGL2* and *BGL3* have a *Xho*I restriction site, the remaining plasmids were digested with *Acc*I, while *ApaL*I was used for cleaving the plasmid backbone (Figures 3.4a, 3.4c, 3.5a, 3,5c).

Unlike laboratory haploid strains of *S. cerevisiae*, wild type isolates are often prototrophic, thus lacking selective genetic markers (Akada, 2002; Baruffini et al., 2009). Thus, screening of recombinant clones relies on dominant selection markers such as *KanMX* for geneticin resistance. The highest geneticin concentration tolerated by the wild type *S. cerevisiae* strains was determined on YPDS medium and on defined YNB medium containing glucose (10 g/L) as sole carbon source (Table 3.4).

The concentration of 200 μg/mL of geneticin was chosen for the selection of recombinants.

S. cerevisiae strain	Fm17		MEL2		M2n		Ethanol Red®	
Geneticin (μg/mL)	YPDS	YNB	YPDS	YNB	YPDS	YNB	YPDS	YNB
0	+++	+++	+++	+++	+++	+++	+++	+++
50	+++	+++	+	+	+++	+++	++	++
100	n.g.	n.g.	n.g.	n.g.	++	++	n.g.	n.g.
200	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.	n.g.

Table 3.4. Dominant selection marker resistance of *S. cerevisiae* strains Fm17, MEL2, M2n, Ethanol Red® grown on YPDS and YNB plates supplemented with increasing concentration of geneticin. (++++: consistent growth; n.g.: no growth)

Yeast cells were prepared as described in *2.7* and transformed through electroporation. In order to obtain the highest number of recombinant clones, optimal voltage, cell density and DNA concentrations were defined to be: 1.4 kV, 200 Ω, 25 μF; 10 mL of the initial inoculum was concentrated and used to prepare 50 μL of competent cells that were transformed with 1 μg of linearized DNA. The electroporated cells were plated on selective YPDS agar supplemented with geneticin. Geneticin-resistant clones were picked with sterile pipette tips and point-inoculated on fresh YPDS plates supplemented with geneticin. About 40% of the about 4000 clones initially grown in presence of geneticin, confirmed to retain the newly acquired antibiotic resistance.

Clones exhibiting resistance to geneticin were further tested for the ability to utilize cellobiose as sole carbon source or for exhibiting β-glucosidase activity, using several methods described in *2.8.* Advantages and disadvantages of the different methods are summarized in Table 3.5.

Screening method	Advantages	Disadvantages
Agar plates with MUG	High throughput	• High number of false positives • Not quantitative
Agar plates with esculin	High throughput	• Long incubation time • Not quantitative
Growth on YNB plate with cellobiose	High throughput	• High number of false positives • Not quantitative
Growth in YNB broth supplemented with cellobiose	Little or no risk of false positives	• Time consuming • Low throughput • Attempts to micronize yeast cultures lead to higher number of false positives
Extracellular ß-glucosidase activity on PNPG in 96-well plates	High throughput	• Requires specific plate-reader spectrophotometer • Requires previous culturing in YPD medium.

Table 3.5 – Advantages and disadvantages of the different screening methods used in this study for identifying ß-glucosidase producing clones.

The use of methods based on MUG, esculin or cellobiose allows to screen high numbers of potential clones, requiring limited amount of time and effort. However, some of these techniques suffered from high number of false positives, rendering them unsuitable for this specific purpose. Another method based on growth in YNB broth supplemented with cellobiose as sole carbon source required high amounts of time and laboratory supplies, despite being extremely accurate in identifying production of ß-glucosidase.

Esculin precipitation in agar plates was chosen as standard method for a qualitative screening for ß-glucosidase producing clones, as it offered the best compromise in terms of time requirements and reliability.

Among the geneticin resistant clones produced, a consistent amount of recombinant clones exhibiting ß-glucosidase phenotype on esculin agar plates was

obtained for each combination of wild type host strains and integrative vectors. The integrated yeasts with the largest esculin precipitation halos were selected and maintained on agar plates for further analysis (Table 3.6).

	Fm17	M2n	MEL2	Ethanol Red®
pBKD1-*BGL1*	0	3	1	2
pBKD1-*BGL2*	5	3	4	0
pBKD1-*BGL3*	2	2	1	3
pBKD2-*BGL1*	4	4	1	2
pBKD2-*BGL2*	5	2	1	3
pBKD2-*BGL3*	2	0	0	4

Table 3.6– Number of recombinant yeast clones exhibiting ß-glucosidase activity on esculin agar plates for each combination of transformed yeast strains and integrative plasmids.

ß-glucosidase activity of these clones was quantified by enzymatic assay on *p*NPG in 96-well plates. Despite requiring the additional step of growing the recombinant clones in YPD medium (Table 3.5), this method resulted particularly suitable for quickly quantifying the enzymatic activity of a limited number of samples.

ß-glucosidase producing clones were compared in terms of enzymatic activity with two different benchmark strains: i) the *S. cerevisiae* T2[pBKD1-*BGL1*] strain, previously constructed by δ-integration of *BGL1* from *S. fibuligera* in a wild type *S. cerevisiae* yeast (Trento, 2013) and ii) the haploid laboratory strain Y294[*Pccbgl1*] (Njokweni et al., 2012).

Extracellular ß-glucosidase activity was detected in all the selected recombinants reported in Table 3.6, with a high variability among the different combinations of engineered yeast and integrated genes, ranging from 0.15 to 3.50

nkat/ (mg DCW) (data not shown). Noteworthy, all the newly engineered strains produced higher enzymatic activities than that of the benchmark T2[pBKD1-*BGL1*] (lower than 0.10 nkat/(mg DCW)). Nevertheless, their enzymatic activities were found to be lower than that showed by the laboratory strain Y294[*Pccbgl1*] (7.20 nkat/(mg DCW)) (Njokweni et al., 2012).

This finding could be explained considering that a lower number of gene copies may be integrated into the chromosome, compared to the high number of gene copies provided by multicopy episomal plasmid in Y294[*Pccbgl1*]. Furthermore, δ-integrations could occur in chromosome regions hardly accessible to the transcriptional machinery. Despite the widespread distribution of δ-sequences within the yeast chromosomes, frequent occurring of integration into one single chromosome was reported (Sakai et al., 1990), although the reasons remain to be elucidated. In addition, the diploid nature of natural isolated and many industrial yeasts strains, including M2n, could be responsible for low gene expression. Since expression of the δ-sequence is governed by haploid-specific transcriptional activation, the expression level of a δ-integrated heterologous gene diploid cells can be much lower than that in haploid cells (Ekino et al., 2002). Successful δ-integration of fungal ß-glucosidases within yeast chromosomes have so far mostly involved engineering of haploid strains (Cho et al., 1999; Yamada et al., 2010a).

Before further characterizing their hydrolytic activities on cellobiose, all 54 clones have been evaluated for mitotic stability according to Favaro et al (2012). Thus, all recombinants were grown in sequential batch cultures using non-selective YPD broth. The majority of the screened clones lost both the phenotypes of resistance to antibiotic and esculin hydrolytic activity. After 120 generations, only one engineered strain was found to be mitotically stable. This strain, named M2n[pBKD1-*BGL3*]-C1, was obtained by expressing *BGL3* in *S. cerevisiae* M2n, under the control of promoter and terminator sequences of the constitutively expressed *PGK1.*

3.4 BGL3 characterization

BGL3 produced by M2n[pBKD1-*BGL3*]-C1 was characterized on *p*NPG-based enzymatic assays, in order to: i) verify the extracellular localization of enzyme, ii) identify the optimal working conditions in terms of pH and temperature, iii) quantify the maximum activity and iv) assess the stability of the enzyme at different incubation temperatures, indicated as the ability to retain the initial activity over time.

Extracellular localization of the BGL3 was studied by quantifying the enzymatic activity on *p*NPG in three different systems: i) a cell culture of the recombinant strain; ii) the supernatant and iii) a resuspension of the yeast cells separated by centrifugation of the initial cell culture, hereafter indicated as cell-bound enzyme. As described in *2.11*, M2n[pBKD1-*BGL3*]-C1 and the parental M2n were cultured for 72 h in YPD broth. Samples were taken every 24 h. Supernatant and cells resuspension were brought to a final volume equal to those of the cell culture, in order to facilitate the comparison of their enzymatic activities. The experiment was performed at the temperature of 50°C at three different pH values. All measurements were conducted in triplicate. The enzymatic activity was expressed as nanokatals per milligram of dry cell weight (nkat/(mg DCW)), which is defined as the enzyme activity required to produce 1 nmol of glucose per second per milligram of dry cell weight.

The highest enzymatic activity was achieved after growing the recombinant strain for 48 h, and is represented in Figure 3.6. At all pH values tested, activity in the supernatant represented about 80% of the total activity, measured on the cell culture (Figure 3.6). The enzymatic activity was maximum at pH 5.0 in each of the three systems evaluated. Activity of the supernatant at pH 4.0 and 6.0 was 50% and 40% of that showed at pH 5.0, respectively.

The higher activity in the supernatant than that exhibited by cell-bound enzyme indicates that BGL3 is mainly secreted extracellularly (Figure 3.6). A significant part of the enzyme, however, remains cell-bound.

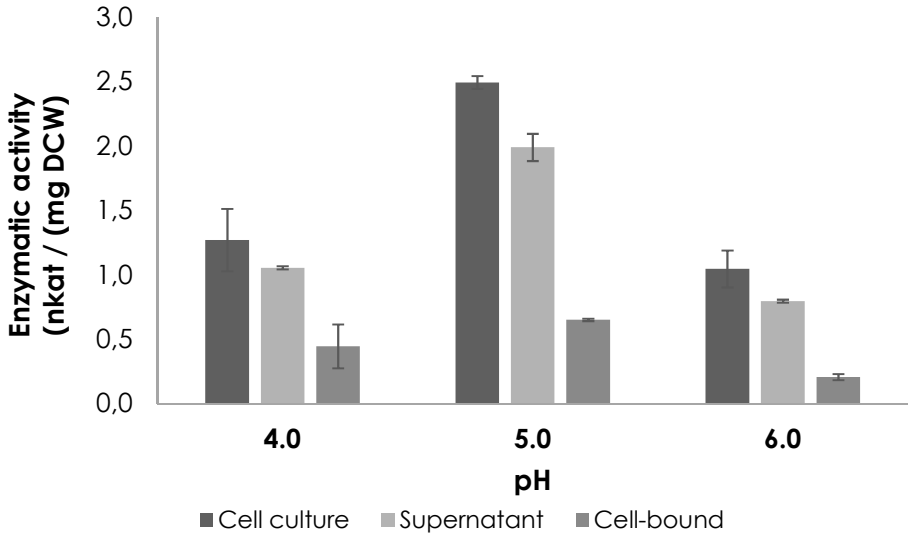

Figure 3.6 – Enzymatic activity of BGL3 secreted by M2n[pBKD1-*BGL3*]-C1 after growing for 48 h in YPD medium, measured at 50°C at different pH values. Data shown are the mean values of three replicates and standard deviations are included.

The optimal conditions for secreted BGL3 were then defined in terms of temperature and pH, using the cell-free supernatant system.

The cell-free supernatant of a liquid culture of M2n[pBDK1-*BGL3*]-C1 grown for 48 h at 30°C was assayed *in vitro* in citrate buffer at different pH values (4.0, 4.5, 5.0, 5.5) at the temperature of 50°C, using *p*NPG as substrate. The activity of recombinant BGL3 from *P. chrysosporium* was the highest at pH 5.0 (Figure 3.7a). Deviations from the optimal pH resulted in marked decrease in the enzymatic activity, which diminished to about 60% and 70% of the highest value at pH 4.0 and 5.5, respectively. The enzymatic activity of the cell-free supernatant was then assayed at different temperatures ranging from 30 to 70°C at the optimal pH of 5.0. The highest enzymatic activity was achieved at 60°C (Figure 3.7b) At higher and lower temperatures, decrease in enzymatic activity is more pronounced than previously discussed in the case of the pH. While only 14% of the activity is lost at 70°C, when compared to the optimal temperature, lowering

incubation temperature results in a stronger decrease. At 40°C and 30°C the activity is diminished by 55 and 78% respectively.

Enzymatic activity of BGL3 from the supernatant was quantified as 3.50 nkat/mg DCW after incubating at 30°C for 48 hours in YPD broth. M2n[pBKD1-*BGL3*]-C1 produced around 6 g/L of dry biomass. The enzymatic activity was also evaluated at the optimal pH and temperature, after growing the yeast for 48 h in defined YNB medium. In these conditions it was quantified as 1.80 nkat/mg DCW.

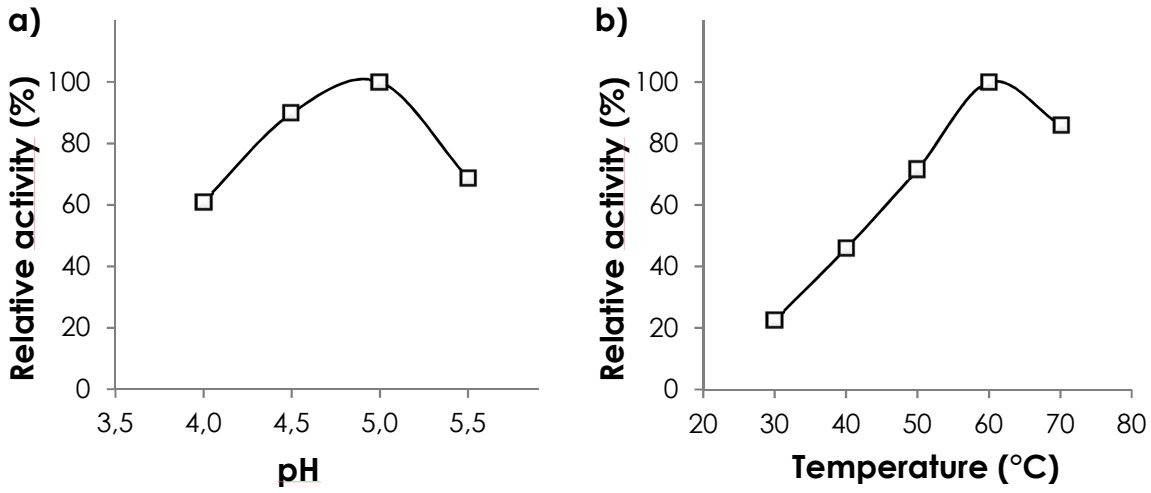

Figure 3.7 - Effect of pH and incubation temperature on ß-glucosidase activity in the supernatant of the recombinant M2n[pBKD1-*BGL3*]-C1. The enzymatic activity was evaluated at pH 4.0, 4.5, 5.0, 5.5 at 50°C (a) and at 30, 40, 50, 60, 70°C at pH 5.0 (b). The strain was previously cultured for 48 hours at 30°C in YPD medium. Activity is expressed as a percentage of the highest value.

The effect of incubation temperature on retaining initial BGL3 activity was assessed by exposing samples of the supernatant of a liquid culture of M2n[pBKD1-*BGL3*]-C1 to different temperatures for increasing amounts of time, as described in *2.12.* In particular, three different temperatures were studied: 30°C, representing the working temperature in industrial large-scale fermenters; 40°C, for evaluating the enzyme performances at the increased temperature that

favors thermotolerant yeast; 60°C, at which the enzyme shows the highest activity. The experiment was conducted at pH 5.0.

Enzymatic activity was maintained nearly stable after exposition at 30 and 40°C for up to 24 hours, while it decreased to zero already after 2 h incubation at 60°C (Figure 3.8a). The experiment was repeated at 60°C, and the supernatant was incubated for shorter time intervals, up to 20 min (Figure 3.8b). BGL3 activity gradually decreases over time, reducing to 44% of the highest value (displayed by the control sample, not exposed to the specific temperature) after 5 min exposure. Incubation for 20 min resulted in no measurable enzymatic activity. The higher activity displayed at 40°C indicates that BGL3 is particularly suited for the expression in thermotolerant yeast. In these conditions, ß-glucosidase would cleave cellobiose into glucose at a 2-fold faster rate (Figure 3.7b) than at 30°C, while its activity would remain stable for up to 24 h after secretion (Figure 3.8a).

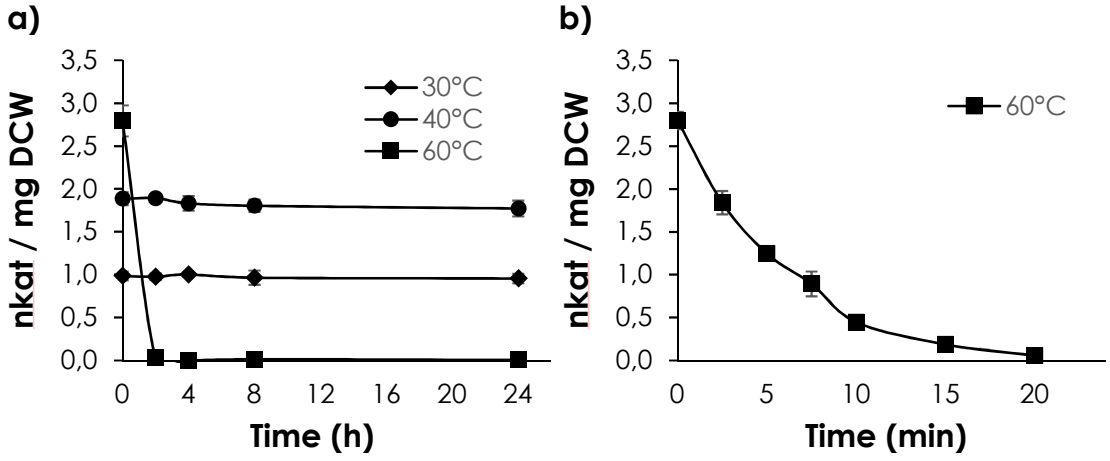

Figure 3.8 – Enzymatic stability of BGL3 from the supernatant of the recombinant M2n[pBKD1-*BGL3*]-C1, previously cultured for 48 hours at 30°C in YPD medium. a) samples of the supernatant were exposed at 30, 40 and 60°C for increasing time intervals, ranging from 0 to 24 hours. b) samples of the supernatant were exposed at 60°C for increasing time intervals, ranging from 0 to 20 minutes. Data shown are the mean values of three replicates and standard deviations are included.

3.5 Growth kinetics on glucose and cellobiose

M2n[pBDK1-*BGL3*]-C1 was evaluated for the newly acquired ability to consume cellobiose. The yeast was grown aerobically in YNB medium containing either glucose (10.53 g/L) or the equivalent amount of cellobiose (10 g/L) as the sole carbon source, and compared to the parental *S. cerevisiae* M2n strain. With the aim to compare the performances of M2n[pBDK1-*BGL3*]-C1 to those of a laboratory strain producing BGL3, growth kinetics of the laboratory strain *S. cerevisiae* Y294[*Pccbgl1*], expressing *BGL3* via multicopy episomal plasmids (Njokweni et al., 2012) were also studied.

M2n[pBDK1-*BGL3*]-C1 reached a final OD_{600} of 4.7 after 100 h (Figure 3.9a), proving the ability of secreting sufficient amounts of ß-glucosidase to sustain growth on cellobiose as sole carbon source. The recombinant strain, however, consumed only 6.7 g/L of cellobiose, representing about two thirds of the available. As expected, the parental M2n did not show any growth on this dimer (Figure 3.9b). When grown on the equivalent amount of glucose, M2n[pBDK1-*BGL3*]-C1 consumed all the carbon source available, reaching a final OD_{600} of 6.8 (Figure 3.9a), as early as after 48 h. Growth kinetics of the recombinant strain is comparable to the one showed by the parental M2n (Figure 3.9b), indicating that yeast transformation and the ß-glucosidase production do not cause any severe metabolic burden to the recombinant yeast.

Laboratory strain Y294[*Pccbgl1*] reached a final OD_{600} of 6.2 when cultured in cellobiose, which is slightly higher than obtained in glucose (final OD_{600} 5.5) (Figure 3.9c). Despite the higher activity this strain exhibits on cellobiose, due to the presence of numerous *BGL3* copies in multicopy episomal plasmids, only 9.5 g/L out of 10 g/L of cellobiose available were consumed by the laboratory strain.

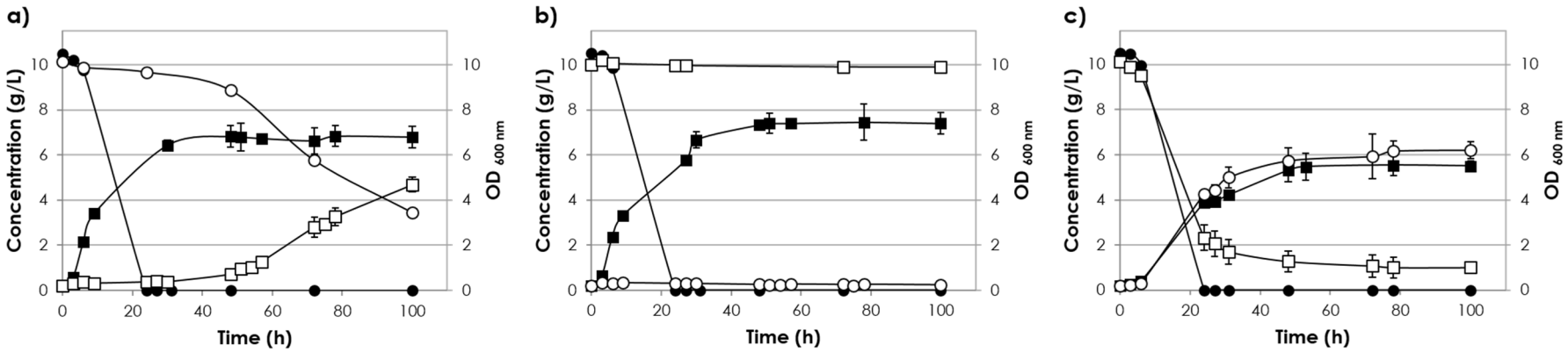

Figure 3.9 - Comparison of growth kinetics of *S. cerevisiae* strains in YNB broth containing glucose or cellobiose as sole carbon source: M2n[pBDK1-*BGL3*]-C1 (a), M2n (b), Y294[*Pccbgl1*] (c). Sugar concentration and optical density measured at 600 nm are represented by ● and ■, respectively. Full and empty symbols indicate growth on glucose and cellobiose, respectively. YNB broth for cultivation of Y294[*Pccbgl1*] was supplemented with amino acids: tryptophan (76 mg/L), hystidine (76 mg/L) and leucine (360 mg/L).

3.6 Enzymatic activity at low pH values

Previous experimental data indicated that recombinant M2n[pBDK1-*BGL3*]-C1 could not completely hydrolyze all cellobiose available in defined medium under aerobic culturing conditions (Figure 3.9a). The same apparently occurred with laboratory strain Y294[*Pccbgl1*] (Figure 3.9c). However, both strains completely consumed the equivalent amount of glucose, yielding to higher optical density.

As discussed in *3.4*, enzymatic activity of secreted ß-glucosidase BGL3 was highest at pH 5.0 and decreased at lower pH values. Since yeast metabolism is known to result in medium acidification (Hahn-Hägerdal et al., 2005), we speculated that decrease in pH during cell growth could negatively affect enzyme performances and be responsible for the incomplete cellobiose utilization.

In order to unravel this hypothesis, the enzymatic activity of BGL3, previously assayed at pH values ranging from 4.0 to 5.5, was quantified at lower pH values (2.5, 3.0, 3.5) at the temperature of 60°C, at which BGL3 activity was known to be highest (Figure 3.7b). The activity of BGL3 from the supernatant of a M2n[pBDK1-*BGL3*]-C1 cell culture grown for 48 h in YPD medium was quantified as described in *2.11*.

BGL3 activity strongly decreased below pH 4.0 resulting in 92% decrement at pH 3.0 when compared to the highest activity, achieved at pH 5.0 (Figure 3.10). At pH 2.5, ß-glucosidase activity was reduced by over 99%.

The experiment was replicated using the supernatant of a cell culture of Y294[*Pccbgl1*], cultured for 48 h in YNB medium supplemented with aminoacids to ensure auxotrophic growth, as described in *2.10*. ß-glucosidase secreted by the laboratory strain exhibited a comparable decrease in enzymatic activity at lower pH (data not shown).

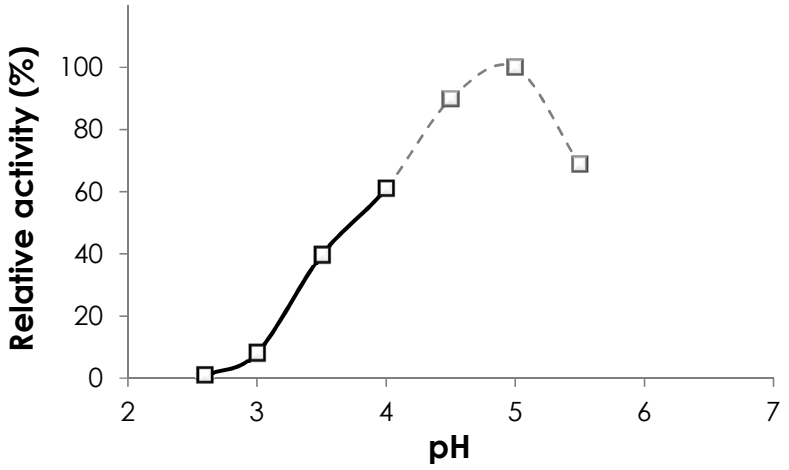

Figure 3.10 - Effect of pH on extracellular ß-glucosidase activity in the supernatant of the recombinant M2n[pBKD1-*BGL3*]-C1 at pH values lower than 4.0 (solid black line). The strain was previously cultured in YPD medium for 48 hours at 30°C. Activity is expressed as a percentage of the highest value (dashed grey line).

These findings clearly indicate that ß-glucosidase BGL3 activity strongly diminishes at low pH. As a result, pH decrease of the medium caused by yeast metabolism is likely to result in the inhibition of secreted ß-glucosidase and in the yeast inability to completely consume the cellobiose available. In the case of Y294[*Pccbg11*], the higher amount of ß-glucosidase produced already in the early growth phases, also indicated by the steady hydrolytic activity on cellobiose (Figure 3.9c), is likely responsible for the broader, yet incomplete, cellobiose consumption.

3.7 Effect of buffered medium on cellobiose consumption

In order to further assess the role of pH on cellobiose consumption, recombinant M2n[pBDK1-*BGL3*]-C1 and the parental M2n were evaluated for the ability to grow aerobically in YNB buffered medium containing 50 mM citrate

buffer pH 5.0 to maintain a stable pH. Either glucose (10.53 g/L) or the equivalent amount of cellobiose (10 g/L) were supplemented as the only carbon source. The growth performances of each strain were compared to those exhibited in unbuffered medium, previously studied. Similarly, growth kinetics of the laboratory strain Y294[*Pccbgl1*], were studied under the same experimental conditions.

M2n[pBDK1-*BGL3*]-C1 and M2n strains exhibited higher growth on glucose in buffered than in unbuffered medium, showing comparable kinetics. The two strains reached a final optical density (OD_{600}) of 12.0 and 11.0 respectively (Figure 3.11a, 3.11b), marking a strong difference over the growth on unbuffered medium (final OD_{600} of 6.8 and 7.4, respectively). In both cases, available glucose was completely depleted. In buffered medium, pH remained almost unaltered (Figure 3.11a, 3.11b). As expected, yeast metabolism resulted in a strong acidification in unbuffered medium, leading to final pH close to 2.3 (Figure 3.11a, 3.11b).

Increased optical density in buffered medium can be explained by considering the diminished necessity to pump protons outside the cell membrane in order to maintain cytoplasmic pH unaltered, which occurs at the expense of ATP molecules (Piper et al., 1998). In fact, a raised need for ATP results in lower resources for biomass synthesis. In addition, acidification of the cytoplasm causes the inhibition of essential metabolic functions, including glycolysis (Bracey et al., 1998; Krebs et al., 1983).

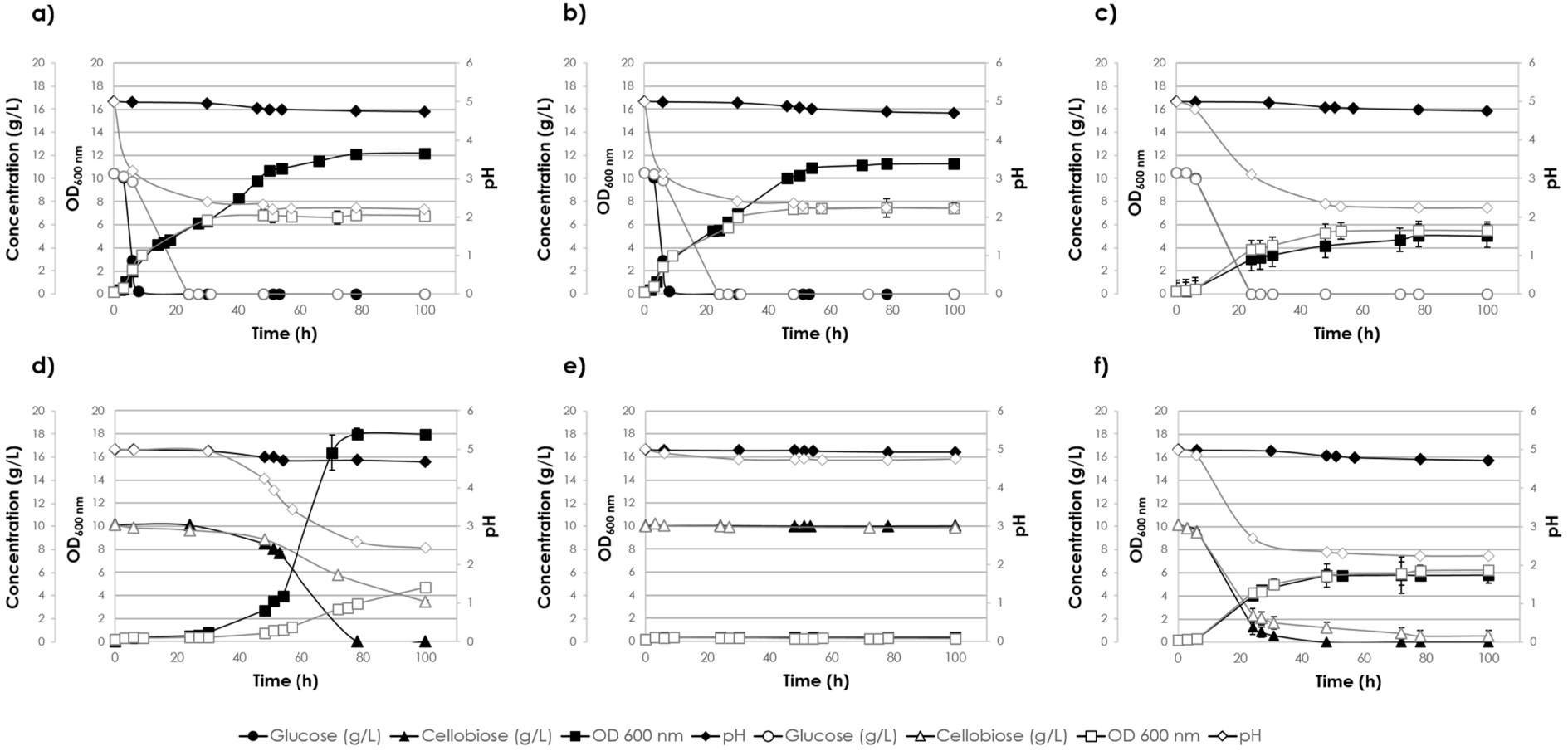

Figure 3.11 – Comparison of growth kinetics of *S. cerevisiae* strains in YNB broth containing glucose (10.53 g/L) or cellobiose (10 g/L) as sole carbon source: M2n[pBDK1-*BGL3*]-C1 (a,d), M2n (b,e), Y294[*Pccbgl1*] (c,f). Glucose and cellobiose concentrations are represented by ● and ▲, respectively. Full and empty symbols indicate buffered and unbuffered medium (citrate buffer 50 mM, pH 5), respectively. YNB broth for cultivation of Y294[*Pccbgl1*] was supplemented with amino acids: tryptophan (76 mg/L), hystidine (76 mg/L) and leucine (360 mg/L).

M2n[pBDK1-*BGL3*]-C1 was able to consume all the cellobiose available in buffered medium. On the contrary, in unbuffered medium, about 3.3 g/L of cellobiose remained still unconsumed (Figure 3.11d). Complete substrate consumption is likely to occur as a result of the buffering activity. As for growth on glucose, pH result almost stable (Figure 3.11d). In these conditions, ß-glucosidase activity is still close to the highest achieved. As a consequence, M2n[pBDK1-*BGL3*]-C1 reached a final OD_{600} of 18.0 on buffered medium, higher than OD_{600} 4.7 obtained without buffer (Figure 3.11d). In buffered medium containing cellobiose (Figure 3.11d), the recombinant strain exhibits over 2-fold higher growth than on glucose (Figure 3.11a). As expected, parental M2n was not capable of using cellobiose as carbon source (Figure 3.11e).

The different growth kinetics shown by M2n[pBDK1-*BGL3*]-C1 in buffered and unbuffered media can be explained as a consequence of the "glucose repression" effect, shown by many *S. cerevisiae* strains. When glucose concentration is higher than a strain-specific threshold, the expression of the structural genes responsible for synthesizing respiratory enzymes is repressed. As a result, most of the pyruvate formed by glycolysis is channeled to ethanol even in aerobic conditions, rather than into the tricarboxylic acid cycle (Barnett and Entian, 2005; Gancedo, 1998). Both occurrence of this phenomenon and the threshold concentration that triggers it are strongly strain dependent. Glucose concentrations as low as 0.15 g/L were shown to cause the glucose repression effect in *S. cerevisiae* strains (Verduyn et al., 1984). Fermentation is a much less efficient mechanism for energy production than respiration and, as a result, less biomass is produced (Gombert et al., 2001; Meijer et al., 1998).

When using glucose as sole carbon source, M2n[pBDK1-*BGL3*]-C1 is likely to convert the sugar into biomass through the least efficient aerobic fermentation, at least in the early growth phases, when glucose concentration is high. Instead, when cellobiose is slowly cleaved into glucose, the sugar may never cross the concentration level that triggers aerobic fermentation. In these conditions, the

sugar is then converted into energy via the more efficient respiration route, thus supporting higher biomass yield.

When cultured in buffered medium containing glucose, the laboratory strain Y294[*Pccbgl1*] reached a slightly lower OD_{600} than the wild type compared strains. The yeast reached final OD_{600} of 5.0 in buffered medium containing glucose and OD_{600} 5.5, on cellobiose (Figure 3.11f), similarly to the growth exhibited in unbuffered medium (Figure 3.11c). In both cases, growth on cellobiose did not result in significant higher final optical density than in glucose, as instead it might be expected, since Y294[*Pccbgl1*] shows glucose repression behavior (Du Preez et al., 2001). A possible explanation may lay in the steady ß-glucosidase expression assured by multicopy episomal plasmids. The enzymatic activity showed by this strain was quantified in 7.20 nkat/mg DCW, after 48 h incubation in YNB medium (Njokweni et al., 2012), amounting to about 4 times higher than M2n[pBDK1-*BGL3*]-C1. Due to the high ß-glucosidase activity shown by this strain, cellobiose may be quickly cleaved into glucose already at the early growth stages. As a result, the glucose concentration could exceed the threshold that favors aerobic fermentation, thus blocking the more efficient aerobic metabolism. HPLC analysis indicated ethanol production consequently to decrease in cellobiose concentration (data not shown) in both buffered and unbuffered medium, further supporting this hypothesis.

3.8 Fermentation performances

The fermentation performances of *S. cerevisiae* M2n[pBDK1-*BGL3*]-C1, M2n and Y294[*Pccbgl1*] were evaluated in small scale fermentations in buffered and unbuffered medium containing glucose and cellobiose as sole carbon sources (formulated as described in *2.10*).

The recombinant M2n[pBDK1-*BGL3*]-C1 exhibited a fermentation pattern comparable to that of the parental strain in unbuffered medium containing glucose (Figure 3.12a, 3.12b). Both yeast consumed all the carbon source

available, yielding 4.3 g/L of ethanol after 6 hours, corresponding to 80% of the theoretical yield. Similarly, final OD_{600} reached about 3.40, corresponding to 1.43 g/L of dry biomass. This finding confirms that yeast transformation and ß-glucosidase secretion did not result in any evident and significant metabolic burden on M2n[pBDK1-*BGL3*]-C1.

M2n[pBDK1-*BGL3*]-C1 could not utilize all cellobiose available in unbuffered medium (Figure 3.12d). About 1 g/L of the 10 g/L available remained unconsumed after 144 h. The strain produced 3.6 g/L of ethanol, corresponding to 67% of the theoretical yield. Recombinant strain produced higher biomass on cellobiose than on glucose. Final OD_{600} was 4.20, corresponding to 1.50 g/L of dry biomass. Parental M2n was not capable of fermenting cellobiose into ethanol (Figure 3.12e).

Laboratory strain Y294[*Pccbgl1*] showed a lower fermentation rate than the other strains in unbuffered medium, thus glucose was completely consumed only over 24 h of fermentation. However, final ethanol yield (4.3 g/L, 80% of the theoretical) was identical to that of both M2n recombinant and parental strains.

Y294[*Pccbgl1*] exhibited similar fermentation performances than M2n[pBDK1-*BGL3*]-C1 when fermenting cellobiose, both in terms of ethanol yield and fermentation rate. The laboratory strain consumed all cellobiose available by 24 h of fermentation, producing 4.2 g/L of ethanol, which corresponds to 78% of the theoretical yield. Thanks to the higher enzymatic activity guaranteed by multicopy episomal plasmids, Y294[*Pccbgl1*] conversion of cellobiose into ethanol was only 2% less efficient than conversion of glucose. In addition, fermentation of cellobiose resulted in slightly higher biomass than produced from glucose. Final OD_{600} was 3.30 on cellobiose, while only 2.90 on glucose.

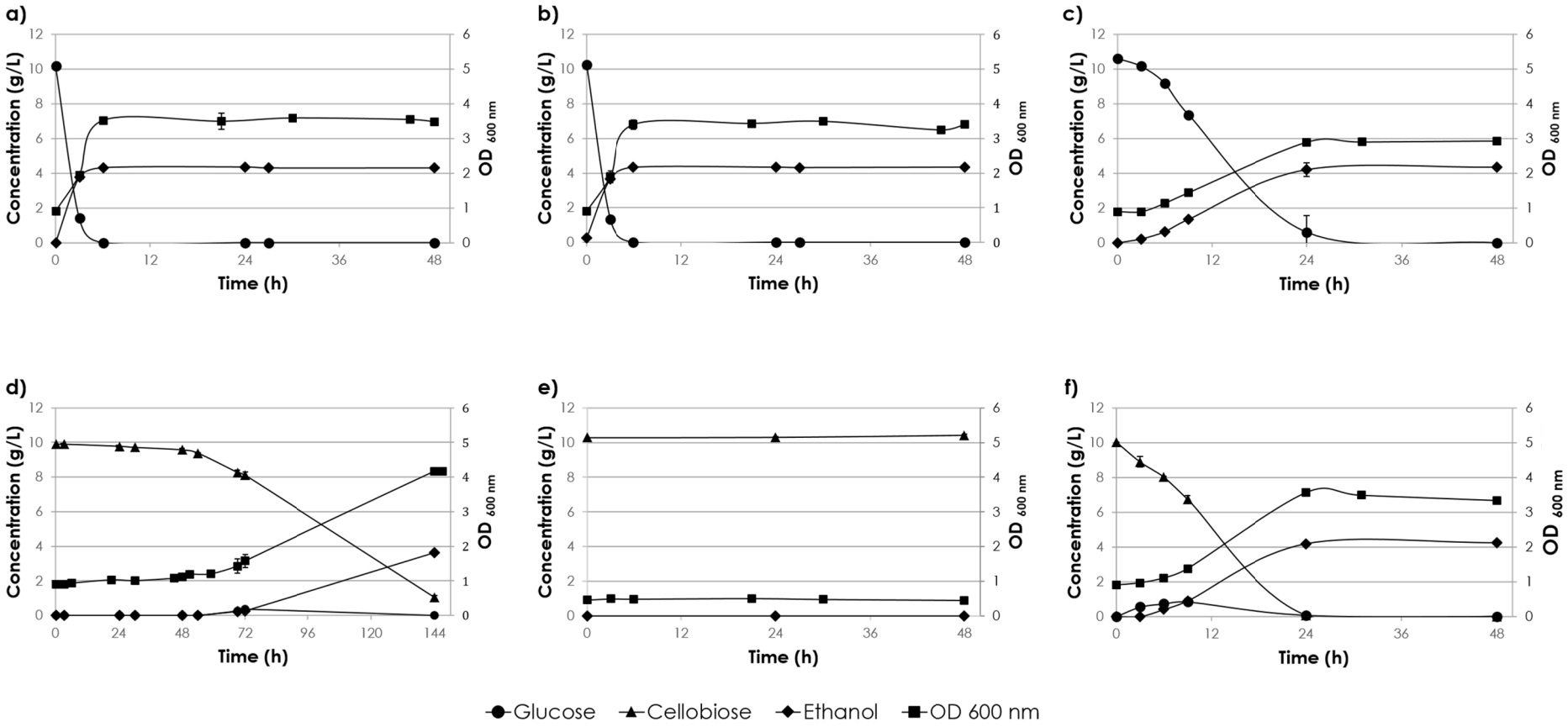

Figure 3.12 – Comparison of fermentation performances of: *S. cerevisiae* strains in YNB broth containing glucose or cellobiose as sole carbon source: M2n[pBDK1-*BGL3*]-C1 (a,d), M2n (b,e), Y294[*Pccbgl1*] (c,f). YNB broth for fermentation by Y294[*Pccbgl1*] was supplemented with amino acids: tryptophan (76 mg/L), hystidine (76 mg/L) and leucine (360 mg/L).

In presence of buffered medium containing glucose, M2n[pBDK1-*BGL3*]-C1 and M2n exhibited no significant difference compared to the fermentation performances shown in unbuffered medium (Figures 3.13a, 3.13b). Glucose anaerobic fermentation did not benefit by the presence of the buffer, in contrast to what observed during aerobic growth. This observation can be explained considering that the concentration of glucose used in aerobic conditions represses aerobic metabolism, while in anaerobic conditions only the fermentation route is available. Instead, presence of buffer allowed complete consumption of cellobiose by M2n[pBDK1-*BGL3*]-C1 (Figure 3.13c). The recombinant strain produced 3.9 g/L of ethanol, corresponding to 73% of the theoretical yield. Cellobiose fermentation in buffered medium resulted in 10% improvement in final ethanol concentration, compared to fermentation in unbuffered medium. As a consequence of complete cellobiose consumption, produced biomass in presence of buffer was higher than in unbuffered medium. Final OD_{600} was 5.20, corresponding to 1.84 g/L of dry biomass. As expected, parental M2n was not capable of fermenting cellobiose into ethanol (Figure 3.13e).

In buffered medium (Figure 3.13c), Y294[*Pccbgl1*] exhibited the same glucose fermentation performances shown in unbuffered medium. When fermenting cellobiose (Figure 3.13f), the laboratory strain showed slightly better biomass production abilities. Final OD_{600} was 3.70, significantly higher than in unbuffered medium (final OD_{600} of 3.30), while ethanol yield remained unaltered.

Preliminary data on glycerol production indicates a glucose concentration of about 10 g/L acts as a stress factor for yeast fermentation, since glycerol is known to be related to redox balancing in stressful metabolic conditions (Scanes et al., 1998). Both in buffered and unbuffered medium supplemented with glucose, all yeast strains studied synthesized about 1 g/L of glycerol (data not shown). Instead, glycerol concentration was lower in presence of cellobiose, ranging from 0.45 g/L for M2n[pBDK1-*BGL3*]-C1 to 0.80 g/L for Y294[*Pccbgl1*] (data not shown).

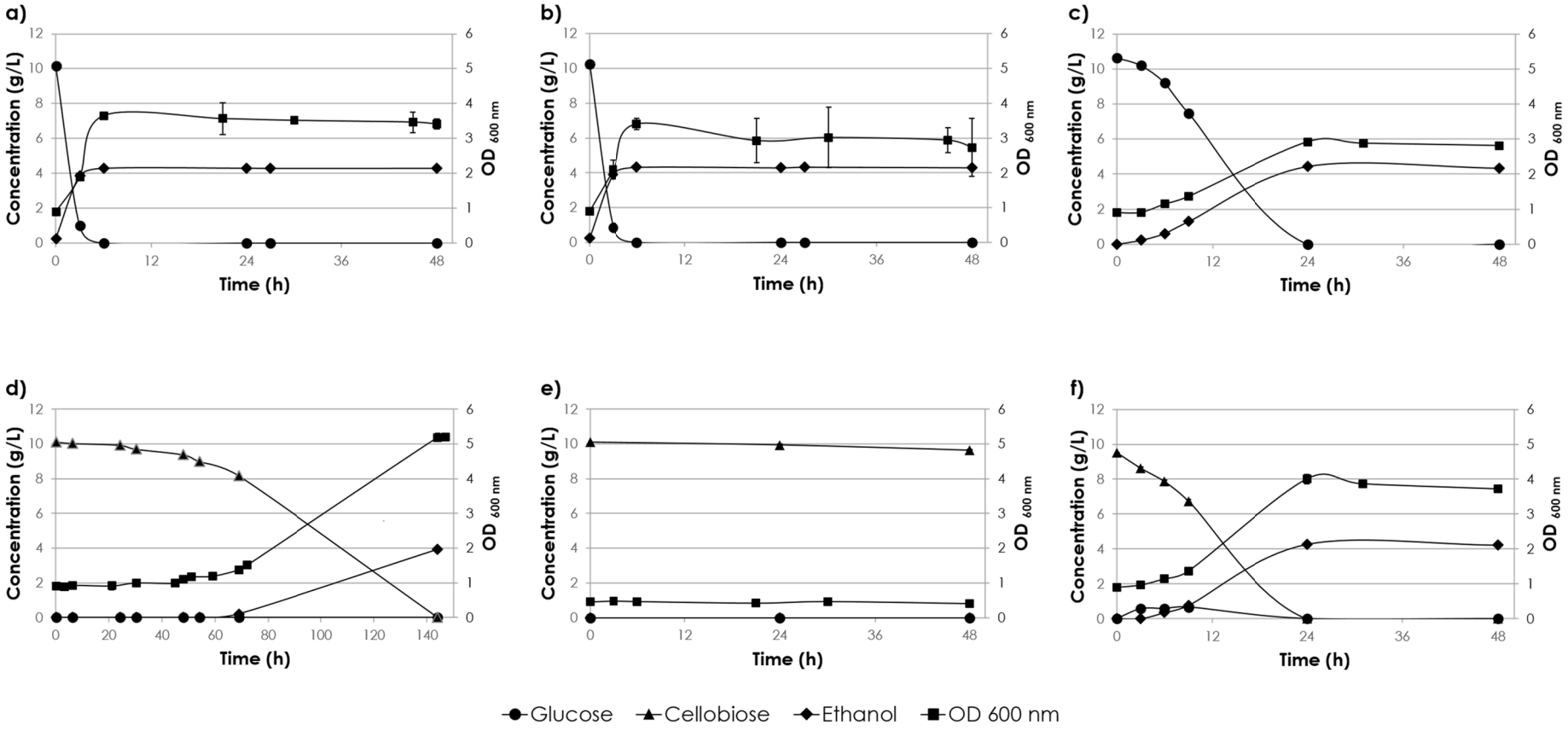

Figure 3.13 – Comparison of fermentation performances of: *S. cerevisiaee* strains in buffered (citrate buffer 50 mM, pH 5) YNB broth containing glucose or cellobiose as sole carbon source: M2n[pBDK1-*BGL3*]-C1 (a,d), M2n (b,e), Y294[*Pccbg11*] (c,f). YNB broth for fermentation by Y294[*Pccbg11*] was supplemented with amino acids: tryptophan (76 mg/L), hystidine (76 mg/L) and leucine (360 mg/L).

4. Conclusions

Consolidated Bioprocessing (CBP) is gaining increasing attention as a promising approach for improving the economic competitiveness of second generation bioethanol. Production costs could be significantly reduced by developing industrial-grade engineered microorganisms able to supply part of the enzymes required for the hydrolysis of the substrate and to tolerate high concentration of the inhibitory compounds present in pre-treated lignocellulose.

In this study, the construction of a robust CBP yeast for lignocellulose conversion into bioethanol was started by integrating fungal ß-glucosidases into the chromosomes of robust wild type yeast. Among the cellulases required for cellulose degradation, ß-glucosidase was chosen as it plays a key role in the process, representing the rate limiting enzyme. In addition, gene expression by chromosomal integration improves the stability of the new phenotypic traits in the recombinant yeast.

In order to identify a suitable yeast for genetic engineering, a collection of wild type strains previously selected for their robustness was screened for their tolerance to high concentration of inhibitors, either formulated as synthetic mixtures or as by-products released in different lignocellulosic pre-hydrolysates. The former provided insights on the overall robustness of each tested yeast. More interestingly, the latter yielded a wide variety of responses, indicating that tolerance to different inhibitory compounds is highly strain-specific.

The yeast strain demonstrating the highest inhibitors tolerance was chosen for the expression of ß-glucosidases, together with a benchmark industrial strain currently used in bioethanol production. Similarly, two wild type yeast, also evaluated in the early phase of the work, were selected for the same purpose, as they previously displayed high fermentative performances on hydrolyzed lignocellulosic material and have been already indicated as suitable platforms for CBP.

A conspicuous number of successfully integrated recombinant clones expressing three ß-glucosidases of fungal origin were obtained from the four wild-type *S. cerevisiae* strains. Among the clones displaying the highest enzymatic activity on esculin plates, one of the engineered strains was able to durably secrete sufficient amount of ß-glucosidase to grow on cellobiose as sole carbon source. The enzymatic activity of the secreted ß-glucosidase was characterized in terms of optimal temperature and pH. In addition, the effect of prolonged exposition at different temperatures on the enzyme stability was also evaluated.

Noteworthy, the engineering event and the constitutive production of recombinant enzyme did not result in any significant metabolic burden for the host, as the engineered yeast retained growth and fermentative performances comparable to the parental strain.

These promising findings indicate that integration of exogenous genes is a suitable approach for developing superior yeast with phenotypes of industrial interest.

In addition, characterization of the produced ß-glucosidase demonstrated that the enzyme can display high activity and steady stability at high temperatures, thus confirming the importance of identifying thermotolerant yeast as platforms for developing highly performant CBP microorganisms.

Small scale fermentation indicated that the recombinant yeast constructed in this work can directly convert cellobiose into ethanol with high fermentative yield. Also, this robust strain showed comparable fermentation performances on cellobiose with a laboratory yeast strain expressing the same ß-glucosidase via multicopy episomal plasmid, despite the remarkable disadvantage caused by the lower number of gene copies integrated into the genome.

The development of a cellobiose-fermenting yeast is of great interest for industrial conversion of lignocellulosic biomass into ethanol. In particular, the results of this research mark one step closer to the realization of engineered yeast suitable for the direct fermentation of pre-treated lignocellulose into bioethanol.

In order to further assess the industrial applicability of this strain, future studies will focus on small scale fermentation of pre-treated lignocellulosic material supplemented with cellobiose, to evaluate the fermentative performances and the inhibitors resistance of the constructed microorganism in inhibitors-rich industrial substrate for second generation bioethanol.

To our knowledge, this work represents the first attempt to produce a CBP microorganism via expression of ß-glucosidases into robust yeast characterized by innate inhibitors tolerance.

5. References

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6. Participation to courses, seminars and congresses; publications; other educational activities.

6.1 Seminars and courses

- Servizio Trasferimento di Tecnologia – Università degli Studi di Padova, *Corso di formazione "Database brevettuale ORBIT"*, March 6, 2014, Padova, Italy.
- Fritz Schweingruber, *Scientific Communication: research analysis and results presentation*, March 11-13, 2014, Legnaro (PD), Italy.
- BioAware, *BioloMICS version 10 course,* October 15, 2015, Perugia, Italy.
- SIMTREA, Società Italiana di Microbiologia Agraria, Alimentare e Ambientale, *"Summer school on computational analysis from genomic diversity to ecosystem structure"*, September 5-9, 2016, Firenze, Italy

6.2 Partecipation to congresses

- Attendance to "*XXI ISAF, International Symposium on Alcohol Fuels*", March 10-15, 2015, Gwangju, Republic of Korea
- Attendance to "*MD2015, 3rd International Conference on Microbial Diversity*", October 13-15, 2015, Perugia, Italy
- Attendance to "*RRB-12, 12th International conference on Renewable Resources and Biorefineries",* May 30-31, June 1, 2016, Ghent, Belgium
- Attendance to "*PGSM, 1st DAFNAE Postgraduate Scientists Meeting",* September 22-23, 2016, Legnaro, Italy

6.3 Papers

- Favaro L, Viktor MJ, Rose SH, Viljoen-Bloom M, van Zyl WH, Basaglia M, **Cagnin L**, Casella S (2015) Consolidated bioprocessing of starchy substrates into ethanol by industrial *Saccharomyces cerevisiae* strains secreting fungal amylases. *Biotechnol Bioeng 112:1751–60*

- Cripwell R, Favaro L, Rose SH, Basaglia M, **Cagnin L**, Casella S, van Zyl W (2015) Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases and amylolytic yeast. *Appl Ener 160:610-7*

- Shah AT, Favaro L, Alibardi L, **Cagnin L**, Sandon A, Cossu R, Casella S, Basaglia M (2016) *Bacillus sp.* strains to produce bio-hydrogen from the organic fraction of municipal solid waste. *Appl Ener 76:116–24*

- Favaro L, Corte L, Roscini L, **Cagnin L**, Tiecco M, Colabella C, Berti A, Basaglia M, Cardinali G, Casella S (2016) A novel FTIR-based approach to evaluate the interactions between lignocellulosic inhibitory compounds and their effect on yeast metabolism. *RSC Adv 6:47981–9*

6.4 Oral communications

- **Cagnin L**, Favaro L, Rose SH, Basaglia M , van Zyl WH, Casella S (2016) Bioethanol production from sugarcane bagasse hydrolysate by inhibitors-tolerant yeast engineered for cellobiose fermentation. *RRB-12, 12th International conference on Renewable Resources and Biorefineries, May 30-31, June 1, 2016, Ghent, Belgium.*

- **Cagnin L**, Favaro L, Rose SH, Basaglia M, van Zyl WH, Casella S (2016) Production of bioethanol from lignocellulosic hydrolisates using inhibitors-tolerant yeast engineered for cellobiose fermentation. *PGSM, 1st DAFNAE Postgraduate Scientists Meeting, September 22-23, 2016, Legnaro, Italy.*

6.5 Abstracts

- Favaro L, **Cagnin L**, Casella S, van Zyl E, Basaglia M (2014) Robust yeast for the production of bioethanol from steam-exploded sugarcane bagasse. *Global Biotechnology congress*, *June 16-19, 2014, Boston, MA, USA.*

- **Cagnin L**, Favaro L, Rose SH, Basaglia M, van Zyl WH, Casella S (2015) Fungal ß-glucosidase expression in industrial *Saccharomyces cerevisiae* strains for the bioethanol production from lignocellulosic biomass. *XXI ISAF, International Symposium on Alcohol Fuels, March 10-15, 2015, Gwangju, Republic of Korea.*

- Dobson R, Favaro L, Rose SH, Basaglia M, **Cagnin L**, Casella S, van Zyl WH (2015) Simultaneous saccharification and fermentation of wheat bran to ethanol using a defined recombinant cellulase cocktail and industrial amylolytic *Saccharomyces cerevisiae* strains. *XXI ISAF, International Symposium on Alcohol Fuels, March 10-15, 2015, Gwangju, Republic of Korea.*

- **Cagnin L**, Favaro L, SH, Basaglia M, van Zyl WH, Casella S (2015) Expression of Phanerochaete chrysosporium ß-glucosidase in industrial *Saccharomyces cerevisiae* yeast for bioethanol production from lignocellulosic biomass. *ISSY32, International Specialized Symposium On Yeasts, September 13-15, 2015, Perugia, Italy*.

- Favaro L, Viktor MJ, Rose SH, Basaglia M, **Cagnin L**, Cripwell R, Viljonen-Bloom M, Casella S, van Zyl WH (2015) Engineering industrial yeast strains for consolidated bioprocessing of starchy substrates and by-products to ethanol. *ISSY32, International Specialized Symposium On Yeasts, September 13-15, 2015, Perugia, Italy*.

- Roscini L, Favaro L, Corte L, **Cagnin L**, Tiecco M, Colabella C, Basaglia M, Cardinali G, Casella S (2015) FTIR stress response assay could lead the development of industrial yeast strains with high tolerance to lignocellulose-to-ethanol inhibitors. *ISSY32, International Specialized Symposium On Yeasts, September 13-15, 2015, Perugia, Italy*.

- **Cagnin L**, Favaro L, SH, Basaglia M, van Zyl WH, Casella S (2015) Development of industrial cellobiose fermenting *Saccharomyces cerevisiae* strains for the bioethanol production from lignocellulosic biomass. *MD2015, 3rd International Conference on Microbial Diversity, October 13-15, 2015, Perugia, Italy*.

- Favaro L, Corte L, Roscini L, Colabella C, Tiecco M, **Cagnin L**, Basaglia M, Casella S, Cardinali G (2015) Exploring and FTIR-ing yeast diversity towards the development of superior strains for lignocellulosic ethanol. *MD2015, 3rd International Conference on Microbial Diversity, October 13-15, 2015, Perugia, Italy*.

- Ali Shah T, Favaro L, Alibardi L, **Cagnin L**, Cossu R, Basaglia M, Casella S (2015) Exploring microbial diversity of a brewery full scale anaerobic digester to look for robust and efficient H2-producing microbes. *MD2015, 3rd International Conference on Microbial Diversity, October 13-15, 2015, Perugia, Italy*.

- **Cagnin L**, Favaro L, Rose SH, Basaglia M, van Zyl WH, Casella S (2016) Fungal ß-glucosidase expression in inhibitor tolerant industrial *Saccharomyces cerevisiae* strains for the bioethanol production from lignocellulosic biomass. *ECO-BIO 2016, Challenges in building a sustainable biobased economy, 6-9 March 2016, Rotterdam, The Netherlands.*

- Favaro L, Basaglia M, **Cagnin L**, Viktor M, Rose SH, Cripwell R, Bloom M, Casella S, van Zyl WH (2016) Consolidated bioprocessing of starchy substrates and residues into bioethanol. *RRB-12, 12th International conference on Renewable Resources and Biorefineries, 30-31 Maggio, 1 June 2016, Ghent, Belgium.*

- Rodriguez Gamero EJ, Favaro L, **Cagnin L**, Povolo S, Romanelli MG, Basaglia M, Casella S (2016) Microbial production of polyhydroxyalkanoates from fatty by-products. *RRB-12, 12th International conference on Renewable Resources and Biorefineries, 30-31 Maggio, 1 June 2016, Ghent, Belgium.*

- **Cagnin L,** Favaro L, Shah AT, Ali S, Sandon A, Cossu R, Casella S, Basaglia M (2016) Selection of *Bacillus* sp. strains for the efficient production of bio-hydrogen from the organic fraction of municipal solid waste. *PGSM, 1st DAFNAE Postgraduate Scientists Meeting, 22-23 September 2016, Legnaro, Italy.*

6.6 Didactic activity

- Co-tutor for the 1st level degree in Biotechnology "Fungal ß-glucosidase cloning into industrial *Saccharomyces cerevisiae* strains for bioethanol production" A.Y. 2013-2014 (Student: Sofia Creuso, Tutor: Prof.ssa Marina Basaglia)

- Co-tutor for the 1st level degree in Biotechnology "Chromosomal integration of BGL3 from *Phanerochaete chrysosporium* in industrial *Saccharomyces cerevisiae* strains" A.Y. 2014-2015 (Student: Irene Bassan, Tutor: Prof. Sergio Casella)

- Teaching assistant for the course "Microbiologia Generale (C.d.L. Triennale in Scienze e Tecnologie Alimentari) A.Y. 2015-2016. Headed by: Prof. Sergio Casella

6.7 Awards

- Awarded a "*Green Chemistry poster prize*". *RRB-12, 12th International conference on Renewable Resources and Biorefineries, 30-31 May, 1 June 2016, Ghent, Belgium.*
- Awarded for "*Best oral presentation". PGSM, 1st DAFNAE Postgraduate Scientists Meeting, 22-23 September 2016, Legnaro, Italy.*
- Awarded a *"Premio soggiorni di ricerca per giovani ricercatori soci SIMTREA non strutturati"* by *Società Italiana di Microbiologia Agraria, Alimentare e Ambientale (SIMTREA), September 2016.*

7. Published papers

ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Consolidated Bioprocessing of Starchy Substrates Into Ethanol by Industrial *Saccharomyces Cerevisiae* Strains Secreting Fungal Amylases

Lorenzo Favaro,[1] Marko J. Viktor,[2] Shaunita H. Rose,[2] Marinda Viljoen-Bloom,[2] Willem H. van Zyl,[2] Marina Basaglia,[1] Lorenzo Cagnin,[1] Sergio Casella[1]

[1]Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), Università di Padova, Agripolis, Viale dell'Università 16 35020, Legnaro (PD), Italy; telephone: +39 049-8272921 (926); fax: +39 049-8272929; e-mail: marina.basaglia@unipd.it

[2]Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

ABSTRACT: The development of a yeast strain that converts raw starch to ethanol in one step (called Consolidated Bioprocessing, CBP) could significantly reduce the commercial costs of starch-based bioethanol. An efficient amylolytic *Saccharomyces cerevisiae* strain suitable for industrial bioethanol production was developed in this study. Codon-optimized variants of the *Thermomyces lanuginosus* glucoamylase (*TLG1*) and *Saccharomycopsis fibuligera* α-amylase (*SFA1*) genes were δ-integrated into two *S. cerevisiae* yeast with promising industrial traits, i.e., strains M2n and MEL2. The recombinant M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] yeast displayed high enzyme activities on soluble and raw starch (up to 8118 and 4461 nkat/g dry cell weight, respectively) and produced about 64 g/L ethanol from 200 g/L raw corn starch in a bioreactor, corresponding to 55% of the theoretical maximum ethanol yield (g of ethanol/g of available glucose equivalent). Their starch-to-ethanol conversion efficiencies were even higher on natural sorghum and triticale substrates (62 and 73% of the theoretical yield, respectively). This is the first report of direct ethanol production from natural starchy substrates (without any pre-treatment or commercial enzyme addition) using industrial yeast strains co-secreting both a glucoamylase and α-amylase.

Biotechnol. Bioeng. 2015;112: 1751–1760.

KEYWORDS: consolidated bioprocessing (CBP); industrial yeast; codon optimization; raw starch; sorghum; triticale

Correspondence to: M. Basaglia

Contract grant sponsor: University of Padova
Contract grant sponsor: Regione Veneto
Grant numbers: PSR 2007-2013; Misura 124; 2307660
Contract grant sponsor: National Research Foundation of South Africa
Grant numbers: 76597; 68955
Received 26 November 2014; Revision received 3 February 2015; Accepted 9 March 2015
Accepted manuscript online 18 March 2015;
Article first published online 14 July 2015 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.25591/abstract).
DOI 10.1002/bit.25591

Introduction

Biofuels, which includes bioethanol, can be obtained from dedicated crops (e.g., sugarcane and corn), by-products of agricultural processing activities (e.g., sugarcane bagasse) or even organic municipal waste. Lignocellulosic biomass is the preferred substrate as it is more abundant and less expensive than sucrose and starch substrates (Demirbas, 2009; Jang et al., 2012). However, the limitations associated with lignocellulosic ethanol production include the slow rate of enzymatic saccharification, high enzyme and pre-treatment cost, and the requirement of inhibitor-tolerant industrial yeast strains (den Haan et al., 2013; Favaro et al., 2013a). Consequently, starch is still the most commonly used feedstock for ethanol production, with a relatively mature technology developed for corn in the USA (Brehmer et al., 2008) that produced about 52.5 billion litres of bioethanol in 2012, an increase from 49.2 billion litres in 2010 (Renewable Fuels Association, Falling walls & rising tides—2012 Ethanol industry outlook, Washington).

Besides wheat and corn grains, starchy by-products such as wasted crop, cereal bran, cassava pulp, and brewery-spent grains, have been proposed as alternative low-cost feedstocks for the production of bioethanol (Apiwatanapiwat et al., 2011; Favaro et al., 2012a,2013b; Kim and Dale 2004). However, current starch-to-ethanol processes require an energy-intensive liquefaction step as well as substantial amounts of exogenous amylases for enzymatic hydrolysis of raw starch; both these significantly impact the economic viability of starch as feedstock (van Zyl et al., 2012).

Starch hydrolysing enzymes are abundant in the animal, microbial and plant kingdoms, but only a selected few are able to hydrolyse raw starch (van Zyl et al., 2012). Efficient raw starch degrading enzymes (RSDE) can significantly reduce the energy requirements and simplify the production of starch-based biofuels (Robertson et al., 2006). However, a limited number of RSDE have been cloned and characterized, e.g., α-amylases from *Lipomyces kononenkoae* (Eksteen et al., 2004; Knox et al., 2004;

Ramachandran et al., 2008), *Streptomyces bovis* (Yamada et al., 2010a), *Cryptococcus* and *Bacillus* (Gupta et al., 2003; Sun et al., 2010), and glucoamylases from *Rhizopus oryzae* (Yamada et al., 2010a), *Corticium rolfsii, Saccharomycopsis fibuligera* (Eksteen et al., 2004; Sun et al., 2010), *Aspergillus awamori* (Favaro et al., 2012b), and *Aspergillus tubingensis* (Viktor et al., 2013).

Cost-effective conversion of raw starch to biofuels requires the production of starch-hydrolysing enzymes by a fermenting yeast to achieve liquefaction, hydrolysis, and fermentation (Consolidated Bioprocessing, CBP) in a single organism. The yeast *Saccharomyces cerevisiae* remains the preferred host for ethanol production due to its high ethanol, osmo- and inhibitor tolerance in industrial processes, but it lacks the enzymes for the hydrolysis of starch (Favaro et al., 2013c; van Zyl et al., 2012). This could be overcome by engineering *S. cerevisiae* strains for heterologous production of the enzymes required for starch utilization. Co-expression of α-amylases and glucoamylases through extracellular secretion or tethering of enzymes on the cell surface of mainly *S. cerevisiae* laboratory strains has previously been reported (reviewed in van Zyl et al., 2012) while only few industrial raw starch CBP yeast have been developed. A polyploid *S. cerevisiae* strain, secreting both the *Aspergillus awamori* GA1 and *Debaryomyces occidentalis* AMY, converted 80% of 200 g/L raw starch with 80 g/L ethanol produced after 6 days, equating to 0.56 g/L/h (Kim et al., 2011). Similarly, Viktor et al. (2013) reported that the semi-industrial *S. cerevisiae* Mnuα1 strain, expressing the *A. tubingensis* α-amylase and glucoamylase genes, completely hydrolysed 200 g/L raw corn starch within 5 days, producing 70 g/L ethanol (0.58 g/L/h). Both recombinant strains were only evaluated on small-scale, whereas bioreactor experiments are essential to proof the concept of raw starch CBP.

The challenge remains to engineer a robust yeast that can liquefy and saccharify high concentrations of raw starch, while simultaneously fermenting the sugars to ethanol (van Zyl et al., 2012). Industrial yeast are more robust than laboratory strains and display more valuable traits, including higher ethanol productivity and yield, thermostability and higher tolerance to acids, ethanol and sugar (Favaro et al., 2014). Their genetic engineering, however, is challenging and the use of episomal plasmids is undesirable as their maintenance depends on selectable markers (Romanos et al., 1992). Reiterated DNA sequences such as δ-sequences of the Ty retrotransposon and ribosomal DNA have been efficiently used as target sites to ensure the integration of multiple gene copies and therefore high expression levels (Favaro et al., 2010; Yamada et al., 2010b).

In this study, two novel robust *S. cerevisiae* strains were engineered to simultaneously produce and secrete the *Thermomyces lanuginosus* glucoamylase, TLG1, and the *S. fibuligera* α-amylase, SFA1, for raw starch hydrolysis and fermentation. The sequences, selected among a number of screened amylases for their high potential in terms of starch hydrolysis, were codon-optimized and the recombinant enzymes partially characterized by extracellular amylolytic activity and SDS-PAGE. The hydrolysis and fermentation of raw corn starch were evaluated in a bioreactor configuration at high substrate loading (200 g/L) and compared to the natural starchy substrates, sorghum, and triticale.

Materials and Methods

Media and Growth Conditions

Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany). Recombinant plasmids were constructed and amplified in *E. coli* DH5α. The bacterial strains were cultured at 37°C on a rotating wheel in Terrific Broth or on LB agar (Sambrook and Russel, 2001). Ampicillin was added to a final concentration of 100 μg/mL for the selection of plasmid-bearing bacteria. The *S. cerevisiae* strains were cultivated in YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). Recombinants were selected on YPD agar plates containing 200–300 μg/mL geneticin (G418, Sigma-Aldrich, UK), and screened for starch hydrolysis on synthetic complete (SC) starch plates containing 6.7 g/L yeast nitrogen base (Sigma-Aldrich, UK), 20 g/L corn starch (Sigma-Aldrich, UK) and 20 g/L agar.

For bioreactor studies, a modified YPD containing 5 g/L glucose, 100 mg/L ampicillin, and 15 mg/L streptomycin (to inhibit bacterial contamination), 3 mL/L ethanol, 3 mL/L Tween 20 and 18 mg/L ergosterol was used. Raw corn starch (Sigma-Aldrich, UK), triticale (*X Triticosecale* Wittmack, cultivar US2007) or sweet sorghum (*Sorghum bicolor* L., cultivar PAN8816) was added at a concentration of 200 g/L. The triticale and sorghum seeds (provided by Dr. Willem Botes, Department of Genetics, Stellenbosch University) were milled and sieved, with the fractions smaller than 500 μm pooled and used as substrate.

Strains and Plasmids

The genotype and origin of plasmids, yeast and bacterial strains used in this work are summarized in Table I.

DNA Manipulations and Plasmid Construction

Restriction enzyme digestion, electrophoresis, DNA ligation, transformation, and DNA preparation from E. coli were performed using standard methods (Sambrook and Russel, 2001). Enzymes for restriction digests and ligations were sourced from Roche Applied Science (Germany) and used as recommended by the supplier. DNA fragments were purified from agarose gels using the Gene Clean kit (Qbiogene Inc., Montreal, Canada).

The synthetically designed *T. lanuginosus TLG1* and *S. fibuligera SFA1* genes (GenBank accession number EF545003.1 and E03536.1, respectively) were codon-optimized (GenArt Corporation, USA) for expression in *S. cerevisiae* (Sharp and Cowe, 1991) with the native secretion signals intact. The *Pac*I and *Asc*I restriction sites were added to the 5' and 3'-ends of the sequences, respectively.

The synthetic *SFA1* gene was subcloned into the *Pac*I and *Asc*I sites of pBKD1 to create plasmid pSFA1, whereas the synthetic *TLG1* gene was subcloned in the same restriction sites on pBKD2 to obtain plasmid pTLG1 (Fig. 1). The $ENO1_P$-*TLG1*-$ENO1_T$ cassette was excized from pTLG1 with *Spe*I and *Not*I digestion and subcloned into the corresponding sites of pSFA1 to generate pSFA1-TLG1 (Fig. 1).

Table I. Summary of plasmids and strains constructed in this study.

Plasmid/Strains	Relevant genotype or phenotype	Source
pDRIVE	*bla*	Qiagen (USA)
pBKD1	*bla* δ-sites-$PGK1_P$-$PGK1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	McBride et al. (2008)[b]
pBKD2	*bla* δ-sites-$ENO1_P$-$ENO1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	McBride et al. (2008)[b]
pSFA1	*bla* δ-sites-$PGK1_P$-*SFA1*-$PGK1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	This work
pTLG1	*bla* δ-sites-$ENO1_P$-*TLG1*-$ENO1_T$ TEF_P-*KanMX*-TEF_T[a]-δ-sites	This work
pTLG1-SFA1	*bla* δ-sites-$PGK1_P$-*SFA1*-$PGK1_T$ TEF_P-*KanMX*-TEF_T[a] $ENO1_P$-*TLG1*-$ENO1_T$ -δ-sites	This work
E. coli XL1-Blue	*MRF' endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [*F'proAB lacq ZΔM15 Tn10(tet)*]	Stratagene (USA)
S. cerevisiae M2n	Semi-industrial strain	Viktor et al., 2013
S. cerevisiae MEL2	Industrial strain with high fermentative vigour	Favaro et al., 2013b
S. cerevisiae M2n[TLG1]	*TLG1 multiple copy integration*	This study
S. cerevisiae M2n[SFA1]	*SFA1 multiple copy integration*	This study
S. cerevisiae M2n[TLG1-SFA1]	*TLG1 and SFA1 multiple copy integration*	This study
S. cerevisiae MEL2[TLG1-SFA1]	*TLG1 and SFA1 multiple copy integration*	This study

[a]*TEF1* promoter and terminator from *Ashbya gossypii*.
[b]McBride JEE, Deleault KM, Lynd LR, Pronk JT. 2008. Recombinant yeast strains expressing tethered cellulase enzymes. Patent PCT/US2007/085390.

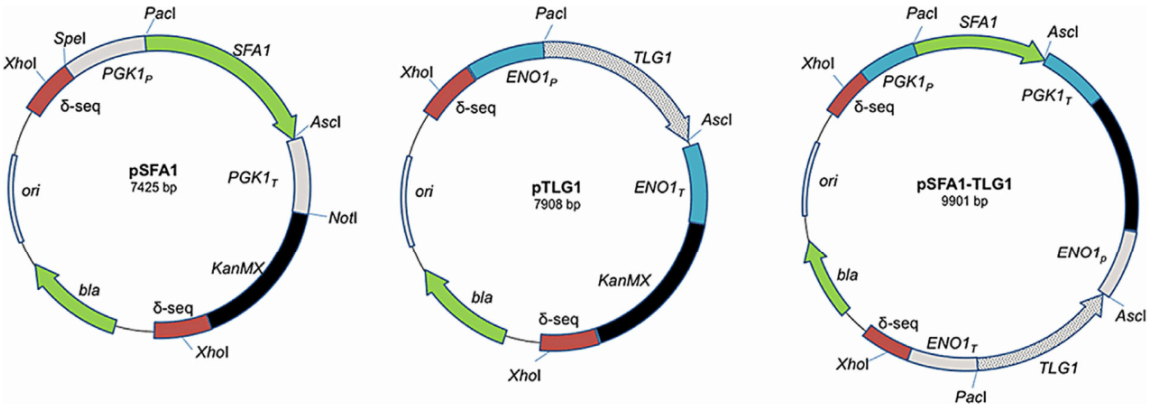

Figure 1. A schematic representation of the final vector constructs used in this study for codon-optimized amylase expression. The *S. fibuligera SFA1* was cloned under the regulation of the *PGK1* promoter and terminator sequences, whereas the *T. lanuginosus TLG1* was cloned between the *ENO1* promoter and terminator sequences. The $ENO1_P$-*TLG1*-$ENO1_T$ cassette was obtained from pTLG1 and subcloned onto pSFA1 to generate plasmid pSFA1-TLG1.

Bacterial and Fungal Transformations

Recombinant plasmids were transformed into chemically competent *E. coli* cells, followed by selection on LB-ampicillin agar plates. The industrial *S. cerevisiae* strains were engineered by means of electroporation (Favaro et al., 2012b). The plasmids were digested with *Xho*I prior to transformation and recombinant yeast cells were selected on YPD-geneticin agar plates supplemented with 1 M sorbitol.

The *S. cerevisiae* strains were transferred onto SC-starch plates and cultured for 4 days at 30°C. Plates were transferred to 4°C to allow precipitation of the residual starch, with a clear zone around the colony indicative of starch hydrolysis.

For quantitative assays, yeast recombinants were aerobically cultivated in 50 mL YPD medium at 30°C with agitation at 200 rpm with sampling at 24 h intervals. The supernatant was obtained by centrifugation (5 min, 2,235*g*) and extracellular enzymatic activities were determined.

The total amylase activity of strains expressing both α-amylase and glucoamylase was determined in liquid assays using the reducing sugar assay with glucose as standard (Miller, 1959). The optimal enzyme pH was assessed at 50°C with 50 μL of the supernatant and 450 μL of the substrate (0.1% soluble potato starch or 2% raw corn starch) suspended in 0.05 M citrate-phosphate buffer at pH values from 3.5 to 6.5.

The concentration of raw starch was specifically 20-fold higher than that of soluble starch in order to improve the accessibility of the substrate for the recombinant enzymes (Favaro et al., 2012b).

The optimal assay temperature was determined at pH 4.5 using temperatures ranging from 30 to 70°C. The enzymatic

reactions were conducted for 10 min and terminated by boiling in a waterbath for 15 min. The colorimetric changes were measured spectrophotometrically at 540 nm with a microtitre plate reader (Tecan Spectrafluor, Milan, Italy). Similar procedures were used to quantify the glucose released from soluble and raw corn starch, with the peroxidase-glucose oxidase method using the D-Glucose assay kit (Boehringer Mannheim-R-Biopharm, Germany).

Enzymatic activities were expressed as nanokatals per gram dry cell weight (nkat/g DCW), which is defined as the enzyme activity required to produce 1 nmol of glucose per second per gram dry cell weight. All experiments were carried out in triplicate.

Electrophoresis and Zymogram Analysis

Recombinant *S. cerevisiae* strains were cultivated in 20 mL SC medium and the supernatant was harvested after 3 days. Two micrograms of lyophilized supernatant were separated by SDS-PAGE using two duplicate 8% separation gels (Laemmli, 1970). Electrophoresis was carried out at 100 V for 90 min at room temperature and protein species on the one gel was visualized with the silver staining method (O'Connell and Stults, 1997). The unstained gel was washed with citrate-phosphate buffer (pH 4.5) for 30 min at room temperature with gentle agitation to remove the SDS before transfer onto a plate containing 2% soluble starch (pH 6). The gel was removed after 24 h at 30°C and the starch plate stained with a 10% iodine solution.

Small-Scale and Bioreactor Fermentation Studies With High Substrate Loading

Small-scale fermentations were conducted in 120 mL serum bottles containing 100 mL YPD 200 g/L glucose inoculated with 50 g/L wet cell weight (corresponding to nearly 6×10^8 CFU/mL) of yeast cultures, grown for 72 h at 30°C. The fermentations were carried out under oxygen-limited conditions and the bottles, equipped with a bubbling CO_2 outlet, were incubated at 30°C on a magnetic stirrer. Samples were taken through a capped syringe needle pierced through the bottle stopper.

For bioreactor experiments, pre-cultures were cultivated in 200 mL YPD medium (in 2 L Erlenmeyer flasks) for 48 h at 30°C on a shaker platform (100 rpm). Bioreactor fermentations were performed in a 2 L MultiGen Bioreactor (New Brunswick Scientific Corporation, Edison, New Jersey) with a wet cell loading of 50 g/L in 1 L modified YPD supplemented with 200 g/L raw corn starch, triticale or sweet sorghum as carbon source. Glucose (5 g/L) was also added to provide an initial carbon source to the cells, thus reducing the lag phase for ethanol production.. The wet cell weight was determined by weighing a cell pellet obtained from centrifugation of the pre-culture at 3000*g* for 5 min. The triticale and sorghum substrates contained 63% and 73.5% starch per dry weight (DW), respectively. Fermentations were carried out at 30°C with stirring at 100 rpm and regular sampling of fermentation broth through a designated sampling port.

Analytical Methods and Calculations

Ethanol, glycerol, maltose, and glucose concentrations were quantified with HPLC (Shimadzu, Japan) equipped with a refractive index detector. A cation-H refill cartridge (Bio-Rad, Hercules, USA) preceding the Aminex HPX-87H column (Bio-Rad, Hercules), which was run at 65°C with 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.5 mL/min.

The ethanol yield (g of ethanol/g of available sugar) was calculated considering the amount of glucose equivalent available at the beginning of the fermentation. The theoretical CO_2 yields were calculated based on the ethanol concentrations, assuming that equimolar ethanol and CO_2 are produced. The percentage starch converted to glucose, maltose, glycerol, ethanol, and CO_2 was calculated on a mole carbon basis. The volumetric productivity (Q) was based on grams of ethanol produced per litre of culture medium per hour (g/L/h) and the maximum volumetric productivity (Q_{max}) was defined as the highest volumetric productivity displayed.

Results

Cloning and Genomic Integration of Amylase Genes Into Industrial Strains

The *T. lanuginosus TLG1* and *S. fibuligera SFA1* genes were codon-optimized for expression in *S. cerevisiae* and cloned individually or combined in pBKD1 and pBKD2-derived plasmids (Fig. 1, Table I). The genes were first integrated individually into the genome of the semi-industrial *S. cerevisiae* M2n strain to evaluate their respective starch hydrolysing activities. Co-expression of *TLG1* and *SFA1* was subsequently evaluated in S. cerevisiae M2n and in the industrial S. cerevisiae MEL2 strain, previously described for its promising industrial fitness (Favaro et al., 2013b). Southern blot analysis of the engineered strains confirmed the chromosomal integrations of heterologous gene(s) (data not shown) and all the recombinant SFA1-strains produced hydrolysis zones (Fig. 2a); zones were neither expected nor observed for M2n[TLG1] expressing the exo-type glucoamylase *TLG1* (Fig. 2a).

Characterization of Recombinant Amylases

Characterization of protein species by SDS-PAGE indicated that the TLG1 protein (predicted molecular size of 67 kDa) was glycosylated to yield a product of 90 kDa, whereas the recombinant SFA1 size was similar to the expected 56 kDa (Fig. 2b). Zymogram analysis confirmed that the recombinant SFA1 was active (clear hydrolysis zones appeared after iodine staining of the starch plate). The TLG1 protein did not produce starch hydrolysis zones, in line with the absence of hydrolysis halos on the soluble starch plate (Fig. 2a).

Both the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains displayed maximum total soluble starch hydrolysis at pH 4.5 (Fig. 2c), with a continuous decrease in activity as the pH values increased above 5.5. At the optimal pH of 4.5, the enzymatic activity peaked at 60°C, with lower temperatures resulted in reduced activities (Fig. 2d). Raw and soluble starch hydrolysis by the recombinant strains was therefore evaluated at pH 4.5 and either

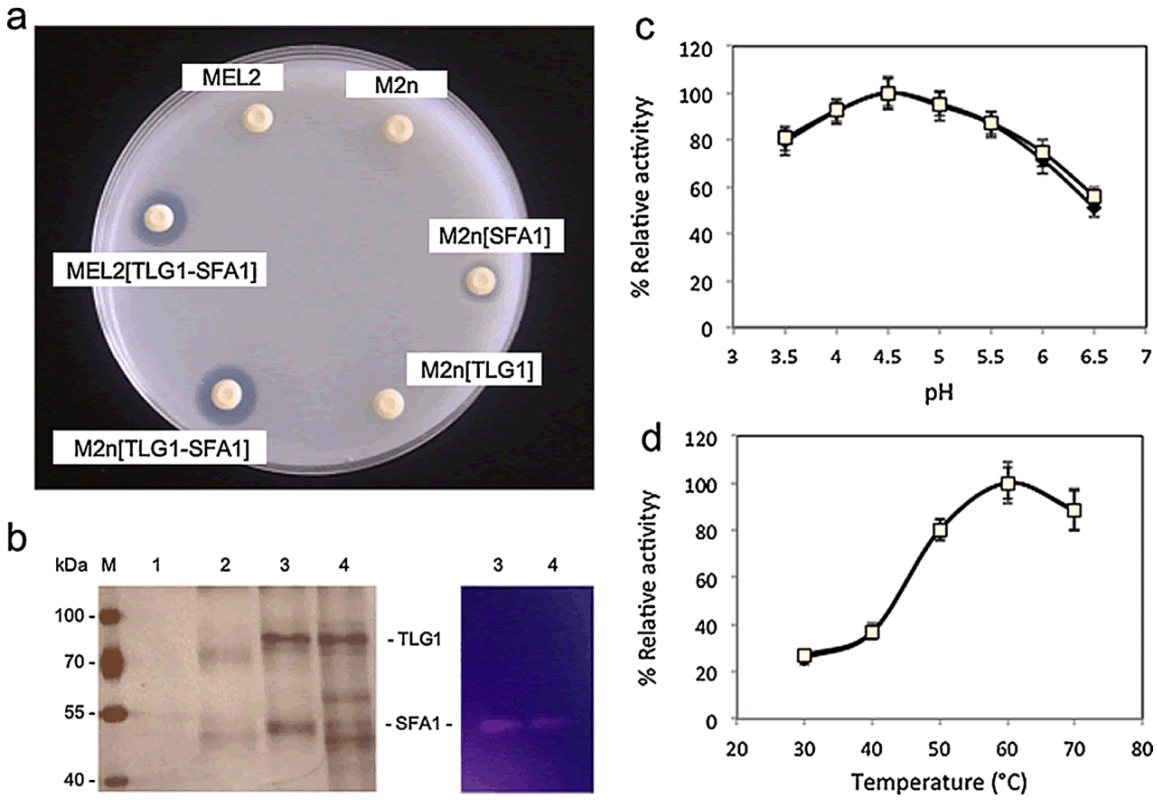

Figure 2. (a) Soluble starch plate assay indicates hydrolysis zones surrounding the *S. cerevisiae* M2n[SFA1], M2n[SFA1-TLG1] and MEL2[SFA1-TLG1] strains, whereas the reference strains (*S. cerevisiae* M2n and MEL2) and *S. cerevisiae* M2n[TLG1] indicated no α-amylase activity. (b) SDS-PAGE of the supernatant of *S. cerevisiae* M2n (lane 1), *S. cerevisiae* MEL2 (lane 2), *S. cerevisiae* M2n[SFA1-TLG1] (lane 3), *S. cerevisiae* MEL2[SFA1-TLG1] (lane 4) after silver staining. On the right the iodine stained starch plate indicating hydrolysis after exposure to the proteins in the SDS-PAGE gel. The protein size marker is depicted on the left hand side. The effect of (c) pH and (d) incubation temperature on the relative amylase activity of (□) *S. cerevisiae* M2n[SFA1-TLG1] and (◆) *S. cerevisiae* MEL2[SFA1-TLG1] grown in YPD medium containing 20 g/L glucose.

Table II. Soluble and raw starch hydrolysing activities (nkat/DCW) of the engineered *S. cerevisiae* strains when grown in YPD broth for 72 h. The assays were performed at 30 and 60°C in citrate-phosphate buffer at pH 4.5 with either 0.1% soluble starch or 2% raw starch. The values are the means of the results obtained from two experiments conducted in triplicate (±SD). Parental strains did not give any starch-degrading activities.

	Soluble starch		Raw starch	
	60°C	30°C	60°C	30°C
Total Amylase activity (Reducing sugar assay[a])				
S. cerevisiae M2n[TLG1-SFA1]	8110 ± 474	2076 ± 168	4461 ± 381	1124 ± 97
S. cerevisiae MEL2[TLG1-SFA1]	7125 ± 335	1817 ± 127	3883 ± 338	971 ± 90
Released Glucose (Glucose kit assay[b])				
S. cerevisiae M2n[TLG1-SFA1]	5061 ± 385	1284 ± 98	2634 ± 239	674 ± 62
S. cerevisiae MEL2[TLG1-SFA1]	4165 ± 300	1037 ± 68	2161 ± 214	541 ± 55

[a]Reducing sugar assay detects all reducing sugars (monosaccharides and oligosaccharides).
[b]Glucose kit assay only detects glucose.

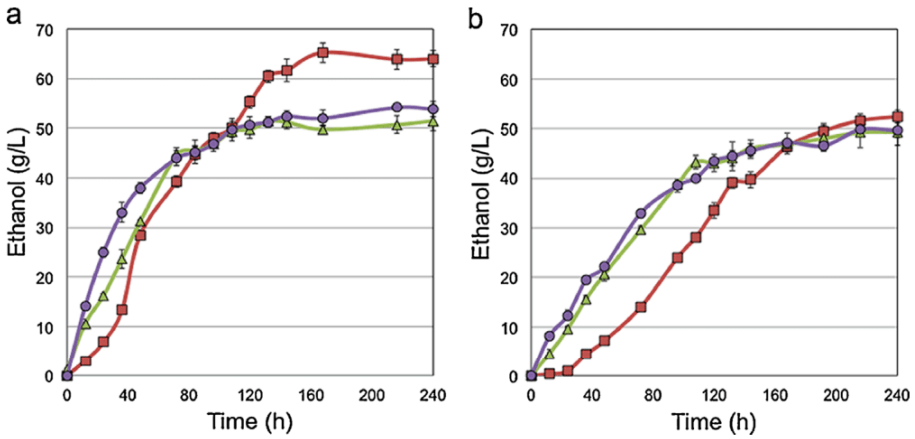

Figure 4. Ethanol production in 2 L bioreactor from YPD broth supplemented with 5 g/L glucose and 200 g/L raw corn starch (■), sorghum (●) or triticale (▲) by *S. cerevisiae* M2n[SFA1-TLG1] (a) and *S. cerevisiae* MEL2[SFA1-TLG1] (b). Values represent the mean of three repeats and error bars represent the standard deviation.

Table III. Conversion of starch to ethanol and by-products by recombinant *S. cerevisiae* strains.

Component	*S. cerevisiae* M2n[TLG1-SFA1]		*S. cerevisiae* MEL2[TLG1-SFA1]	
Substrate: 200 g/L raw starch + 5 g/L glucose = a glucose equivalent of 227 g/L				
Product (g/L)	120 h	240 h	120 h	240 h
Glucose	–	–	–	–
Maltose	–	0.69 ± 0.02	1.40 ± 0.04	–
Glycerol	2.50 ± 0.20	2.90 ± 0.60	2.47 ± 0.17	3.29 ± 0.03
Ethanol	55.81 ± 0.10	64.00 ± 0.10	33.46 ± 1.52	52.43 ± 1.03
CO_2	52.97	61.30	32.05	50.22
Total carbon	111.28	128.89	69.38	105.95
Carbon conversion (mol C)	65%	75%	40%	62%
Ethanol (% theoretical)	48%	55%	29%	45%
Q (g/L/h)	0.47	0.27	0.28	0.22
Q_{max} (g/L/h)	0.59 after 48 h		0.30 after 132 h	
Substrate: 147.5 g/L sorghum starch + 5 g/L glucose = a glucose equivalent of 169.0 g/L				
Product (g/L)	120 h	240 h	120 h	240 h
Glucose	–	–	–	–
Maltose	–	–	0.45 ± 0.09	–
Glycerol	2.84 ± 0.25	3.07 ± 0.05	3.42 ± 0.12	4.30 ± 0.03
Ethanol	50.67 ± 1.75	53.87 ± 1.55	43.46 ± 0.80	49.58 ± 1.42
CO_2	48.54	51.60	41.63	47.49
Total carbon	102.05	108.54	88.97	101.37
Carbon conversion (mol C)	80%	85%	69%	79%
Ethanol (% theoretical)	59%	62%	50%	57%
Q (g/L/h)	0.42	0.22	0.36	0.21
Q_{max} (g/L/h)	0.78 after 24 h		0.46 after 36 h	
Substrate: 126.0 g/L triticale starch + 5 g/L glucose = a glucose equivalent of 145.0 g/L				
Product (g/L)	120 h	240 h	120 h	240 h
Glucose	–	1.32 ± 0.09	–	–
Maltose	0.81 ± 0.45	1.93 ± 0.05	1.31 ± 0.14	0.27 ± 0.03
Glycerol	2.76 ± 0.04	2.86 ± 0.07	4.07 ± 0.08	4.17 ± 0.18
Ethanol	49.73 ± 1.75	51.48 ± 1.99	43.02 ± 1.78	49.24 ± 2.62
CO_2	47.64	49.31	41.21	47.17
Total carbon	100.94	106.91	89.62	100.85
Carbon conversion (mol C)	92%	99%	81%	91%
Ethanol (% theoretical)	67%	73%	59%	67%
Q (g/L/h)	0.41	0.21	0.36	0.21
Q_{max} (g/L/h)	1.04 after 24 h		0.58 after 36 h	

Q: Ethanol productivity; Q_{max}: Maximum ethanol productivity

60°C (optimal temperature for enzyme activity) or 30°C (yeast cultivation temperature). Both total amylase and glucoamylolytic assays indicated that starch hydrolysis at 30°C corresponded to 26% of the activity at 60°C (Table II). Furthermore, the activity on raw corn starch was approximately 53% of that obtained on soluble starch. The *S. cerevisiae* M2n[TLG1-SFA1] strain displayed higher enzymatic values than the MEL2[TLG1-SFA1] strain under all the assay conditions (Table II).

Fermentation Studies

The parental and recombinant yeast strains were first evaluated for their ability to ferment glucose at a high substrate loading under oxygen-limited conditions in 120 mL fermentation bottles (Fig. 3). Parental strains performed slightly better than the recombinant yeasts, with a noticeable difference for the MEL2. After 96 h, the wild types MEL2 and M2n strains produced 96.45 and 94.60 g/L ethanol, respectively, while the recombinant counterparts yielded 91.00 and 92.31 g/L (Fig. 3).

The *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains were subsequently evaluated for the direct conversion of raw corn starch to ethanol in 2 L bioreactor batch fermentations through a simulated CBP of 200 g/L raw starch and 5 g/L glucose (Fig. 4). The *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] yeast produced 64.00 and 52.43 g/L of ethanol, respectively (corresponding to 55 and 45% of the theoretical yield) after 240 h of fermentation (Fig. 4, Table III). As expected, the parental yeast strains did not utilise the raw starch for ethanol production (data not shown). Raw starch conversion by *S. cerevisiae* MEL2[TLG1-SFA1] strain was slower than *S. cerevisiae* M2n[TLG1-SFA1], probably due to the 36 h lag phase observed for the former (Fig. 4). The low residual levels of glucose and maltose in the fermentation broth indicate a rapid sugar uptake by the engineered strains (Table III).

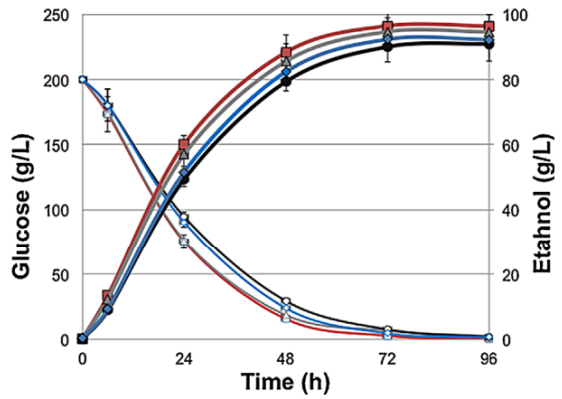

Figure 3. Ethanol production (closed symbols) and glucose consumption (open symbols) by (▲) *S. cerevisiae* M2n, (■) *S. cerevisiae* MEL2, (◆) *S. cerevisiae* M2n [SFA1-TLG1] and (●) *S. cerevisiae* MEL2[SFA1-TLG1] were monitored over time under oxygen-limited conditions.

As reported in Table III, although the final volumetric productivity (Q) was comparable between the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains (0.27 and 0.22 g/L/h, respectively), the Q_{max} of M2n[TLG1-SFA1] (0.59 g/L/h after 48 h) was approximately 1.8-fold higher than that of MEL2[TLG1-SFA1] (0.30 g/L/h after 132 h). Starch conversion by *S. cerevisiae* M2n[TLG1-SFA1] was also superior, with almost 75% of the polysaccharide converted compared to 62% by MEL2[TLG1-SFA1] (Table III).

Sorghum and triticale were subsequently evaluated as potential CBP substrates for the recombinant yeast (Fig. 4, Table III). The *S. cerevisiae* M2n[TLG1-SFA1] strain converted 80% of the raw starch (147.5 g/L) present in 200 g/L sorghum within 5 days (Fig. 4a, Table III) with the production of 50.67 g/L ethanol, whereas *S. cerevisiae* MEL2[TLG1-SFA1] only reached similar ethanol levels after 10 days (Fig. 4b, Table III). The volumetric productivity of *S. cerevisiae* M2n[TLG1-SFA1] was therefore higher, peaking at 0.78 g/L/h after 24 h, compared to *S. cerevisiae* MEL2[TLG1-SFA1] that only achieved 0.46 g/L/h after 36 h (Table III). At the end of the fermentation, starch conversion by *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] was 85 and 79%, respectively, with ethanol yields of 62 and 57% of the theoretical, respectively (Table III).

Triticale was effectively converted into ethanol with both the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains producing similar levels of ethanol, i.e., 51.48 and 49.24 g/L, respectively from 200 g/L triticale (126.0 g/L raw starch) after 10 days (Fig. 4). However, the volumetric productivity for M2n[TLG1-SFA1] was higher after 5 days (Table III), with a maximum of 1.04 g/L/h observed after 24 h, about 1.8-fold greater than the highest volumetric productivity (0.58 g/L/h) for MEL2 [TLG1-SFA1] (Table III). It was therefore clear that the *S. cerevisiae* M2n strain was superior in terms of starch utilization and ethanol yields, being able to convert 99% of the available starch and produce 73% of the theoretical ethanol yield. The higher conversion of triticale starch relative to sorghum and corn starch can partly be ascribed to high levels of native plant amylolytic enzymes present in triticale (Pejin et al., 2009).

Discussion

Sorghum and triticale are important cereal grains due to their drought resistance and the relatively low input costs required for cultivation thereof. However, both cereals have a relatively low cash value if sold directly as feed grain (Hoseney et al., 1981; Rooney and Awika 2005) and new industrial applications should be developed to improve their market significance. Given the relatively high starch content of the two grains, they can be considered as a potential feedstock for bioethanol production (Wang et al., 1997; Rooney et al., 2007). This would, however, require CBP to produce ethanol.

The development of a CBP yeast towards the starch-to-ethanol route requires robust strains to be engineered for the production of raw starch hydrolysing enzymes in adequate quantities. The *S. cerevisiae* MEL2 and M2n strains that displayed promising industrial fitness (Favaro et al., 2013b, Viktor et al., 2013) were therefore chosen as hosts for the production of the recombinant enzymes.

Since codon optimization can significantly improve gene expression levels and the subsequent functionality of the enzymes (Favaro et al., 2012b), the *TLG1* (*T. lanuginosus* glucoamylase) and *SFA1* (*S. fibuligera* α-amylase) genes were codon-optimized for expression in *S. cerevisiae*. The synthetic sequences were cloned individually (Fig. 1) and expressed in *S. cerevisiae* M2n (creating strains M2n[TLG1] and M2n[SFA1]) with their respective activity confirmed on soluble starch (Fig. 2a). This was followed by the construction of the raw starch fermenting *S. cerevisiae* M2n[TLG1-SFA1] and *S. cerevisiae* MEL2[TLG1-SFA1] strains that displayed clearing zones on starch plates (Fig. 2a), as opposed to a smaller halo for *S. cerevisiae* M2n[SFA1] and none for M2n[TLG1].

Based on the deduced amino acid sequences, the predicted molecular weights of 56 and 67 kDa for the recombinant SFA1 and TLG1, respectively, were similar to those of native α-amylases and glucoamylases characterized in *S. fibuligera* and *T. lanuginosus* (Hostinová et al., 2010; Thorsen et al., 2006). As reported in Figure 2b, SDS-PAGE analysis of the supernatant indicated that only TLG1 was glycosylated in both strains, having a molecular mass of about 90 kDa instead of the predicted 67 kDa.

The combined amylase activity of the recombinant strains performed well between pH 3.5 and 5.5 with only 53% residual activity detected at pH 6.5 (Fig. 2c). The amylases acted effectively between 50 and 70°C, with less than 30% relative activity at the optimal fermentation temperature (30°C). These conditions are in agreement with those reported for other raw starch degrading α-amylases and glucoamylases (Robertson et al., 2006; Sun et al., 2010). Moreover, the optimal pH value of 4.5 detected for the codon-optimized amylases was similar to those of the native TLG1 of *T. lanuginosus* (Thorsen et al., 2006) and SFA1 of *S. fibuligera* (Hostinová et al., 2010) whereas their optimal temperature of 60°C was slightly different since the native enzymes were described by the same authors for temperature optimum of 70 and 50°C, respectively.

The enzymatic activity was influenced by the incubation temperature and nature of the substrate (Table II). As expected, the hydrolytic activities were significantly lower on the more recalcitrant raw starch compared to soluble starch, whereas the higher temperature of 60°C increased the enzyme activity approximately 4-fold irrespective of the strain and substrate. The *S. cerevisiae* M2n[TLG1-SFA1] strain performed slightly better than *S. cerevisiae* MEL2[TLG1-SFA1] at both 30 and 60°C on either soluble or raw starch. This could be ascribed to different copy numbers or site(s) of integration for the synthetic genes, but further genetic studies are required to confirm these hypotheses.

Delta-integration of the synthetic *TLG1* and *SFA1* genes slightly affected the fermentation ability of the recombinant strains (Fig. 3). This is in agreement with previous reports by Favaro et al. (2012b) and Kang et al. (2003) indicating that the high number of integrations targeted to the δ-elements did not significantly impair the growth rate of the recombinant strains on glucose.

This study is one of only a few that demonstrated the concept of CBP raw starch to ethanol in fermenters using a high gravity feed of 200 g/L raw starch, but it represents the first report on CBP of unprocessed starchy substrates with recombinant industrial yeast strains at a bioreactor scale. Other researches were based mainly on laboratory strains, which make direct comparison with the current work difficult. The *S. cerevisiae* YF237 laboratory strain, displaying the *R. oryzae* glucoamylase on its surface and secreting the *Streptococcus bovis* α-amylase, produced 51 g/L of ethanol from 100 g/L of raw corn starch after 60 h of fermentation (Khaw et al., 2006). The laboratory *S. cerevisiae* YF207, co-expressing the *R. oryzae* glucoamylase and *S. bovis* α-amylase on the cell surface, yielded about 55 g/L of ethanol from 200 g/L of raw corn starch after 10 days of fermentation (Chen et al., 2008). The latter compared well with the 64 and 52 g/L ethanol obtained by the *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains (Fig. 4, Table III) from 200 g/L raw corn starch after 10 days. In contrast to the reports mentioned above, the enzymes in this study were not tethered to the cell wall, but secreted during cultivation on raw corn starch.

Sorghum and triticale were selected as natural starchy substrates to evaluate the fermentative capabilities of the recombinant *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1] strains. The starch component of both materials has similar properties to corn starch and should therefore be suitable as feedstock for an integrated bioethanol process. Both grains were efficiently converted to ethanol (Fig. 4), in particular by the M2n[TLG1-SFA1] strain, with starch conversion rates and ethanol production (relative to theoretical yield) exceeding those from raw corn starch (Table III).

This could be attributed to the different structural composition and native amylolytic enzymes content of the three substrates. Digestion of starch is dependent on starch properties such as granule size, architecture, degree of crystallinity and polymerization, surface pores or channels, non-starch components and their interactions with starch, and amylose:amylopectin ratio. Considering that all the starchy materials used in this work have similar amylose:amylopectin ratio, shape and distribution of granules (Tester et al., 2004, 2006), the different ethanol yields described in this study could be mainly linked to protein matrices surrounding starch granules: corn and sorghum grains have dense proteins, limiting the access of the amylolytic enzymes to starch, meanwhile the protein matrices in triticale are more diffuse and do not impede the hydrolysis (McAllister et al., 1990). Moreover, the presence of relatively high concentrations of metal ions in triticale and sorghum would stabilize α-amylase in the presence of high ethanol concentrations (Abdel-Aal and Wood, 2005; Yamada et al., 2011). Such stabilization would ensure the continued functioning of SFA1 and may account for greater and more rapid saccharification of the starch, thus resulting in higher ethanol yields. Furthermore, native amylolytic enzymes (mainly α-amylase) in both grains will supplement the recombinant enzymes. Results from the MEL2[TLG1-SFA1] strain seem to confirm this hypothesis as ethanol was readily detected after 12 h of incubation from both triticale and sorghum, whereas ethanol production from corn starch, which does not contain native amylases, was delayed (Fig. 4b).

The *S. cerevisiae* M2n[TLG1-SFA1] strain displayed comparable and high volumetric productivities on all the three substrates towards the end of the fermentation (Fig. 4a), confirming that the high enzymatic activities (Table II) supported the effective saccharification of all three starchy substrates. The *S. cerevisiae* MEL2[TLG1-SFA1] strain was inferior to the M2n[TLG1-SFA1]

strain (Fig. 4b) due to lower levels of enzymatic activity (Table II) and produced approximately 20% less glucose on raw corn starch at 30°C, which hampered the fermentation process.

To our knowledge, only Yamada et al. (2011) have thus far reported CBP of real starchy biomass applying the tetraploid amylolytic MNIV/δGS strain (combining δ-integration and polyploidization of laboratory strains) on brown rice. The reported ethanol yield and volumetric productivity were about 100% and 0.65 g/L/h, respectively, and compared well with those achieved by the diploid semi-industrial *S. cerevisiae* M2n[TLG1-SFA1] strain for a similar time frame. Considering the higher ploidy of the MNIV/δGS laboratory strain, the recombinants constructed in this study might be further improved upon by polyploidization (Yamada et al., 2010b).

In conclusion, this is the first report of the simultaneous expression of codon-optimized genes of *TLG1* and *SFA1* in a foreign host. The resulting recombinants demonstrated ethanol production in excess of 60 g/L using a high gravity feed of 200 g/L corn starch, triticale, and sorghum substrates without any pre-treatment or exogenous enzyme addition. For the first time, industrial strains, co-producing glucoamylase and α-amylase enzymes were described for efficient CBP of natural starchy biomass at a bioreactor scale.

The engineered strains' ethanol performance will be evaluated on other starch-containing substrates, such as wheat bran or potato peels, and repeated fermentations are likely to further enhance the efficiency of the recombinant strains. Since these feedstocks also contain other polysaccharides such as cellulose and hemicellulose, the addition of cellulases and hemicellulases would further improve the release of fermentable sugars and therefore the ethanol yield from cereal grains. Bioethanol production from such substrates by means of an amylolytic yeast strain will thus benefit from the addition of these enzymes via heterologous expression or exogenous addition.

This work was partially supported by the research project funded by the University of Padova entitled 'Engineering Consolidated Bioprocessing yeasts for the one-step conversion of cellulosic substrates into bioethanol'; the research project "BioRivaluta" funded by Regione Veneto (PSR 2007-2013, Misura 124, n. 2307660); the bilateral joint research project N. ZA11MO2 entitled 'Development of robust yeast strains for bioethanol production from starchy and cellulosic plant biomass' and the National Research Foundation of South Africa (76597 to MVB and 68955 to WHvZ). Dr Favaro is a recipient of an 'Assegno di ricerca Senior' grant from the University of Padova (Padova, Italy). Lorenzo Cagnin (Padova, Italy) is recipient of a PhD bursary from the University of Padova. Dr Maryna Saayman (Stellenbosch University, SA) is acknowledged for technical assistance. Dr Willem Botes (Stellenbosch University, SA) provided and prepared the triticale and sorghum feedstock.

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Applied Energy 160 (2015) 610–617

Contents lists available at ScienceDirect

Applied Energy

journal homepage: www.elsevier.com/locate/apenergy

Utilisation of wheat bran as a substrate for bioethanol production using recombinant cellulases and amylolytic yeast

Rosemary Cripwell [a], Lorenzo Favaro [b,*], Shaunita H. Rose [a], Marina Basaglia [b], Lorenzo Cagnin [b], Sergio Casella [b], Willem van Zyl [a]

[a] Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland, Stellenbosch, South Africa
[b] Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), Università di Padova, Agripolis, Viale dell'Università 16, 35020 Legnaro, Padova, Italy

HIGHLIGHTS

- A cocktail of recombinant cellulases was proposed for wheat bran hydrolysis.
- Optimal conditions for enzymatic hydrolysis of wheat bran were determined.
- Recombinant amylolytic strains completely hydrolysed the starch in wheat bran.
- Addition of cellulases to SSF with amylolytic strains enhanced ethanol yield.

ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 16 August 2015
Accepted 12 September 2015
Available online 28 September 2015

Keywords:
Bioethanol
Wheat bran
Recombinant cellulase cocktail
Industrial engineered amylolytic yeast
Simultaneous saccharification and fermentation

ABSTRACT

Wheat bran, generated from the milling of wheat, represents a promising feedstock for the production of bioethanol. This substrate consists of three main components: starch, hemicellulose and cellulose. The optimal conditions for wheat bran hydrolysis have been determined using a recombinant cellulase cocktail (RCC), which contains two cellobiohydrolases, an endoglucanase and a β-glucosidase. The 10% (w/v, expressed in terms of dry matter) substrate loading yielded the most glucose, while the 2% loading gave the best hydrolysis efficiency (degree of saccharification) using unmilled wheat bran. The ethanol production of two industrial amylolytic *Saccharomyces cerevisiae* strains, MEL2[TLG1-SFA1] and M2n [TLG1-SFA1], were compared in a simultaneous saccharification and fermentation (SSF) for 10% wheat bran loading with or without the supplementation of optimised RCC. The recombinant yeast *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] completely hydrolysed wheat bran's starch producing similar amounts of ethanol (5.3 ± 0.14 g/L and 5.0 ± 0.09 g/L, respectively). Supplementing SSF with RCC resulted in additional ethanol production of about 2.0 g/L. Scanning electron microscopy confirmed the effectiveness of both RCC and engineered amylolytic strains in terms of cellulose and starch depolymerisation.

This study demonstrated that untreated wheat bran could be a promising ready-to-use substrate for ethanol production. The addition of crude recombinant cellulases improved ethanol yields in the SSF process and *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] strains can efficiently convert wheat bran's starch to ethanol.

1. Introduction

Lignocellulosic biomass is the preferred substrate for bioethanol as it is more abundant and less expensive than sucrose and starch substrates [1]. However, the limitations associated with lignocellulosic ethanol production include the slow rate of enzymatic degradation, high enzyme cost and the requirement of inhibitor-tolerant industrial yeast strains [2–4]. Consequently, starch is still the most commonly used feedstock for ethanol production, with a relatively mature technology developed for corn in the USA [5] that produced about 52.5 billion litres of bioethanol in 2012, an increase from 49.2 billion litres in 2010 [6].

Current starch-to-ethanol processes require an energy-intensive liquefaction step, as well as substantial amounts of exogenous amylases for enzymatic hydrolysis of raw starch; both these significantly impact the economic viability of starch as feedstock [7]. In order to implement the large scale ethanol production from raw starch, the development of an industrial yeast

* Corresponding author. Tel.: +39 049 8272926; fax: +39 049 8272929.
E-mail address: lorenzo.favaro@unipd.it (L. Favaro).

http://dx.doi.org/10.1016/j.apenergy.2015.09.062
0306-2619/

that converts starch to ethanol in one step (called consolidated bioprocessing – CBP) is needed [8–11].

Recently, few studies reported the use of *Saccharomyces cerevisiae* strains for the fermentation of natural starchy substrates at a bioreactor scale. Favaro and colleagues described the direct ethanol production from natural starchy substrates (corn, sorghum and triticale), using industrial yeast strains co-secreting glucoamylase and α-amylase enzymes [12]. Yamada et al. [13] achieved the CBP of brown rice by the amylolytic laboratory strain MNIV/δGS producing almost 80 g/L of alcohol from 200 g/L of brown rice after 120 h. Although the above reports pave the way for the industrial CBP of raw starch to ethanol, their focus was on substrates composed only of starch, meanwhile many industrial starch-rich by-products are available in great quantities with different compositions in terms of cellulose and hemicellulose. These polysaccharides first have to be converted into sugars, in order to achieve high ethanol efficiencies and make the overall process economically viable. This is the case with wasted crop, cereal bran, cassava pulp, sago pith residues and brewery-spent grains, which have been proposed as low-cost materials for bioethanol, mainly by means of chemical pre-treatment, commercial cellulases, xylanase and amylases addition and subsequent fermentation [14–19]. The previously mentioned studies, though achieving promising results, demonstrate that the total exploitation of such substrates still needs to be addressed and that there is an opportunity to further increase the hydrolysis and fermentation yields from agricultural by-products containing different polysaccharides. Cheap and plentiful residual biomass has been investigated as renewable material to be converted into fuels, polymers, enzymes and bulk chemicals [20–23].

This research focused on wheat bran as an abundant and inexpensive starchy substrate, with a high potential for bioethanol due to its low pre-treatment cost [14,15]. In addition to the starch content (15–30% dry matter), the hemicellulose and cellulose fractions can also be used for bioethanol production [24]. Although wheat bran does not require costly pre-treatments for hydrolysis [15,25], not many studies have used this substrate for ethanol production [26]. Therefore, there is scope to optimise current technologies.

The hydrolysis of cellulose, starch and hemicellulose requires commercial enzymes that are very costly and not feedstock specific. Banerjee and colleagues [27] have developed a core set of recombinant enzymes for the hydrolysis of ammonia fibre expansion (AFEX) treated corn stover, using *Trichoderma reesei* enzymes produced in *Pichia pastoris*. However, there is still limited information available on the use of feedstock specific recombinant enzyme cocktails. An advantage of recombinant cocktails over commercial cocktails is that they are defined mixtures and do not contain unnecessary proteins.

In this present study, we examine the use of recombinant cellulolytic enzymes and engineered amylase-secreting strains for the hydrolysis and saccharification of wheat bran's cellulose and starch. The first objective was to investigate the simultaneous hydrolysis of cellulose using a recombinant cellulase cocktail (RCC) produced by engineered yeast and fungal strains. For the first time, the crude enzymes secreted in the supernatant were directly used to optimise the hydrolysis of wheat bran in terms of glucose yield. Once the optimisation of hydrolysis was achieved, the industrial *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] strains (both secreting the *Thermomyces lanuginosus* glucoamylase, TLG1, and the *Saccharomycopsis fibuligera* α-amylase, SFA1) were utilised for the simultaneous saccharification and fermentation (SSF) process in the presence of RCC resulting in high ethanol yields. This is the first report describing the conversion of starchy and cellulosic substrate into ethanol using crude recombinant enzymes and engineered amylolytic strains.

2. Material and methods

2.1. Strains, media and cultivations

The genotype and origin of strains used in this work are summarised in Table 1. The wild type *S. cerevisiae* MEL2 and M2n, with their respective recombinant strains MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], were utilised for wheat bran fermentation. The engineered strains contained the *TLG1* gene (glucoamylase from *T. lanuginosus*) expressed under the control of the *ENO1* promoter and the *SFA1* gene (α–amylase from *S. fibuligera*) expressed under the control of the *PGK1* promoter sequences [12]. Both genes were codon optimised for expression in *S. cerevisiae* and integrated into the delta sequences on the genomes of the industrial *S. cerevisiae* MEL2 and M2n strains [12].

Unless stated otherwise, all chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

The recombinant *S. cerevisiae* strains were maintained on either solid SC^{-URA} agar plates (containing 6.7 g/L yeast nitrogen base without amino acids [Difco Laboratories], 20 g/L glucose and yeast synthetic drop-out medium supplements (Sigma–Aldrich (Germany) or solid YPD (Yeast Peptone Dextrose) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar).

Culture medium (6.7 g/L yeast nitrogen base, 20 g/L peptone and 20 g/L glucose, 0.05 mM citric acid buffer, pH5) was used to prepare the yeast inocula for the fermentation studies. Fermentation medium is similar to the cultivation medium, but contained 0.5 g/L glucose and 10% (w/v) unmilled wheat bran. The *Aspergillus niger* D15[EgA] strain was maintained on spore plates and

Table 1
Strains and recombinant enzymes used in this study.

Strains	Relevant enzyme[a]	Source organism	Reference
RCC[b]			
S. cerevisiae Y294[CbhI]	Cellobiohydrolase I (CbhI)	*Talaromyces emersonii*	[28]
S. cerevisiae Y294[CbhII]	Cellobiohydrolase II (CbhII)	*Chrysosporium lucknowense*	[28]
Aspergillus niger D15[EgA]	Endoglucanase I (EgA)[c]	*Aspergillus niger*	[29]
S. cerevisiae Y294[Pcbgl1B]	β-glucosidase (Bgl)	*Phanerochaete chrysosporium*	[30]
SSF			
S. cerevisiae MEL2	–	Industrial strain for bioethanol	[15]
S. cerevisiae M2n	–	Semi-industrial strain	[31]
S. cerevisiae MEL2[TLG1-SFA1]	Glucoamylase (TLG1) α-Amylase (SFA1)	*T. lanuginosus* *S. fibuligera*	[12]
S. cerevisiae M2n[TLG1-SFA1]	Glucoamylase (TLG1) α-Amylase (SFA1)	*T. lanuginosus* *S. fibuligera*	[12]

[a] All enzymes were secreted using their native secretion signal, with the exception of Pcbgl1B (using the *T. reesei* Xyn2 secretion signal).
[b] RCC (recombinant cellulase cocktail) [31].
[c] EgA was expressed using the native DNA sequence, whereas all other genes were codon optimised for expression in *S. cerevisiae*.

cultivated in double strength minimal media (2× MM, with 100 g/L glucose, lacking uridine) [32].

2.2. Chemical analysis of wheat bran

Wheat (*Triticum aestivum* L.) was grown in the area of Rovigo (Italy, 45°4′51″N, 11°47′38″E), harvested at 6 months, processed by Grandi Molini Italiani (Rovigo, Italy) and stored in plastic bags at 4 °C. The wheat bran had a geometric mean diameter of 0.79 mm [14]. The dry matter content (903.4 g/kg) was obtained by drying triplicate samples for 48 h in an oven at 100 °C. Wheat bran was analysed in terms of ash, starch, hemicellulose, cellulose, lignin and protein content according to international standard methods [33]. The same procedures were adopted to determine the content in terms of starch, hemicellulose, cellulose, lignin in the spent SSF wheat bran samples.

2.3. Pre-treatment of wheat bran

Raw wheat bran was homogenised to a geometric mean diameter of 0.45 mm, using a laboratory knife mill to obtain milled wheat bran. Unmilled and milled wheat bran were pre-treated with 1% sulphuric acid (w/w dry wheat bran) at 121 °C. Dry matter concentration was adjusted to 51 g/kg with deionised water. Pre-treatment vessels were filled with 100 mL of the resulting slurry and autoclaved at 121 °C for 30 min [15].

2.4. Enzymes

A recombinant cellulase cocktail (RCC) (Table 1), with a protein ratio of 114:102:1:637 (CbhI:CbhII:EgA:Bgl) [34] was used for wheat bran hydrolysis. The total activity (on carboxymethyl cellulose (CMC)) and protein concentration for RCC was 7.45 nkat/mL and 16.11 mg/mL, respectively.

2.5. Determination of protein content

The protein content was determined with the Bio-Rad protein reagent (BioRad, USA), as directed by the manufacturer with bovine serum albumin (BSA) as standard. Protein concentration was expressed as milligram of protein per mL.

2.6. Enzymatic hydrolysis

Hydrolysis trials were carried out to study the effect of pre-treatment, substrate loading, and enzyme loading on the enzymatic hydrolysis of wheat bran. The extent of bran's starch hydrolysis with the amylolytic enzymes secreted by *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] was also investigated. Hydrolysis trials were performed in a 5 mL working volume in McCartney bottles, with 0.05 M citric acid buffer (pH 5), 0.02% NaN_3 (to prevent contamination), 2%, 5%, 10% (w/v) substrate loading and the RCC cocktail. Reactions were incubated at 30 °C in a laboratory rotary-shaker-incubator (10 rpm), with sampling (0.1 mL) at time zero and at regular intervals. All substrate loadings are expressed as w/v, based on dry weight.

In the case of bran starch hydrolysis, yeast cultures of *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] were sampled after 72 h cultivation in YPD broth and their supernatant collected after centrifugation at 16,000*g* for 3 min. The glucose content of the samples was determined (in duplicate) using the Roche D–Glucose Kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Absorbance was measured by a spectrophotometer at 340 nm (Boehringer Mannheim/R-Biopharm). All the experiments were performed in triplicate. Data was analysed by three ways factorial ANOVA (Analysis Of Variance) using Duncan test *post hoc* means differentiation.

2.7. Fermentation studies on wheat bran

Inocula for *S. cerevisiae* strains were prepared in 200 mL culture medium in 500 mL Erlenmeyer flasks and incubated on a rotary shaker (30 °C) at 150 rpm for 60 h. An SSF was performed using fermentation medium containing 10% (w/v) unmilled wheat bran and an initial inoculum of 0.3 g dry weight/L. Control fermentations (without enzyme addition) were run in parallel to the SSF reactions using the fermentation medium, supplemented with 30 g/L glucose, since wheat bran typically contains 10% cellulose and 20% starch. In addition, hydrolysis controls with RCC and wheat bran were run in parallel to the SSF reactions under the same conditions except for the inoculum.

Unmilled wheat bran was used as the substrate and different filter-sterilised enzyme combinations were compared: (1) no enzymes and (2) RCC. Fermentations and control reactions were conducted at a working volume of 50 mL (pH 5) in a 55 mL serum bottle for 10 days at 30 °C on a magnetic stirrer. Serum bottles were equipped with a bubbling CO_2 outlet and fermentations were carried out under oxygen-limited conditions. Ampicillin (100 mg/L) and streptomycin (75 mg/L) were added to prevent contamination.

Samples were taken daily during the course of the fermentation and analysed for glucose, cellobiose and ethanol content, using ultra High Performance Liquid Chromatography (Nexera – Shimadzu Italia SRL, Milan, Italy) with a hydrogen column (Rezex R0A) at 60 °C and 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The compounds were detected with a refractive-index detector (RID 6A; Shimadzu, Kyoto, Japan). Experiments were performed in triplicate.

2.8. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) images were obtained from hydrolysis and SSF samples of wheat bran. Wheat bran was dehydrated in ethanol solutions at increasing concentrations (10%, 20%, 30%, 50%, 70%, 80%, 90%, 95% and absolute) and applied to a specimen stub. Samples were then coated with gold and observed using a Jeol JSM-6490 Scanning Electron Microscope at 15 kV.

2.9. Calculations

Glucose concentrations were used to calculate the degree of saccharification (DS). DS_{glucan} represents the soluble glucose released after hydrolysis (soluble sugars determined at time zero were deducted). DS_{starch} was based on the total sugar concentration in the hydrolysate (corrected for glucose concentration measured at time zero) with respect to the initial starch concentrations. A conversion factor of 0.9 (162/180) was applied due to the difference in the mass between the anhydroglucose ring and glucose, as a water molecule is added during the hydrolysis.

$$DS_{glucan} = \frac{[\text{glucose g/L}] \times 0.9}{[\text{cellulose g/L}]} \times 100\%$$

$$DS_{starch} = \frac{[\text{glucose g/L}] \times 0.9}{[\text{starch g/L}]} \times 100\%$$

The ethanol yield, $Y_{E/S}$, (g of ethanol/g of utilised glucose/polysaccharide) was calculated considering the amount of glucose/cellulose/starch consumed during the fermentation and

compared to the maximum theoretical yield of 0.51 g of ethanol/g of consumed glucose and 0.56 g of ethanol/g of consumed starch and/or cellulose. The volumetric productivity (Q) was based on grams of ethanol produced per litre of culture medium per hour (g/L/h) and the maximum volumetric productivity (Q_{max}) was defined as the highest volumetric productivity displayed by the *S. cerevisiae* strains.

3. Results and discussion

3.1. Wheat bran composition

The composition of the bran used in this work is reported in Table 2. Other than starch and cellulose (both nearly 11% of dry matter), the substrate was particularly rich in hemicellulose, with a value (39%) quite similar to those previously reported [35]. Interestingly, starch content was low if compared to that of other reports [14,15,26] indicating different and variable efficiency of starch extraction during milling processes.

Bran is also composed of a large protein fraction (17.9%). The values agree well with recently published results [14,15] and lignin content (about 5%) was similar to that reported by Palmarola-Adrados et al. [26]. This study focused on the conversion of wheat bran's hexose-containing polysaccharides into ethanol meanwhile the hydrolysis and fermentation of bran's hemicellulose is currently being addressed towards the complete exploitation of wheat bran for bioethanol production.

3.2. Cellulose wheat bran hydrolysis by crude recombinant cellulase cocktail (RCC)

In order to achieve high yields in the hydrolysis of wheat bran cellulose, several recombinant enzymes were screened for their saccharifying activities (data not shown). The following four cellulases were selected for their high hydrolytic potential, confirming their promise in terms of cellulose depolymerisation, as previously reported in our research outcomes [28–30]: namely, the cellobiohydrolase I (CbhI) of *Talaromyces emersonii*, the cellobiohydrolase II (CbhII) of *Chrysosporium lucknowense* and the β-glucosidase (Pcbgl1B) of *Phanerochaete chrysosporium* secreted by *S. cerevisiae* Y294 together with the endoglucanase I (EgA) of *A. niger* heterologously produced by *A. niger* D15[EgA]. The enzymes were found to be effective once formulated in a cocktail, hereafter referred as RCC, with the protein concentration ratio of 114:102:1:637 (CbhI:CbhII:EgA:Bgl). The influence of chemical pre-treatment, substrate and enzymatic loading on hydrolysis yield was then tested.

3.2.1. Effect of pre-treatment

Milled and unmilled wheat bran was pre-treated at 121 °C for 30 min with or without low sulphuric acid addition (1% w/w dry wheat bran) and RCC applied to the resulting pre-treated materials in order to select the most promising substrates.

As expected, the structural analysis conducted on the four different substrates revealed that, after the mild pre-treatment, most of the cellulose was still intact and limited solubilisation of hemicellulose also took place mainly in the sulphuric acid pre-treated materials with the highest degree of depolymerisation detected in the milled wheat bran (data not shown). However, no significant differences in terms of glucose levels and degree of saccharification (DS_{glucan}) were measured after the hydrolysis with RCC of the four materials (data not shown). As a result, since physico-chemical pre-treatment adds extra cost to the process, unmilled wheat bran, not-sulphuric acid pre-treated, was used for the remainder of the study.

Table 2
Composition (% of the dry matter) of unmilled and milled wheat bran used in this study.

Component	Unmilled (%)	Milled (%)
Hemicellulose	39.06	38.99
Starch	11.01	11.01
Cellulose	10.68	10.91
Protein	17.94	17.88
Lignin	4.98	5.08
Ash	0.05	0.04

3.2.2. Effect of substrate loading

Hydrolysis trials on unmilled wheat bran were subsequently performed with different substrate loadings (Fig. 1a). As expected, higher substrate loadings resulted in greater levels of glucose released ($p < 0.001$). However, the lower wheat bran concentrations, the higher saccharification yields were achieved: the DS_{glucan} obtained after 144 h was 34%, 24% and 18% for the 2%, 5% and 10% substrate loadings, respectively (Fig. 1b).

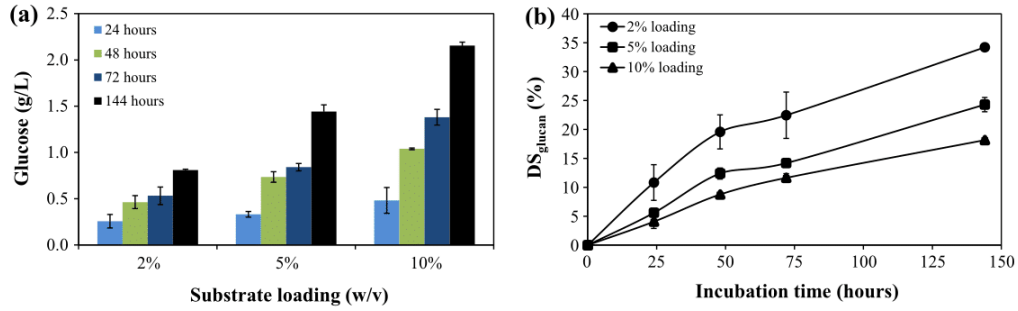
Overall, as reported in Fig. 1, the increase in glucose release and DS_{glucan} is not linear indicating a plateauing effect. The lower DS_{glucan} obtained for the higher substrate loadings corresponds to previous observations on several substrates [36–38] and can be ascribed to possible inhibition of the enzymes as a result of the accumulating glucose, and/or reduced accessibility of the cellulose. However, the amount of glucose released using a 10% substrate loading (Fig. 1a) is the highest ($p < 0.001$) and enough to support the growth of *S. cerevisiae*. Therefore, such a loading would be better suited for SSF process. Increasing the substrate concentration above 10% was not possible, as the reaction mixture would became too viscous, compromising proper mixing.

3.2.3. Effect of enzyme loading

The effect of enzyme dosages was investigated on 5% and 10% substrate loading (Fig. 2a). When the enzyme loading was doubled (2x RCC), the glucose yield after 24 h increased by 86% and 49% for the 5% and 10% substrate loadings, respectively. At 144 h, the increase was 51% and 9%, respectively (Fig. 2a). The highest DS_{glucan} (37%) was achieved with a 2x RCC and 5% substrate loading (Fig. 2b), which was nearly 13% higher than for the reaction with 5% substrate loading and RCC. A slight increase (<2%) in DS_{glucan} was observed when the enzyme concentration was doubled using a 10% substrate loading, however, this was not statistically relevant and possibly ascribed to the accumulation of glucose in 2x RCC condition, thus inhibiting the enzymes activity. The ANOVA test revealed a significant improvement of the glucose yield when the substrate loading, the enzyme loading, or treatment time increased (Fig. 2c).

3.3. Wheat bran's starch hydrolysis using crude recombinant amylases secreted by the engineered amylolytic strains

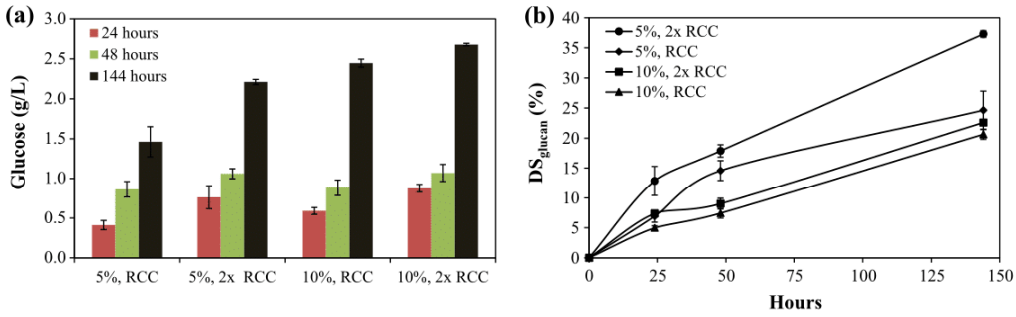
The amylolytic enzymes secreted by *S. cerevisiae* M2n [TLG1-SFA1] and MEL2[TLG1-SFA1], to be used in the SSF of wheat bran, were assessed in terms of hydrolysis on wheat bran's starch in trials with three different substrate loadings: 2%, 5% and 10% (Fig. 3). The recombinant amylases secreted by both industrial strains were effective in hydrolysing the starch content of wheat bran and, at the tested substrate dosages, displayed similar glucose release which appears to be linear (Fig. 3). After 90 h of incubation, the DS_{starch} was approximately 49% and 42% in all the substrate loadings for MEL2[TLG1-SFA1] and M2n[TLG1-SFA1], respectively, suggesting a slightly higher saccharification ability for the former yeast.



(c)

Parameter	*F* value	Probability of *F* value	Significance
Substrate loading	235.74	<0.001	**
Incubation time (h)	256.24	<0.001	**
Substrate loading x h	26.12	<0.001	**

Fig. 1. Effect of three substrate loadings (2%, 5% and 10%) on the hydrolysis of wheat bran cellulose using the RCC. Released glucose (a) and degree of saccharification (DS_{glucan}), (b) were calculated for wheat bran hydrolysis at 2%, 5% and 10% substrate loadings. Statistical evaluation (c) by ANOVA of the effect of different substrate loadings, time (h) and their interaction on hydrolysis after 144 h (**$p < 0.01$).



(c)

Parameter	*F* value	Probability of *F* value	Significance
Substrate loading	28.60	<0.001	**
Enzyme loading	216.26	<0.001	**
Incubation time (h)	470.42	<0.001	**
Substrate loading x Enzyme loading	<0.001	0.957	ns
Substrate loading x h	15.25	<0.001	**
Enzyme loading x h	19.56	<0.001	**

Fig. 2. Effect of substrate and enzyme loadings on enzymatic hydrolysis of wheat bran cellulose. Experiments were carried out with 5% and 10% substrate loading (w/v) of unmilled wheat bran and two different enzyme loadings: 1x RCC and a 2x RCC. Released glucose (a) and degree of saccharification (DS_{glucan}), (b) were calculated. Statistical evaluation (c) by ANOVA of the effect of substrate loading, enzymatic loading and incubation time (h), as well as their interactions on hydrolysis (ns: not significant; **$p < 0.01$).

3.4. Fermentation studies on wheat bran

A substrate loading of 10% was used for the wheat bran SSF, as it gave the highest glucose levels in the hydrolysis trials (Figs. 1a, and 3). As described in Section 2.7, reference fermentations were performed with both recombinant (*S. cerevisiae* M2n [TLG1-SFA1] and MEL2[TLG1-SFA1]) and wild type (*S. cerevisiae* M2n and MEL2) strains in broth containing 30 g/L glucose to simulate wheat bran composition (Fig. 4a, Table 3).

The yeast showed similar fermentative performances: all the glucose was metabolised within 18 h and the maximum ethanol concentrations ranged from 13.92 to 14.29 g/L, with an average ethanol yield of about 93% of the theoretical (Table 3). Moreover, as reported in Table 3, both maximum and final volumetric productivities were comparable for the two parental and recombinant yeast.

During SSF of wheat bran without RCC addition, only the engineered strains were able to produce ethanol (Fig. 4b, Table 3). The recombinant yeast MEL2[TLG1-SFA1] yielded, after 72 h, 5.26 g/L ethanol (Fig. 4b) while *S. cerevisiae* M2n[TLG1-SFA1], displaying similar volumetric productivity, produced up to 5.01 g/L ethanol in the same timeframe (Table 3). Starch was not detected

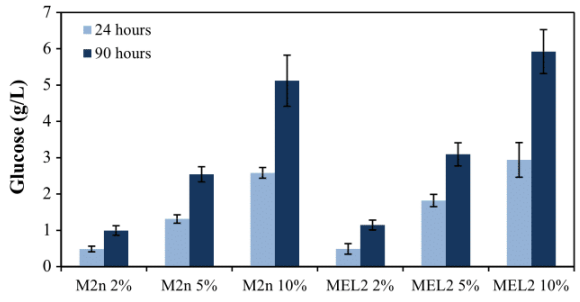


Fig. 3. Glucose (g/L) released during wheat bran's starch hydrolysis using the supernatant of recombinant *S. cerevisiae* M2n[TLG1-SFA1] and MEL2[TLG1-SFA1]. Three different substrate loadings were used (2%, 5% and 10% w/v). Data shown are the mean values of three replicates and standard deviations are included.

by the chemical analysis performed on spent wheat bran at the end of the SSF, indicating that both strains completely hydrolysed the polysaccharide (Table 3). The resulting ethanol yield per gram of consumed starch was higher than 85% and 81% for MEL2 [TLG1-SFA1] and M2n[TLG1-SFA1], respectively, with productivity values comparable for the engineered strains (Table 3). Their starch-to-ethanol conversion efficiencies were similar to those recently described for the same engineered strains from raw corn starch, sorghum and triticale [12].

SEM of wheat bran samples during the SSF confirmed the ability of the recombinant yeast to break down the starch granules, which were abundantly present at the beginning of the fermentation (Fig. 5a), limited in number but still visible after 44 h of incubation (Fig. 5b) and completely disappeared after 72 h of fermentation by MEL2[TLG1-SFA1] (Fig. 5c).

Supplementing the SSF with the optimised RCC was effective for cellulose hydrolysis, since high glucose levels were released by the enzymes (data not shown). As a result, both wild type and engineered strains were supported for ethanol production and, after 72 h, the ethanol level by MEL2[TLG1-SFA1] exceeded 7.30 g/L, which was 1.4-fold of the amount produced in the absence of the RCC (Table 3). On the other hand, the parental MEL2, unable to produce ethanol from wheat bran in the absence of external enzymes addition, obtained up to 2.30 g/L thanks to RCC. As reported in Table 3, similar ethanol levels were achieved by the wild type M2n and the engineered M2n[TLG1-SFA1].

Overall, the use of RCC and engineered amylolytic strains proved to be strategic, since additional ethanol production was achieved by the recombinant strains and, in the case of MEL2 [TLG1-SFA1] and M2n[TLG1-SFA1], alcohol levels were above 3-fold those of the parental yeast strains (Table 3). The ethanol

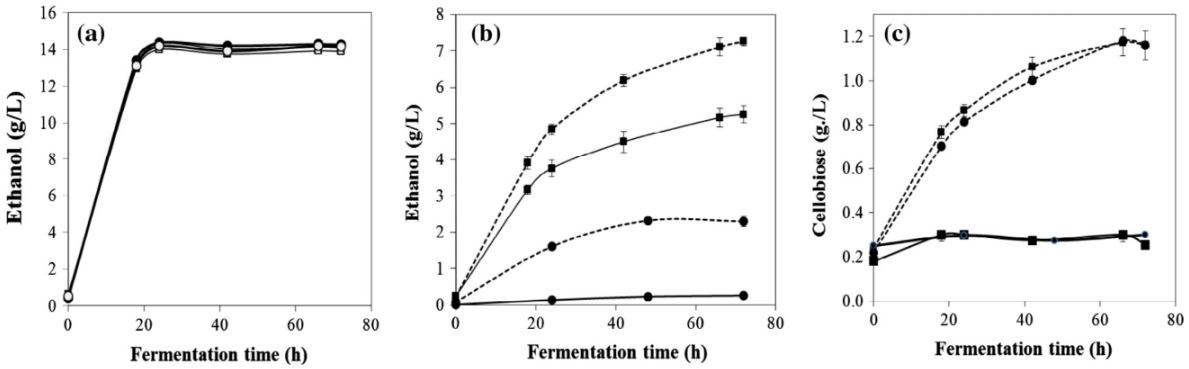


Fig. 4. Fermentation products during SSF of 10% (w/v) unmilled wheat bran. Ethanol levels by wild type *S. cerevisiae* MEL2 (●) and M2n (○) and their respective recombinant *S. cerevisiae* MEL2[TLG1-SFA1] (■) and *S. cerevisiae* M2n[TLG1-SFA1] (□) in control fermentation with 30 g/L glucose (a). Ethanol levels (b) and cellobiose accumulation (c) from wheat bran by *S. cerevisiae* MEL2 (●) and MEL2[TLG1-SFA1] (■) with (dash lines) or without (continuous lines) RCC addition. The results obtained for *S. cerevisiae* M2n and M2n[TLG1-SFA1] were not reported in (b and c) as the data were similar to those of the MEL2 and MEL2[TLG1-SFA1]. Data shown are the mean values of three replicates and standard deviations are included.

Table 3
Conversion of glucose and wheat bran's starch and/or cellulose to ethanol by wild type *S. cerevisiae* yeast (MEL2 and M2n) and their respective engineered strains: MEL2[TLG1-SFA1] and M2n[TLG1-SFA1]. SSF of wheat bran (10% w/v) was conducted with or without RCC (recombinant cellulase cocktail).

Strain	Highest ethanol concentration (g/L)	Glucose utilisation (%)	Starch utilisation (%)	Cellulose utilisation (%)	$Y_{E/S}$ (g/g)	Q (g/L/h)	Q_{max} (g/L/h)
Glucose (30 g/L) medium							
MEL2	14.29	100	–	–	0.48 (94%)	0.22	0.74
MEL2[TLG1-SFA1]	14.12	100	–	–	0.47 (93%)	0.21	0.73
M2n	14.18	100	–	–	0.47 (93%)	0.21	0.73
M2n[TLG1-SFA1]	13.92	100	–	–	0.47 (91%)	0.21	0.72
Wheat bran without RCC							
MEL2	0.18	–	0	0	–	–	–
MEL2[TLG1-SFA1]	5.26	–	100	0	0.48 (85%)	0.07	0.18
M2n	0.23	–	0	0	–	–	–
M2n[TLG1-SFA1]	5.01	–	100	0	0.45 (81%)	0.07	0.17
Wheat bran with RCC							
MEL2	2.30	–	0	41	0.50 (89%)	0.03	0.09
MEL2[TLG1-SFA1]	7.30	–	100	37	0.50 (89%)	0.10	0.22
M2n	2.29	–	0	40	0.50 (89%)	0.03	0.09
M2n[TLG1-SFA1]	7.00	–	100	37	0.49 (88%)	0.10	0.20

$Y_{E/S}$, ethanol yield per gram of consumed substrate calculated on the highest ethanol production and % of theoretical maximum indicated in brackets.

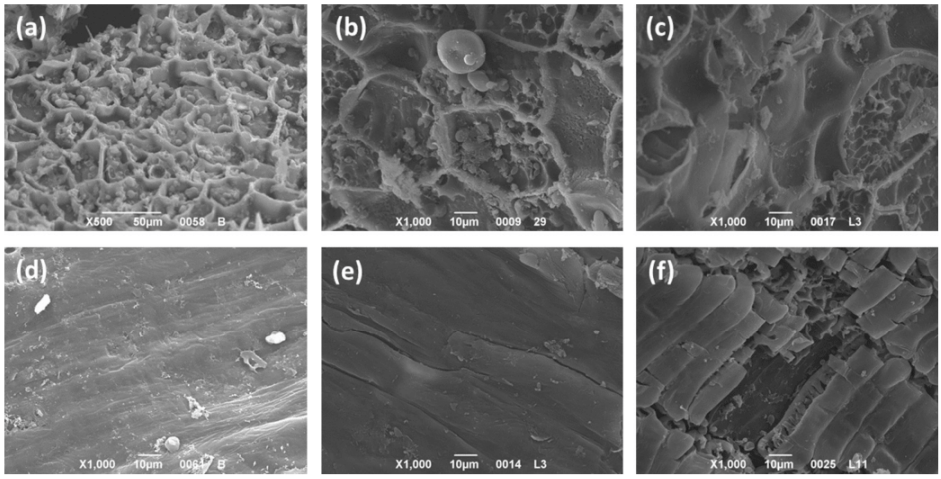


Fig. 5. SEM micrographs of wheat bran at the beginning (a and d), after 44 h (b and e) and 72 h (c and f) of SSF with RCC and *S. cerevisiae* MEL2[TLG1-SFA1].

yield were higher than 88% of the theoretical for all the strains and compared well with those reported for SSF of other cellulosic materials, such as wheat straw, willow and paper sludge [39]. Furthermore, the volumetric productivity values were significantly greater for the recombinant yeast, exhibiting a Q_{max} of about 0.21 g/L/h instead of 0.09 g/L/h as detected for the parental strains (Table 3).

Efficient biomass hydrolysis is dependent on β-glucosidase, as this enzyme is needed for the final step of hydrolysis by converting the cellobiose to glucose [40]. However, an increase of about 1.17 g/L cellobiose was observed after RCC addition to the fermentation with both *S. cerevisiae* MEL2[TLG1-SFA1] and MEL2 (Fig. 4b) indicating insufficient β-glucosidase activity of Bgl from *P. chrysosporium*. In order to avoid commercial β-glucosidase supplementation (which is costly), recombinant β-glucosidase needs to have improved abilities such as increased specific activity [41] and further investigations are in progress to enhance the β-glucosidase activity in RCC.

Despite the suboptimal cellobiose-splitting activity, RCC was able to hydrolyse about 37% of the cellulose content as pointed out by the chemical analysis of wheat bran fermented by the engineered amylolytic strains. The efficiency of cellulose hydrolysis was similar also in the SSF of wheat bran using the parental yeast (Table 3). Considering that RCC was composed by crude supernatant and not purified enzymes, this efficiency has to be considered high and further improvable.

Cellulose depolymerisation was verified by SEM conducted during the wheat bran SSF of all the strains in the presence of RCC. At the beginning of the experiment, the structure of wheat bran was still intact with a rough surface (Fig. 5d), while cellulose damages increased with the incubation time (Fig. 5e after 44 h) and were clearly evident at the end of the SSF (Fig. 5f); thus the RCC was successful in hydrolysing the cellulose and simultaneously exposing the starch to the recombinant amylases secreted by *S. cerevisiae* MEL2[TLG1-SFA1] and by *S. cerevisiae* M2n[TLG1-SFA1]. Overall, SEM analysis showed that significant changes occurred in the structure of wheat bran after SSF with the RCC and amylolytic yeast, proving their effectiveness in terms of starch and cellulose depolymerisation (Fig. 5).

4. Conclusions

In this study, we demonstrated an SSF whereby the cellulose component of wheat is hydrolysed by recombinant cellulases, while at the same time the starch fraction is depolymerised by amylolytic yeast. These results pointed out that recombinant enzyme cocktails and recombinant strains, both tailored for a given substrate, play a key role for the efficient ethanol production from agricultural by-products. Crude enzyme and substrate loading were optimised to define a proficient SSF of wheat bran. *S. cerevisiae* MEL2[TLG1-SFA1] and M2n[TLG1-SFA1] completely converted wheat bran starch to ethanol with high yields and RCC supplementation resulted in additional alcohol production. This research showed that untreated wheat bran can be a ready-to-use substrate for ethanol production by SSF and further techno-economical evaluations will be undertaken to determine the actual feasibility of the whole process for the conversion of such by-product into bioethanol.

Acknowledgements

This work was partially supported by: the research project funded by the University of Padova entitled 'Engineering Consolidated Bioprocessing yeasts for the one-step conversion of cellulosic substrates into bioethanol' (GRIC120EG8); the research project "BioRivaluta" funded by Regione Veneto (PSR 2007-2013, Misura 124, n. 2307660); the bilateral joint research project N. ZA11MO2 entitled 'Development of robust yeast strains for bioethanol production from starchy and cellulosic plant biomass'; and the National Research Foundation (South Africa). Mr. Federico Fontana (Padova University, Italy) is acknowledged for HPLC analysis.

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Applied Energy

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Bacillus sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste

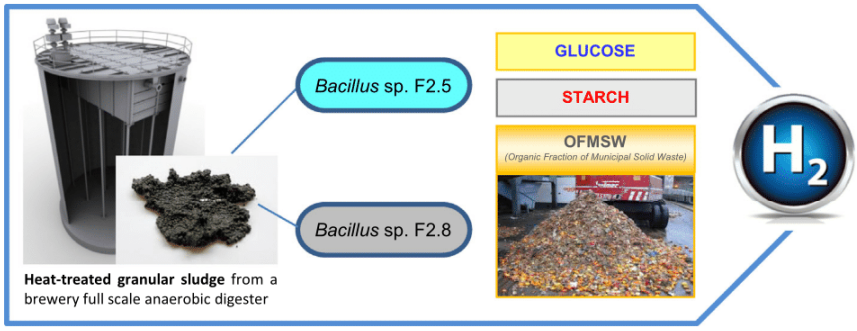
A.T. Shah [a,b], L. Favaro [a,*], L. Alibardi [c,1], L. Cagnin [a], A. Sandon [d], R. Cossu [c], S. Casella [a], M. Basaglia [a]

[a] Department of Agronomy Food Natural resources Animals and Environment, University of Padova, Viale dell'Università 16, 35020 Legnaro, PD, Italy
[b] Fermentation Technology Group, Industrial Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), 38000 Faisalabad, Pakistan
[c] Department of Industrial Engineering, University of Padova, Via Gradenigo, 6/a, 35131 Padova, Italy
[d] Department of Civil, Environmental and Architectural Engineering, University of Padova, via Marzolo 9, 35131 Padova, Italy

HIGHLIGHTS

- For the first time pure microbial cultures produced bio-hydrogen from organic waste.
- Two *Bacillus* sp. strains were selected for high amylolytic activities.
- The strains produced high H_2-yields from glucose and soluble starch.
- Promising H_2 production was confirmed also from organic waste.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 February 2016
Received in revised form 19 April 2016
Accepted 6 May 2016
Available online 14 May 2016

Keywords:
Bio-hydrogen
Dark fermentation
Organic fraction of municipal solid waste
Bacillus sp.
Pure cultures
Strain selection

ABSTRACT

Bio-hydrogen, obtained by fermentation of organic residues, is considered a promising source of renewable energy. However, the industrial scale H_2 production from organic waste is far to be realized as technical and economical limitations have still to be solved. Low H_2 yields and lack of industrially robust microbes are the major limiting factors.

To look for bacteria with both interesting hydrogen fermentative traits and proper robustness, granular sludge from a brewery full scale Upflow Anaerobic Sludge Blanket (UASB) digester was selected as trove of microbes processing complex substrates. One hundred and twenty bacterial strains, previously isolated from heat-treated granular sludge and genetically identified by 16S rDNA sequencing, were screened for extracellular hydrolytic enzymes on cellulose, hemicellulose, starch, pectin, lipids, protein. The most interesting hydrolytic strains were assessed for their H_2 production from glucose and soluble starch. Two *Bacillus* sp. strains, namely F2.5 and F2.8, exhibited high H_2 yields and were used as pure culture to convert Organic Fraction of Municipal Solid Waste (OFMSW) into hydrogen. The strains produced up to 61 mL of H_2 per grams of volatile solids and could be considered as good candidates towards the development of industrially relevant H_2-producing inoculants. This is the first successful application of pure microbial cultures in bio-hydrogen production from OFMSW.

1. Introduction

Biological hydrogen production from organic waste represents both an energy production process and a first stage of stabilization for organic biomass since it degrades complex substrates to readily

* Corresponding author at: Department of Agronomy Food Natural resources Animals and Environment (DAFNAE), Agripolis – University of Padova, Viale dell'Università, 16, 35020 Legnaro, Padova, Italy.
E-mail address: lorenzo.favaro@unipd.it (L. Favaro).
[1] Present address: Cranfield Water Science Institute, Cranfield University, Bedford MK43 0AL, UK.

http://dx.doi.org/10.1016/j.apenergy.2016.05.054
0306-2619/

biodegradable compounds or to metabolites of commercial interest (i.e. organics acids and solvents) [1–3].

Organic waste and low-cost organic by-products of food-processing industry have been already investigated as promising renewable materials to be converted into hydrogen and other fuels, polymers, enzymes and bulk chemicals [4–13]. However, to guarantee the economical sustainability of the organic waste-to-hydrogen route, one of the main requirements is linked to the availability of efficient H_2 producing microbes with proper robustness to be used at industrial scale [1]. In order to obtain suitable inoculants, methanogens and hydrogen-consuming bacteria should be inhibited. To this purpose, several methods for pre-treatment of inocula have been proposed, including heat-treatment, aeration, irradiation, freezing, addition of chemical inhibitors such as acid, alkali, chloroform, etc., as extensively reviewed in [14–18].

The Organic Fraction of Municipal Solid Waste (OFMSW), characterized by high moisture and high biodegradability due to a large content of food waste, kitchen waste and leftovers from residences, cafeterias and markets, has been previously evaluated for H_2 production through the addition of heat-treated inocula [5,19–21]. Although heat shock pre-treatment contributed to good H_2 performances in short lab scale operations, increasing evidences show that a stable H_2 production and methanogens repression is not possible for long-term continuous mode [1,14,22]. Further research is also needed to establish whether the additional technical complexity of heat-treating the inoculum at industrial scale is cost-effective. Pragmatically thinking, heat shock of inocula is technologically more difficult during scale-up as compared to other pre-treatments [16]. Moreover, the use of exogenous inocula does not allow to guide properly the fermentation process [5,14]. To address this issue, recent research advances have been reported indicating that OFMSW itself could produce high H_2 yields, without any external inoculum supplementation [5]. Natural decomposition occurs to food waste when left for few days at room temperature due to the presence of indigenous microorganisms. In case of no or very low oxygen concentration, fermentation of organic matter takes place and methane production may also occur with time. Therefore, some species of indigenous microbial population of organic waste may have good characteristics for the hydrolysis of complex substrates and for an efficient conversion into H_2. As a result, food waste could serve both as substrate and source for H_2 production and H_2-producing bacteria, respectively [5,23]. This novel approach paves the way for the development of inoculants to produce H_2 from OFMSW relying on the indigenous microbes.

Another recent research strategy is the use of selected microbe (s) for the conversion of organic waste into H_2 [21,24]. The main advantages of using pure cultures over mixed microflora are that metabolic changes are easier to detect/tune and more information on the conditions that promote H_2 production can be disclosed [17,18,25]. Furthermore, even in non-sterile environments, pure cultures may be useful in bioaugmentation to achieve higher gas outputs [16,18,23,26]. The possibility to select strain(s) for their hydrolytic and fermenting abilities according to the main complex substrates available in the food waste makes this avenue very effective. However, it remains still unexplored as pure cultures have been so far mostly applied for H_2 production from simple sugars (i.e., glucose, sucrose and xylose) or laboratory-grade soluble starch [14,17,27]. Thus, more researches using pure cultures for H_2 production from organic waste are recommended [17,18,25].

In this paper, to look for microbes with both high hydrogen production potential and proper robustness, granular sludge from a brewery full scale Upflow Anaerobic Sludge Blanket (UASB) digester was selected as promising source because of processing complex substrates at industrial scale. One hundred and twenty bacterial strains, previously isolated from heat-treated granular sludge and selected for their high H_2 production [28], were screened for extracellular hydrolytic profile on cellulose, hemicellulose, starch, pectin, lipids and protein. The isolates exhibited a broad range of hydrolytic activities and the most interesting strains were assessed for their H_2 production from glucose. The top H_2-performing microbes were evaluated using starch as main carbon source. Two *Bacillus* sp. strains showed high H_2 levels and were evaluated also on OFMSW, mainly composed by starch, lipids and protein. The microbes gave promising H_2 yields and could be considered as good candidates towards the future development of industrially relevant microbes for the processing of organic waste into H_2. This is the first successful application of pure microbial cultures in bio-hydrogen production from OFMSW.

2. Materials and methods

2.1. Microbial strains

One hundred and twenty microbial strains were previously isolated from granular sludge samples heat-treated (100 °C) with increasing residence times in order to inhibit indigenous methanogenic bacteria. All the strains were identified by 16S rDNA sequencing [28].

2.2. Screening for the production of extracellular hydrolytic enzymes

Calibrated suspensions (A_{600} = 0.9, corresponding to an average concentration of 10^6 cells per mL) of bacterial cells, grown for 24 h at 37 °C in NB (Nutrient Broth) broth at 100 rpm, were used to inoculate plates containing the appropriate media described below and purified agar (Sigma, Italy). Petri dishes were checked for the presence of enzymatic activity described below, after aerobic incubation at 37 °C for 3 days. No discrepant results were recorded in repeated experiments.

2.2.1. Cellulase activity (CelA)

Cellulase production was detected on Hankin and Anagnostakis Medium containing 5 g/L carboxymethyl-cellulose (CMC). After cell growth, the presence of cellulolytic activity (CelA) was detected by Congo red method [29].

2.2.2. Lipolytic activity (LipA)

Strains were tested on tributyrin agar medium containing (g/L): peptone, 5; yeast extract, 3; tributyrin, 10; agar, 15; pH 6.0. Lipase activity (LipA) of the strains were indicated by a clear halo around the colony in an otherwise opaque medium as previously described [30].

2.2.3. Pectinolytic activity (PecA)

The secretion of extracellular pectic enzymes was tested on polygalacturonic acid medium (g/L): yeast nitrogen base, 6.7; glucose, 5; polygalacturonic acid (Fluka, Italy), 7.5; pH 7.0 [31]. The screening was performed using polygalacturonic acid medium with or without glucose (10 g/L). After cell growth, plates were flooded with a solution of 6 N HCl. The appearance of a degradation halo around bacterial colony was considered an indication of the polygalacturonic acid hydrolysis [32].

2.2.4. Proteolytic activity (PrA)

Extracellular protease production was determined on protein medium with skim milk (Difco, Italy), pH 6.5. A clear zone around the colony indicated protease activity (PrA) as described in literature [31,33].

2.2.5. Starch-degrading activity (StA)

Microbial strains were screened for the ability to hydrolyze soluble potato starch (Sigma, Italy) on Wollum medium containing (g/L): Yeast Extract (Difco), 1; Na_2NO_3, 1; KCl, 0.5; $MgSO_4$, 0.5; starch, 10; agar, 17 [32]. After incubation, Petri dishes were flooded with iodine solution. A pale yellow zone around colonies in a blue medium indicated starch degrading activity (StA) [34,35].

2.2.6. Xylan-degrading activity (XylA)

Cultures were screened for xylan degrading activity by growth on modified Hankin and Anagnostakis Medium containing 0.5% xylan from oat–spelt (Fluka, Italy). Colonies showing xylan-degrading activity (XylA) were identified by a clear hydrolysis zone around the colony after treatment with Congo Red.

2.3. Amylolytic enzymes characterization

The starch degrading strains were tested for their amylolytic activity once cultivated in NB with 20 g/L soluble starch or Starch Production Medium (SPM) supplemented with (g/L): peptone, 5; soluble starch, 20; Na_2HPO_4, 2; KH_2PO_4, 1. The pH was set to 7.0 for both media. The strains were aerobically grown at 37 °C for up to 168 h. Ten mL samples were withdrawn at 24 h intervals and, after centrifugation (10 min, 5500*g*), the supernatant was used for enzymatic assays.

Total amylase activity was determined in liquid assays using the reducing sugar method with glucose as standard [36]. The optimal enzyme pH was assessed at 50 °C with 50 μL of the supernatant and 450 μL of the substrate (0.1% soluble potato starch) suspended in 0.05 M citrate-phosphate or sodium-phosphate buffer at pH values ranging from 5.5 to 8.0. The optimal assay temperature was determined at pH 6.0 and 7.0 using temperatures ranging from 30 to 60 °C. The enzymatic reactions were conducted for 10 min and terminated by boiling in a waterbath for 15 min.

Enzymatic activities were expressed as unit (U) per mL of supernatant, which is defined as the amount of enzyme which releases 1 μmol of reducing end groups per min. All experiments were carried out in triplicate.

2.4. Batch test for hydrogen production from glucose

To evaluate the H_2 potential from glucose of the twenty strains with the most promising hydrolytic phenotype, 100 mL Pyrex vessels, were filled with 50 mL of NB (Oxoid, pH 6.0) with or without glucose (5 g/L) and sterilized by autoclave (121 °C, 20 min). Each strain was pre-grown overnight in NB and inoculated into the batch reactors at an optical density (600 nm) value of 0.2. After inoculation, the reactors were hermetically closed using a silicon plug. Once flushed with N_2 gas for 3 min, the vessels were incubated without stirring in a thermostatic chamber at 37° C.

The amount of biogas produced was recorded daily, using the water displacement method [28]: the biogas accumulated in reactors headspace is released in a second bottle filled with an acidified (pH < 3) and saline (NaCl 25%) solution, which avoids the dissolution of gas into the liquid. The biogas moves an equivalent volume of liquid that was subsequently measured with a graduated cylinder. Biogas composition in terms of hydrogen, carbon dioxide and methane were measured by gas chromatography as indicated in the "Analytical methods and calculations" paragraph.

At the end of fermentation, liquid samples were kept at −20 °C to analyse the volatile fatty acids (VFAs) concentration and the amount of residual glucose or starch as described below in the "Analytical methods and calculations" paragraph.

All experiments were carried out in triplicate and the results averaged.

2.5. Batch test for hydrogen production from soluble starch and OFMSW

The most promising starch-hydrolyzing strains were evaluated for their ability to convert soluble starch into H_2. The strains were grown in SPM for 72 h and then used to inoculate 50 mL fresh SPM into Pyrex bottles as described above. Sodium phosphate buffer (pH 6.0 and 7.0) was used.

In the case of H_2 production from OFMSW, each vessel was supplemented with 10 g VS/L (which corresponds to 150 g/L of fresh weight), instead of soluble starch. OFMSW was sterilized by autoclave (121 °C, 20 min) to suppress the indigenous microbes [5]. The experiments were monitored until biogas production stopped. At the end of H_2 fermentation, liquid samples were withdrawn and kept at −20 °C for further analysis. All the experiments were carried out in triplicate and the results averaged.

The sample of OFMSW used for batch tests was obtained in May 2015 from separate collection of MSW in Padova (Italy). Approximately 200 kg of organic waste was manually sieved, sorted and divided into the following fractions: fruits (F), vegetables (V), meat–fish–cheese (MFC), bread–pasta–rice (BPC), undersieve 20 mm (U) and rejected materials. Undersieve 20 mm was composed of materials smaller than 20 mm. The rejected materials were shoppers, plastics, metals, glass, bones, paper and cardboard, shells and fruit kernels. Results of manual sorting procedure are reported in Table 1.

Using the sorted fractions, a sample of organic waste was prepared maintaining the same proportion of the single fractions without the rejected materials. The prepared sample of OFMSW was ground in a kitchen mill prior to be used as substrate for H_2 production. The shredded OFMSW had total solid (TS) concentration of 146 ± 11 g TS/L and volatile solid (VS) and total organic carbon (TOC) concentration of 93 ± 1% and 45 ± 1%, respectively, referred to dry weight. Total Kjeldahl nitrogen (TKN), ammonium and total phosphorus concentrations were 2861 ± 113 mg N/L, 408 ± 35 mg N/L and 375 ± 18 mg P/L, respectively. Concentrations (of dry weight) of lipids, proteins, cellulose, hemicellulose, lignin, starch and pectin in OFMSW sample were also detected as follows: 18 ± 1, 17 ± 1, 5.0 ± 0.6, 6.0 ± 0.5, 2.0 ± 0.2, 19 ± 1, 8.0 ± 0.7, respectively.

2.6. Analytical methods and calculations

TS, VS, TKN, ammonium and total phosphorous concentrations were analysed according to standard methods [37]. TOC values were obtained by difference between Total carbon (TC) and inorganic carbon (IC). TC and IC were analysed by a TOC analyser (TOC-V CSN, Shimadzu). Concentration of lipids, proteins, pectin, lignin, cellulose, hemicellulose and starch were analysed according to official methods [38].

VFAs concentrations (acetic, propionic, iso and *n*-butyric, iso and *n*-valeric, iso and *n*-caproic and heptanoic acids) were analysed by a gas chromatograph (Varian 3900) equipped with a CP-WAX 58 WCOT fused silica column (25 m × 0.53 mm ID, Varian) and a Flame Ionization Detector (FID). Nitrogen was used as carrier gas at a flow of 4 mL/min in column. The oven temperature programme was initially set at 80 °C for a min, then increased at a rate of 10 °C/min to 180 °C (finally maintained for 2 min). Injector and detector temperatures were both set to 250 °C.

Residual glucose and soluble starch in the NB or SPM broths were measured using the peroxidase–glucose oxidase method with the D-glucose and starch assay kit, respectively (Boehringer Mannheim).

Biogas composition in the headspace of reactors, in terms of hydrogen (H_2), carbon dioxide (CO_2) and methane (CH_4) concentrations, was analysed by gas chromatography using a micro-GC (Var-

Table 1
Results from manual sorting procedure of the OFMSW used in this study.

Fraction	Weight (Kg)	Percentage (%)
Fruit	52.01	25.9
Vegetable	42.21	21.0
Meat–Fish–Cheese	8.95	4.5
Bread–Pasta–Rice	44.44	22.1
Rejected materials	33.52	16.7
Undersieve 20 mm	19.67	9.8
Total	200.80	100

ian 490-GC) equipped with (i) a 10-m MS5A column (to analyse H_2 and CH_4), (ii) a 10-m PPU column (to analyse CO_2) and (iii) two Thermal Conductivity Detectors (TCDs). Helium was used as carrier gas at a pressure of 150 kPa in columns. Injector and column temperatures were both set to 80 °C.

Data on biogas and hydrogen productions was expressed at a temperature of 0 °C and pressure of 1 atm. Hydrogen volumes produced in the time interval between each measurement $[t-(t-1)]$ during dark fermentation batch tests, were calculated using a model considering (i) the hydrogen gas concentration at times t and $t-1$, together with the total volume of biogas produced at time t, (ii) the concentration of the specific gas at times t and $t-1$, and (iii) the volume of the head space of reactors [15]. The following equation was applied:

$$V_{H_2,t} = C_{H_2,t} \cdot V_{BG,t} + V_{HS} \cdot (C_{H_2,t} - C_{H_2,t-1})$$

where

$V_{H_2,t}$: volume of hydrogen produced in the interval between t and $t-1$;
$C_{H_2,t}$, $C_{H_2,t-1}$: hydrogen concentrations measured at times t and $t-1$;
$V_{BG,t}$: volume of biogas produced between time t and $t-1$;
V_{HS}: volume of the headspace of reactors.

Cumulative hydrogen production (V_{H_2cum}) was calculated as sum of hydrogen productions between each measurement ($V_{H2,t}$) during dark fermentation batch tests, according to the following equation:

$$V_{H_2cum} = \sum_{t=1}^{n} V_{H_2,t}$$

where

V_{H_2cum}: cumulative hydrogen production at the end of the dark fermentation test;
$V_{H_2,t}$: hydrogen production between times t and $t-1$.

Hydrogen yields, expressed as NmL H_2/g VS and mol H_2/mol glucose, were calculated according to the following equations:

$$\text{Hydrogen yield}(\text{NmL } H_2/\text{g}) = \frac{V_{H_2cum}}{W_{sub}}$$

where

V_{H_2cum}: cumulative hydrogen production at the end of the dark fermentation test;
W_{sub}: weight of added VS.

$$\text{Hydrogen yield}(\text{mol } H_2/\text{mol glucose}) = \frac{\frac{V_{H_2cum}}{22.414\ \text{L/mol}}}{\frac{W_{glucose}}{180\ \text{g/mol}}}$$

where

V_{H_2cum}: cumulative hydrogen production at the end of the dark fermentation test;
22.414 L/mol: volume occupied by 1 mole of ideal gas at 1 atm pressure and 0 °C;
$W_{glucose}$: weight of glucose equivalent added at the beginning of the batch test;
180 g/mol: weight of 1 mole of glucose equivalent.

The volumetric productivity (Q) was based on NmL H_2/g VS per litre of culture medium per day (NmL H_2/L/d) and the maximum volumetric productivity (Q_{max}) was compared as the highest volumetric productivity displayed by the strains.

3. Results and discussion

3.1. Screening for extracellular enzymatic activities

One hundred and twenty microbial strains were previously isolated and identified from samples of heat-treated granular sludge used to perform hydrogen production batch tests [28]. The heat-treatment (100 °C for increasing residence times of 0.5, 1, 2 and 4 h) strongly affected the microbial viability in the sludge and the heat-treated sludges produced high and variable hydrogen yields from glucose. The microbial consortia surviving after 2 and 4 h boiling times had the most promise [28]. All isolates were screened for the production of industrially relevant extracellular enzymes and exhibited a broad range of hydrolytic activities (Table 2).

Fifty-seven strains were found proteolytic with a great majority of positive isolates belonging to *Bacillus* genus. A high number of pectinolytic strains has been also detected: the fact that only four out of 34 strains confirmed their potential once grown in the presence of both glucose and polygalacturonic acid (PecA + glucose) clearly indicates that, in the screened microbial collection, the production of pectinolytic enzymes is mainly not constitutive. This finding is in accordance with the related literature on microbial pectinases [39]. Twenty-seven microbes gave positive results for starch-degrading activities. As reported in Table 2, three strains produced active xylanases meanwhile only a *B. licheniformis* isolate was found to be cellulolytic. No lipolytic microbes were recovered.

The majority of the catalytic activities were found to be protease, amylase and pectinase. This outcome could be explained considering that the strains have been isolated from an anaerobic digester of a brewery whose fed by-products are usually rich in starch, pectin and protein [40].

Overall, the isolates belonging to *Bacillus* sp. genus displayed the highest number of hydrolytic activities. They are attractive species for the industry as they are rarely pathogenic, grow fast and secrete high amounts of proteins. These properties make bacilli very useful in industrial applications where they contribute up to 50% of the enzyme market [41].

3.2. Hydrogen potential from glucose by selected microbial strains

The presence of different extracellular enzymatic activities in many screened isolates was considered promising towards the definition of a proper inoculum for the conversion of complex organic waste into hydrogen. In literature, indeed, *Bacillus* species are known as strong candidates for biological H_2 production because (i) they can survive under harsh conditions, hence could compete with other microbes, (ii) they have large and versatile enzymatic activities, therefore a diverse range of bio-waste could be used as substrate for bio-hydrogen production, (iii) they do not require light for H_2 production, (iv) *Bacillus* sp. spores are being used as

Table 2
Extracellular enzymatic activity of 120 microbial strains isolated from samples of heat-treated granular sludge (CelA: cellulolytic activity; LipA: lipolytic activity; PecA: pectinolytic activity; PecA + glucose: pectinolytic activity screened in the medium supplemented also with glucose; PrA: proteolytic activity; StA: starch-degrading activity; XylA: xylan-degrading activity).

Strains	No of strains	Number of positive strains						
		CelA	LipA	PecA	PecA + glucose	PrA	StA	XylA
Bacillus sp.	31	–	–	8	–	16	6	1
Bacillus badius	20	–	–	7	–	11	5	–
Bacillus berjingensis	6	–	–	3	–	2	–	–
Bacillus farraginis	8	–	–	–	–	–	–	–
Bacillus flexus	1	–	–	–	–	–	1	–
Bacillus licheniformis	3	1	–	2	1	1	3	1
Bacillus megaterium	3	–	–	3	–	3	3	–
Bacillus subtilis	3	–	–	3	–	1	3	–
Bacillus tequilensis	4	–	–	2	3	1	4	1
Brevibacillus sp.	3	–	–	–	–	–	–	–
Brevibacillus agri	3	–	–	–	–	1	–	–
Brevibacillus brevis	2	–	–	–	–	1	–	–
Brevibacillus parabrevis	1	–	–	–	–	–	–	–
Enterobacter sp.	2	–	–	–	–	–	–	–
Enterobacter cloacae	1	–	–	–	–	–	–	–
Lysinibacillus sp.	16	–	–	5	–	5	3	–
Paenibacillus sp.	6	–	–	2	–	–	–	–
Paenibacillus cookii	3	–	–	–	–	–	1	–
Sporosarcina sp.	4	–	–	–	–	–	–	1
Total no of strains	120							
Total no of positive strains		1	–	35	4	42	29	4

probiotics in humans and animals; thus, they may not pose environmental health concerns [41,42].

Twenty strains belonging to *Bacillus* sp. and *Brevibacillus* sp. were selected for their hydrolytic activities and evaluated for H_2 potential. Firstly, the microbes were screened in NB supplemented with 5 g/L glucose and compared in terms of hydrogen yield and glucose consumption after 48 h of incubation. The microbes produced H_2 with variable yields (0.16–1.53 mol of H_2 per mol of consumed glucose) which were in agreement with the yield range so far reported in literature by *Bacillus* sp. under dark fermentative conditions (0.20–2.04 mol/mol glucose used) [42]. The most proficient microbes are reported in Table 3 together with other H_2-performances recently described for *Bacillus* sp. grown on the same amount of glucose.

Interestingly, the glucose-to-H_2 conversion efficiencies of the newly isolated bacteria were comparable to those of the literature and the highest yields were exhibited by two *Bacillus* sp. strains (namely F2.5 and F2.8) with 1.53 and 1.47 mol of H_2 per mol of used glucose, respectively. The majority of the microbes investigated in this study completely utilize the glucose available in the system meanwhile other *Bacillus* sp. strains, although exhibiting high H_2 yields, did not convert all the substrate [45]. This finding is of great interest since a microbial strain should have both high substrate utilization and H_2 yield for being implemented in the industrial bio-hydrogen technology.

As reported in Table 3, the strains selected in this study showed one to three hydrolytic capabilities whereas only few *Bacillus* sp. microbes with high H_2 potential were described in literature also for enzymatic activities. The most efficient strains, *Bacillus* sp. F2.5 and F2.8, were selected for further studies. Their amylolytic enzymes could be very useful for the H_2-conversion of food waste, where starch can account up to 30% of the TS [20,47,48].

3.3. Characterization of amylolytic enzymes secreted by Bacillus sp. F2.5 and F2.8

To study the starch-degrading activity of *Bacillus* sp. F2.5 and F2.8, the strains were grown both in NB and SPM supplemented with 20 g/L soluble starch. The highest enzymatic activities were detected in SPM broth after 72 h of incubation at 37 °C (data not

Table 3
Comparison of hydrogen production potential of *Bacillus* sp. and *Brevibacillus* sp. strains from glucose (5 g/L) as carbon source.

Strain	Enzymatic profile	H_2 yield (mol/mol glucose)	Residual glucose (%)	Reference
Bacillus sp. F2.5	StA	1.53	nd	This study
Bacillus sp. F2.7	PrA, StA	0.88	2.9	This study
Bacillus sp. F2.8	PrA, StA	1.47	nd	This study
B. farraginis F4.10	PrA, StA	0.31	nd	This study
B. megaterium F1.22	PectA, PrA, StA	0.57	nd	This study
B. tequilensis F2.16	PectA, StA, XylA	0.36	2.5	This study
Brevibacillus sp. F4.12	PectA, PrA	0.75	nd	This study
Brevibacillus sp. F4.16	PrA	0.69	nd	This study
Bacillus sp. EGU444	PrA	0.35	na	[43]
B. thuringiensis EGU378	LipA, StA	0.26	na	[43]
B. megaterium ATCC15374	StA	0.60	1.0	[44]
B. thuringiensis EGU45	nd	1.67	24.0	[45]
B. cereus EGU44	nd	1.92	23.2	[45]
B. cereus EGU43	PrA	1.12	21.6	[45]
B. cereus EGU3	nd	0.96	22.4	[45]
Bacillus sp. FS2011	nd	2.04	0.5	[46]

na: not available; nd: not detectable.

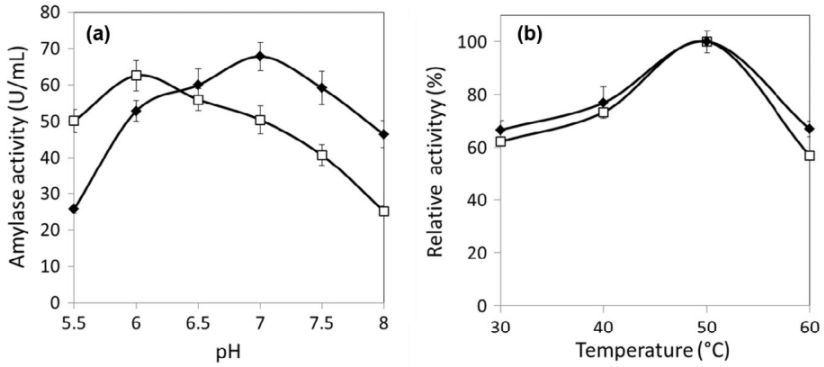


Fig. 1. The effect of pH (a) and incubation temperature (b) on the amylase activity of *Bacillus* sp. F2.5 (□) and *Bacillus* sp. F2.8 (◆) grown for 72 h in SPM containing 20 g/L soluble starch.

shown), thus this medium was selected to deeply investigate their amylolytic abilities. The activity of both microbes after 72 h incubation in SPM was firstly assessed at 50 °C using different pH values (Fig. 1a). The two strains displayed comparable amylase activities: *Bacillus* sp. F2.8 showed the most promise with the highest enzymatic activities (67.8 U/mL) detected at pH 7.0 meanwhile the uppermost catalytic ability of *Bacillus* sp. F2.5 was found at pH 6.0 (62.5 U/mL). pH greatly influenced the enzymes of both strains: the total amylase activity of *Bacillus* sp. F2.5 at higher pH progressively dropped to 25.1 U/mL at pH 8.0, which stand for almost 40% of the highest value. The amylase activity of *Bacillus* sp. F2.8 was high in the pH range of 6.0–8.0.

These findings are in accordance with those described in literature regarding *Bacillus* sp. amylases, where the optimal pH values were reported to be within the broad range of 3.5–12 and the pH was found to deeply affect their catalytic activity on starch [49–51].

The amylolytic enzymes were assayed at temperatures from 30 to 60 °C at the optimal pH for each strain, namely pH 6.0 and 7.0 for *Bacillus* sp. F2.5 and F2.8, respectively. Enzyme activity increased with temperature up to 50 °C, which was found to be the optimum for the two microbes (Fig. 1b).

At 60 °C, the enzymatic values were lower, 57% and 67% of the highest activity detected at 50 °C for *Bacillus* sp. F2.5 and F2.8, respectively. Both microbes had high relative activity at 30 and 40 °C (on average 64% and 74%, respectively) and their optimal temperature values were inferior than those usually reported for other *Bacillus* sp. amylases (60–70 °C) [50,52–54]. Overall, *Bacillus* sp. F2.5 and F2.8 produced amylase with high potential with enzymatic activities comparable to those recently reported by efficient amylolytic *Bacillus* sp. strains [42,50]. Moreover, the high enzymatic activities registered at thermal levels near to those optimal for growth (37 °C) could be beneficial for the saccharification of starchy substrates into glucose during the starch-to-H_2 fermentation.

3.4. Hydrogen production from glucose and soluble starch by Bacillus sp. F2.5 and F2.8

Considering that OFMSW is usually quite rich in starch [20,47], with the final aim of assessing their ability to convert OFMSW into H_2, *Bacillus* sp. F2.5 and F2.8 were firstly evaluated for their H_2 potential from soluble starch (20 g/L) at pH 6.0 and 7.0, selected as the optimal values for the amylase secreted by each strain (Fig. 1a). The microbes were also cultivated in the presence of the equivalent amount of glucose (22 g/L), as reference medium.

No methane was detected throughout the experiments whereas the strains were able to produce H_2 from glucose and soluble starch (Fig. 2a and b).

The two microbes completely utilized glucose within five days yielding high levels of hydrogen. *Bacillus* sp. F2.5 obtained the uppermost H_2 concentrations both at pH 7.0 and 6.0, with 114 and 101 mL of H_2, respectively, whereas *Bacillus* sp. F2.8 produced lower volumes: 101 and 85 mL at pH 7.0 and 6.0, respectively. As a result, the top fermenting abilities were achieved at pH 7.0, with the H_2 yield of 0.91 and 0.81 mol per mol of consumed sugar for *Bacillus* sp. F2.5 and F2.8, respectively. Lowering the pH resulted in a reduced efficiency, mostly for *Bacillus* sp. F2.8 whose yield was 0.69 mol per mol of consumed sugar meanwhile the other strain produced 0.82 mol of H_2 per mol of used glucose. *Bacillus* sp. F2.8 displayed the most efficient fermenting profile with the highest H_2 productivity attained at pH 7.0 (26.8 mL of H_2 per day), which was 1.12-fold that of *Bacillus* sp. F2.5 (24.0 mL of H_2 per day). Relative H_2 concentration was found to be similar (about 45%) for the two strains (Table 4).

In the presence of soluble starch, *Bacillus* sp. F2.5 and F2.8 produced high H_2 levels, too (Fig. 2), consuming all the available polysaccharide. At pH 7.0, *Bacillus* sp. F2.8 confirmed the most efficient hydrolyzing ability, obtaining the highest amount of H_2 (51.8 per gram of consumed starch) in a shorter timeframe (Fig. 2b). Similar performances but with lower productivity were detected for *Bacillus* sp. F2.5 (Fig. 2a): in the first days, higher amounts of hydrogen were produced at pH 6.0 while, at the end of incubation, pH 7.0 supported slightly better the H_2 potential of *Bacillus* sp. F2.5. This finding could be explained considering that, for this strain, pH 6.0 and 7.0 were found to be optimal for amylases and H_2 yield, respectively.

The relative concentration of H_2 was similar for the two microbes (Table 4): 44% and 45% for *Bacillus* sp. F2.8 and F2.5, respectively, and the highest H_2 efficiencies were found at pH 7.0: 0.42 and 0.41 mol of H_2 per mol of consumed starch for *Bacillus* sp. F2.8 and F2.5, respectively.

Their yields from soluble starch were 51% (0.81/0.42) and 44% (0.91/0.41), respectively, of those above presented in the same broth from glucose. Interestingly, although the two strains had similar starch-to-H_2 efficiency, *Bacillus* sp. F2.8 showed H_2 potential from glucose lower than *Bacillus* sp. F2.5 (Tables 3 and 4, Fig. 2). This could be associated with the most efficient starch-degrading activity described for *Bacillus* sp. F2.8 at pH 7.0 (Fig. 1a). Nevertheless, both strains exhibited promising H_2 yields which were found to be comparable with those described in literature mainly by mixed consortia [55,56]. The highest H_2 yield from starch reported so far by a strain belonging to the *Bacillus* genus

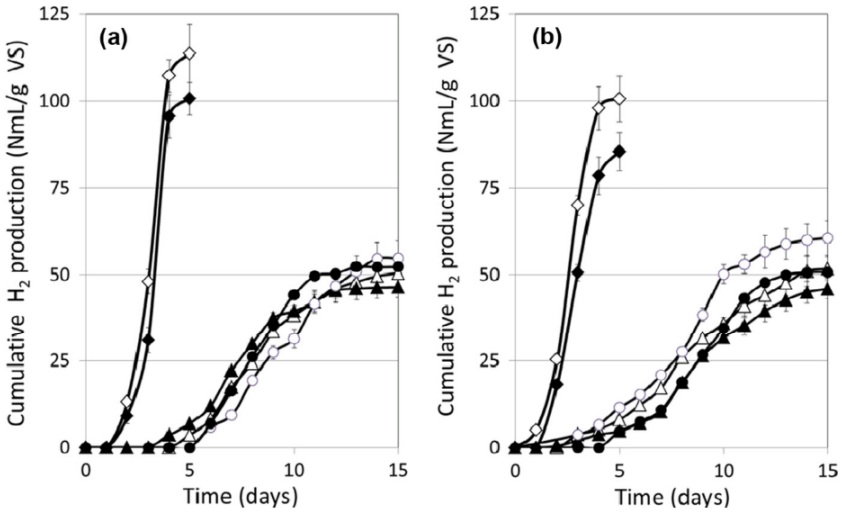


Fig. 2. Cumulative hydrogen productions of *Bacillus* sp. F2.5 (a) and *Bacillus* sp. F2.8 (b) grown in SPM supplemented with 22 g/L of glucose (◆), 20 g/L soluble starch (▲) or 10 g VS/L of OFMSW (●). Filled and empty symbols report values obtained at pH 6.0 and 7.0, respectively. Data shown are the mean values of three replicates and standard deviations are included.

Table 4
VFAs profiles (mg/L and % TVFA, Total Volatile Fatty Acid), maximum volumetric H_2 productivity (Q_{max}), (NmL/L/d), and relative H_2 concentration (%) of the biogas produced on different substrates. Data shown are the mean values of three replicates and standard deviations are included.

Substrate	Strains	pH	H_2 %	Q_{max}	TVFA	Acetate		Propionate		Butyrate	
				mL/L/d	mg/L	mg/L	%	mg/L	%	mg/L	%
Glucose	F2.5	6	45	23.9	1774	973 ± 85	55	247 ± 48	14	554 ± 60	31
		7	45	26.8	2115	1134 ± 99	54	367 ± 26	17	613 ± 62	29
	F2.8	6	45	19.6	1548	873 ± 68	56	182 ± 18	12	493 ± 48	32
		7	45	24.0	1861	1087 ± 99	58	205 ± 16	11	569 ± 55	31
Starch	F2.5	6	45	3.7	896	490 ± 69	55	123 ± 25	14	282 ± 30	31
		7	45	3.9	1058	568 ± 99	54	183 ± 40	17	307 ± 51	29
	F2.8	6	45	3.8	774	437 ± 45	57	91 ± 19	12	247 ± 45	31
		7	44	4.1	1106	637 ± 29	58	123 ± 27	11	345 ± 43	31
OFMSW	F2.5	6	38	4.2	1117	625 ± 71	56	158 ± 20	14	334 ± 39	30
		7	38	3.9	1131	601 ± 55	53	199 ± 17	18	331 ± 28	29
	F2.8	6	39	4.0	945	527 ± 49	56	122 ± 15	13	296 ± 30	31
		7	39	5.0	1277	737 ± 58	58	144 ± 13	11	396 ± 35	31

was recently disclosed as 0.70 mol H_2 per mol of reducing sugar [57]. On the other hand, as reported in Table 4, both *Bacillus* sp. strains described in the present work showed productivity (about 4 mL of H_2 per day) lower than those found in other studies on H_2 production from starch. However, their limited H_2 production rate, which could be mainly influenced by their low inoculum size and static incubation, are likely to be improved by optimizing the growth conditions and other environmental factors such as micronutrients availability, buffers and temperature which were reported as key parameters to boost H_2 productivity [55,56,58].

3.5. Hydrogen potential from OFMSW

The fractions analysis of the OFMSW obtained from manual sorting procedure (Table 1) revealed a composition similar to those of other OFMSW recently described in literature [5,20]. Fruit, vegetable and bread–pasta–rice were the most abundant shares on wet weight basis, meanwhile, as reported in Materials and Methods (Section 2.1), starch, protein and lipids were found to be the main components of TS, with 19%, 18% and 17% of TS, respectively.

From OFMSW, no methane was detected whereas H_2 production was found to be feasible with both strains: H_2 concentrations were slightly higher for *Bacillus* sp. F2.8 (Fig. 2b), which produced almost 61 mL of H_2 per g VS, at pH 7.0. At pH 6.0, the strain achieved mildly lower H_2 levels and productivity. On the other hand, *Bacillus* sp. F2.5 exhibited fermenting abilities comparable for both tested pH values and H_2 production was found 55 and 53 mL per g VS for pH 6.0 and 7.0, respectively (Fig. 2a).

Bacillus sp. F2.8 confirmed the most efficient productivity already described from soluble starch. At pH 7.0, the strain produced 5.0 mL of H_2 per day whereas 4.0 mL of H_2 were daily produced at lower pH (Table 4). *Bacillus* sp. F2.5 had similar H_2 productivity at pH 6.0 (4.3 mL of biogas and H_2) while, at pH 7.0, its productivity was lower resulting in 3.9 mL of H_2 (Table 4). Both strains produced comparable relative H_2 concentrations (nearly 38%) which were inferior than those above reported from soluble starch and glucose (Table 4).

Hydrogen levels produced in this study were consistent with those previously described for batch H_2 fermentation of OFMSW or food waste by using pure or mixed cultures (Table 5). Further, in the present study, inoculum pre-treatment was not required. Moreover, as described in Table 5, this is one of the earliest accounts on a single microbe capable of converting organic waste into H_2 with a high rate and yield. Only recently, Marone and colleagues described few Enterobacteriaceae strains, isolated by the bioaugmentation of vegetable waste (*Rahnella* sp. 10, *Buttiauxella*

Table 5
Comparison of hydrogen production from OFMSW achieved in this study and other performances previously reported from OFMSW and food waste.

Feedstock	Inoculum	Pre-treatment inoculum	Pre-treatment feedstock	Temperature (°C)	Yield (mL H_2/g VS)	Reference
OFMSW	*Bacillus* sp. F2.5	NO	Sterilized	35	61	This study
OFMSW	*Bacillus* sp. F2.8	NO	Sterilized	35	55	This study
OFMSW	Pre-adapted H_2-producing bacteria	NO	NO	37	180	[21]
OFMSW	Pre-treated digested sludge	100 °C 15 min	NO	37	140	[21]
OFMSW	NO	NO	NO	35	42	[5]
OFMSW	Granular sludge	100 °C 4 h	NO	35	70	[5]
OFMSW	Granular sludge	100 °C 4 h	Sterilized	35	57	[5]
OFMSW	Granular sludge	100 °C 4 h	NO	35	25–85	[20]
Food waste	Anaerobic sludge	na	NO	35	39	[59]
Food waste	Anaerobic sludge	na	NO	50	57	[59]
Food waste	Grass compost	180 °C 3 h	NO	35	77	[60]
Food waste	NO	NO	NO	35	4	[61]
Food waste	Food waste	90 °C 20 min	60–90 °C 20 min	35	26–149	[61]
Vegetal waste	Vegetal waste	NO	NO	28	22	[24]
Vegetal waste and potato peels	Vegetal waste and potato peels	NO	NO	28	18	[24]
Vegetal waste	*Rahnella* sp. 10	NO	NO	28	47	[24]
Vegetal waste	*Buttiauxella* sp. 4	NO	NO	28	71	[24]
Vegetal waste	*Raoultella* sp. 47	NO	NO	28	70	[24]

na: not available.

sp. 4 and *Raoultella* sp. 47), for their promise in producing H_2 from vegetable kitchen waste collected from a cafeteria [23]. However, this is the first successful application of pure microbial cultures in bio-hydrogen production from OFMSW. Furthermore, both *Bacillus* sp. strains exhibited high starch-degrading activities meanwhile the above reported microbes did not produce any relevant hydrolytic enzymes [23].

Their spore-forming ability and their being isolated from granular sludge of a full scale UASB anaerobic digester are two additional noteworthy benefits which count for the potential development of *Bacillus* sp. F2.5 and F2.8 as efficient and robust inoculants.

3.6. VFAs profiles from glucose, soluble starch and OFMSW fermentations

H_2 production is coupled with production of VFAs and/or solvents. The composition of VFAs generated is a useful indicator for monitoring the H_2 production pathways. The high VFAs concentrations achieved in this study indicate that favourable conditions for the growth and the activity of both strains were established during the course of the experiments (Table 4). The detected soluble metabolites were acetate, butyrate and propionate. In all batch experiments the acetate was the major component (53–58%) with butyrate as the second most abundant acid (29–32%). This finding proved that similar metabolic pathways were involved and the acetate-butyrate was the predominant fermentation mode, which was reviewed as favouring H_2 production [1,14]. As a result, supplementing different substrates significantly changed only the VFAs quantity rather than their shares: the highest amount of Total VFA (TVFA) was obtained from glucose meanwhile starch and OFMSW supported similar TFVA values. The higher the level of VFA accumulation (Table 4), the higher H_2 production was achieved (Fig. 2a and b)

4. Conclusions

This study demonstrated for the first time the effective conversion of OFMSW into H_2 by using pure cultures of *Bacillus* sp. strains properly selected for both their proficient enzymatic activities and their high fermenting abilities from glucose and starch. Future studies will further increase their H_2 performances and techno-economical evaluations will determine the actual feasibility of the whole process. Taken together, the results of this work gave advances in knowledge towards the development of microbial inoculants for the industrial processing of organic waste in H_2.

Acknowledgements

This work was financed by the Progetto di Ateneo 2010 N. CPDA102570 entitled "Selection and characterization of microbial inoculants for the optimization of biological hydrogen production from organic wastes" (University of Padova). Ali Shah Tawaf was recipient of a scholarship by EMMA-WEST 2013 2nd Cohort Project. Lorenzo Cagnin is recipient of a PhD bursary from the University of Padova.

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Cite this: *RSC Adv.*, 2016, **6**, 47981

A novel FTIR-based approach to evaluate the interactions between lignocellulosic inhibitory compounds and their effect on yeast metabolism

Lorenzo Favaro,[a] Laura Corte,*[b] Luca Roscini,[b] Lorenzo Cagnin,[a] Matteo Tiecco,[c] Claudia Colabella,[b] Antonio Berti,[a] Marina Basaglia,[a] Gianluigi Cardinali[bc] and Sergio Casella[a]

Inhibitors commonly found in lignocellulosic hydrolysates impair yeast metabolism and growth, reducing the productivity of the overall bioethanol production process. FTIR spectroscopy was used to analyze the metabolomic alterations induced by acetic and formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (HMF) on yeast metabolism, using three *Saccharomyces cerevisiae* strains with different sensitivities. IR spectrum alterations were summarized with synthetic descriptors to rapidly visualize the kinds of molecules displaying the more intense reactions and to evaluate the type of interaction between inhibitors in a mixture, at concentrations close to those found at the industrial scale. The four inhibitors induced different levels of mortality and metabolomic changes. The metabolomic response was proportional to the different strain resistance level, further supporting their original classification. Inhibitor mixtures severely hindered the cell viability with the exception of the lowest concentration tested, which was partially biocidal. Furthermore, for the first time, this study revealed antagonistic interactions exerted by inhibitor mixtures on microbial metabolism, closely strain- and dose-dependent. This confirms that yeast strain resistance to single inhibitors cannot be used to predict behaviour on exposure to mixtures. This finding is worth further studies to explain the underlying antagonistic mechanism and to support the selection of highly tolerant strains.

Received 6th April 2016
Accepted 9th May 2016

DOI: 10.1039/c6ra08859g

www.rsc.org/advances

Introduction

The depletion of fossil fuels together with increased environmental awareness has resulted in a strong drive towards developing eco-friendly biofuel technologies. Bioethanol is considered one of the most promising routes.[1,2] The ideal raw substrate for bioethanol is represented by non-edible lignocellulosic biomass, such as energy crops, spruce or birch, as well as agricultural by-products.[3–8] Because lignocellulose is highly refractory to degradation, pre-treatments are needed to make the cellulose more accessible to subsequent enzymatic saccharification.[1,9] However, pre-treatments also result in the co-production of inhibitory compounds from hemicellulose (mainly furfural and acetic acid), lignin (phenolic compounds) and cellulose (5-hydroxymethyl-2-furaldehyde, HMF). The amount and nature of degradation products is directly related to the pre-treatment method and conditions. Nevertheless, the most common and abundant inhibitors are furans, like HMF and furfural, and weak acids, such as acetic and formic acid.[10–12]

Inhibitors cause multiple negative effects on yeast cells by (i) suppressing the biosynthesis of macromolecules, (ii) denaturing the cytoplasmic proteins, (iii) reducing the activity of glycolytic enzymes, disturbing the processes of ion and metabolite transport across the plasma membrane and (iv) altering the lipid composition of the membranes,[13] thus reducing the productivity of the overall process.[10,13]

A variety of detoxification strategies have been developed to lower the inhibitor concentration from pre-treated lignocellulose. Nevertheless, these methods are far from being techno-economically feasible.[14] Several alternatives to detoxification were proposed, such as the selection or the development of less recalcitrant feedstock, the application of mild pre-treatment settings[5,15] and the development of yeast strains with high inhibitors tolerance.

Advanced improvements in the optimisation of yeast robustness may require novel metabolic engineering tools, such as protein engineering and rational metabolic engineering, already elegantly described.[16] However, strains exhibiting multiple tolerance to high temperature and inhibitors levels have not been developed yet. Furthermore, the majority of the

[a]*Department of Agronomy Food Natural Resources Animals and Environment (DAFNAE), University of Padova, Agripolis, Italy*

[b]*Department of Pharmaceutical Sciences-Microbiology, University of Perugia, Borgo XX Giugno, 74, I-06121 Perugia, Italy. E-mail: laura.corte@unipg.it; Fax: +39 075 585 6470; Tel: +39 075 585 6476*

[c]*CEMIN, Centre of Excellence on Nanostructured Innovative Materials, Department of Chemistry, Biology and Biotechnology, University of Perugia, Italy*

engineered and/or evolved strains is obtained so far in haploid laboratory yeast, which generally display suboptimal fermentation performances and poor robustness, making them unsuitable for use in industrial applications.[17]

Screening or selection surveys for wild type tolerant *Saccharomyces cerevisiae* strains were mainly focused on single stress condition, such as high temperature,[18,19] weak acids or furans and phenolics.[13] The search for multiple tolerant yeast has received much less attention.[6,20,21] The resistance to weak acids, furans and phenolics in *S. cerevisiae* is strain-specific and highly dependent on tested concentrations.[13,22] This requires laborious and time-consuming screening procedures.[23] Moreover, these costly researches often focused on single and different physiological parameters (mainly growth, ethanol or biomass yield in the presence of inhibitors) to estimate the ability of the strain to withstand inhibitors, making difficult the comparison between the studies. Therefore, a complete dataset of the responses given by yeast strains exposed to inhibitory compounds is needed for the selection process.

Checkerboard-based methods, normally employed in pharmacology and toxicology to assess the combined effects of two drugs, cannot be employed for this purpose because they (i) do not distinguish the difference between mortality and inhibition, (ii) properly show synergic or additive effects only at lower concentrations than those typically present in lignocellulosic ethanol plants, (iii) give only a synthetic result without any hint of the metabolomic compartments involved.

Over the last few years, Fourier transform infrared (FTIR) spectroscopy has become a powerful high-throughput technique in biophysical and biochemical research, for its sensitivity in the detection of metabolomic changes of cells and tissues.[24] Furthermore, FTIR has been successfully applied for the development of quick bioassays to evaluate the stress-induced cell status in response to different chemicals or to various environmental signals.[25–27]

In the current study, we propose a FTIR-based approach to characterize the metabolomic alterations induced by inhibitory compounds on *S. cerevisiae* metabolism and to evaluate inhibitors interactions at concentrations close to those found in the industrial bioethanol production. Four well-known inhibitors, alone and in quaternary mixtures, were employed to test the possibility offered by this method: acetic acid, formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (HMF). The analysis was carried out using three *S. cerevisiae* strains, chosen among 160 previously screened, as representative for the uppermost, medium and low inhibitors tolerance.

Experimental

Cultures and growth conditions

The *S. cerevisiae* Fm17 and Fp84 were isolated and characterized in terms of inhibitor tolerance together with the type strain *S. cerevisiae* DSM70449, as benchmark.[6,28] Each strain was inoculated at Optical Density (OD_{600}) = 0.5 in 500 mL bottles containing 50 mL YPD medium (yeast extract 1%, peptone 1% and dextrose 2% Difco Laboratories, USA) and grown for 18 h at 25 °C, with 150 rpm shaking.

Stressing agents

Acetic acid, formic acid, furfural and HMF were all obtained from Sigma and used at increasing concentrations in distilled sterile water (Table 1). Each tested concentration was reported as relative concentration (RC) of the third assessed level considered as the highest concentration of the studied inhibitors in lignocellulosic hydrolysates. Inhibitors were formulated also into four mixtures (RC_{25}, RC_{50}, RC_{100}, RC_{200}) obtained adding increasing doses of every toxic compound (Table 1).

FTIR and UV-vis spectrophotometers

The FTIR experiments were carried out with a TENSOR 27 FTIR spectrometer, equipped with HTS-XT accessory for rapid automation of the analysis (Bruker Optics GmbH, Ettlingen, Germany). Cell densities were measured with a Jasco V-530 Spectrophotometer (http://www.jascoinc.com).

FTIR analysis and spectra preprocessing

Cells suspensions, prepared as detailed in "Cultures and growth conditions" section, were centrifuged (3 min at 5300 × *g*), washed twice with distilled sterile water and re-suspended in polypropylene tubes with an appropriate amount of distilled water (standardized OD_{600} = 12). Inhibitors were added to the test tubes in order to obtain the relative concentrations reported in Table 1. The control (0% relative inhibitor concentration) was obtained by re-suspending the cells directly in distilled sterile water. All tests were carried out in triplicate. Tubes were incubated 1 h at 25 °C in a shaking incubator set at 50 rpm. After the incubation, 1.5 mL suspension was taken from each sample, centrifuged (5 min at 5300 × *g*) washed twice with distilled sterile water and re-suspended in 1.5 mL HPLC (High Performance Liquid Chromatography) grade water. 105 μL suspension was sampled for three independent FTIR readings (35 μL each, according to the technique suggested by Essendoubi and colleagues).[29] FTIR measurements were performed in transmission mode. All spectra were recorded in the range between 4000 and 400 cm^{-1}. Spectral resolution was set at 4 cm^{-1}, sampling 256 scans per sample in order to adequately study band intensities and shifts. The software OPUS version 6.5 (BRUKER Optics GmbH, Ettlingen, Germany) was used to carry

Table 1 Inhibitors concentrations used in this study as single compounds or as quaternary mixtures

	Concentration (mM)			
Inhibitor	RC_{25}	RC_{50}	RC_{100}	RC_{200}
Acetic acid[a]	30	60	120	240
Formic acid[a]	13	27	53	106
Furfural[b]	7	14	28	56
HMF[b]	7	15	30	59

[a] pH values of each solution of single acid ranged between 2.4 and 2.8. [b] HMF or furfural formulations have pH values of 6.5. pH values of inhibitors mixtures RC_{25}, RC_{50}, RC_{100}, were 2.6, 2.5, 2.4, 2.2, respectively.

out the quality test, baseline correction and vector normalization.

Spectra analyses

The script MSA (Metabolomic Spectral Analysis) employed for stress analysis was developed in "R" language to carry out the following operations on the matrices of spectral data exported as ASCII text from OPUS 6.5. The analytical procedure could be outlined as follows:

(1) Each single spectrum was normalized in order to have the range spanning from 0 to 1 in a way already suggested in ref. 24. Average spectra from the three repetitions were calculated.

(2) Response spectra (RS) were calculated as difference between each average spectrum and the average spectrum of the same cells maintained in water (defined as control RS). Response spectra of each agent were found to be positive and plotted with the exclusion of the control RS, which is by definition a straight line with RS = 0.

(3) Synthetic stress indexes (SI) were calculated as Euclidean distances of the RS under stress and the control RS. SI of the whole spectrum and of the five different spectral regions individuated by Kümmerle and colleagues[30] were calculated. The five regions were defined as follows: fatty acids (W1) from 3000 to 2800 cm^{-1}, amides (W2) from 1800 to 1500 cm^{-1}, mixed region (W3) from 1500 to 1200 cm^{-1}, carbohydrates (W4) from 1200 to 900 cm^{-1} and typing region (W5) from 900 to 700 cm^{-1}. The typing region was not considered in the analysis because its response did not correlate with the specific stressing conditions tested.

Biocidal activity test

The biocidal activity tests were carried out in parallel with the FTIR-based stress bioassay to compare the metabolomic damages with the loss of viability. 100 μL of each cells suspension prepared for the FTIR analysis were serial diluted to determine the viable cell counting, in triplicate, on YPDA + chloramphenicol (0.5 g L^{-1}) plates. The biocidal effect of the tested compounds was highlighted as cell mortality induced at different concentrations. The cell mortality (M) was calculated as $M = (1 - C_v/C_t) \times 100$, where C_v is the number of viable cells in the tested sample and C_t the number of viable cells in the control suspension.

Measure of the effects of inhibitors mixtures on yeast viability and metabolism

The mortality values induced by the inhibitors mixtures (observed mortality, OM) were compared with that expected at the same concentration (expected mortality, EM).

Since OM values were distributed in a hyperbolic dose–effect curve in all the tested conditions, the fractional product method[31] has been used to estimate EM values, using the following equation:

$$EM_{RC\text{-}th} = 1[(1 - m_1)(1 - m_2)\ldots(1 - m_n)] \quad (1)$$

where $EM_{RC\text{-}th}$ is the EM of each inhibitors mixture (RC_{25}, RC_{50}, RC_{100}, RC_{200}) and m_i is the mortality of the i_{th} inhibitor.

Data obtained were subjected to one-way analysis of variance (ANOVA) and pair comparison was achieved by Tukey's procedure. Additive effect could be postulated with OM = EM, synergistic when OM > EM and antagonistic when OM < EM.

A similar approach was adopted to assess how the yeast metabolome reacts to inhibitors mixture. An Absolute Reduction Indicator (ARI) was calculated as difference between the sum of the metabolomic responses induced separately by each inhibitor and that of the mixture. Positive ARI values indicate antagonism, negative synergism and figures close to 0, additivity.

Study of inhibitors chemical interaction(s) and reaction(s)

The inhibitors potential reactions were studied in water solutions at concentrations of 240, 106, 56 and 59 mM for acetic acid, formic acid, furfural and HMF, respectively. All the combinations of the two acids with furfural and HMF were analyzed. The mixtures were prepared dissolving the inhibitors in water and then left at room temperature for 24 h under magnetic stirring. The samples were then diluted for adequate UV-vis readings (maximum absorbance < 1 a.u.). The spectra registered were superimposed and compared with the spectra of the pure compounds at the same concentrations. The experiments with HCl were performed dissolving furfural and HMF in HCl water solutions at different acid concentrations (pH = 6, 5, 4, 3, 2, 1). The spectral readings were performed in the same manner of the previous experiments and acquired with a Jasco V-530 Spectrophotometer (http://www.jascoinc.com).

Results and discussion

Metabolomic analysis

FTIR spectroscopy was used to characterize the ability of three different strains of *S. cerevisiae* (Fm17, Fp84 and DSM70449) to withstand increasing concentrations of inhibitors at their early stationary phase, as it is in lignocellulosic bioethanol processes, either during SSF (Simultaneous Saccharification and Fermentation) or CBP (Consolidated BioProcessing) systems.[1,32]

Cells were exposed for 1 hour to four dosages of formic acid, acetic acid, furfural and HMF, alone and in quaternary mixtures. The alterations induced by these chemicals on the IR spectrum were summarized with synthetic stress metrics (Stress Indexes, SIs), allowing to rapidly and simply visualize which spectral regions, and therefore what types of molecules, displayed the most intense responses in each specific stressing condition. SIs were obtained as normalized Euclidean distances between the response spectra of cells under stress and those of cells maintained in water, as previously described in Materials and methods section – Spectra analyses. These metrics have been calculated for the whole spectrum (GSI) and for each specific spectral areas involved in the stress response, namely: fatty acids (W1), amides (W2), mixed region (W3) and carbohydrates (W4).[24]

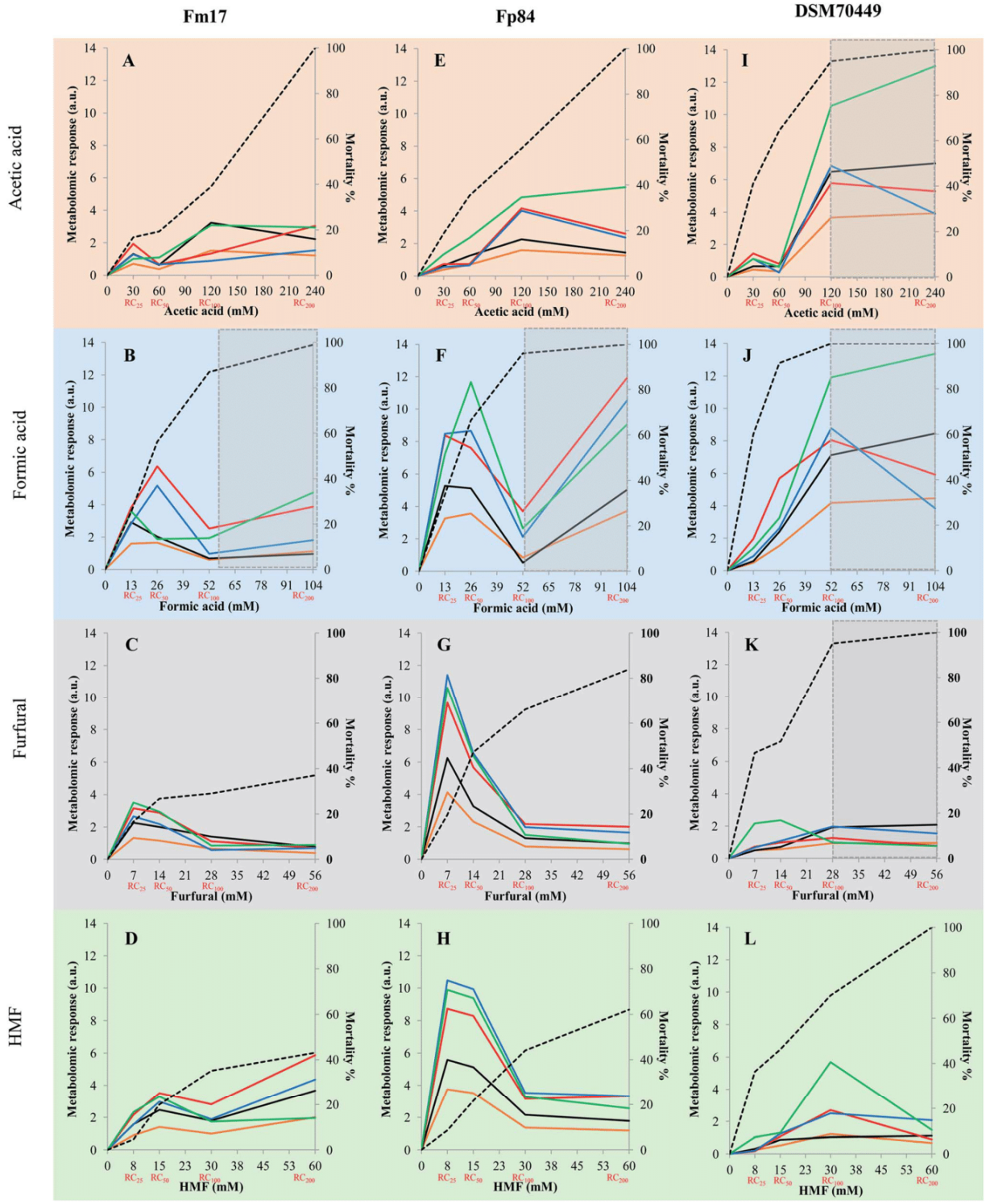

Fig. 1 Stress indexes of *S. cerevisiae* Fm17, Fp84 and DSM70449 cells subjected to increasing RCs of acetic acid, formic acid, furfural and HMF. Orange line represents the whole spectrum (GSI), black line W1 region, red line W2 region, blue line W3 region, green line W4 region, dashed line represents mortality. The degree of variability between replicas throughout the FTIR spectra ranged around 2.7×10^{-2}. Figures in red on the *x* axes represent relative concentrations in respect to the corresponding mmol concentration. *Post mortem* chemical reaction of cellular components is indicated by a grey box. a.u. stands for "arbitrary units".

Mortality and spectral alterations due to single inhibitors. Cellular stress induces very fast changes in terms of cell metabolites, all detectable through an accurate metabolomic analysis, such as FTIR, as early as in the first hours of exposure.[25] The four inhibitors considered in this study caused different levels of mortality and metabolomic alterations on the tested *S. cerevisiae* strains, as shown by the SIs evolution of Fig. 1. The metabolomic response was dissected in two components on the basis of the indication provided by the mortality data: the response of living cells that actively react to the stressing agent (*pre mortem* response) and that typical of dead cells, hereafter referred to as *post mortem*, resulting from an increase of membrane permeability after cell death or by an enzymatic activity consequent with the loss of cell compartmentalization, as already suggested by Corte and colleagues.[36]

Fm17 strain (Fig. 1A–D) displayed the least mortality and metabolomic response. Most of the metabolomic changes were due to 25% and 50% of each inhibitor Relative Concentration (RC), hereinafter named as RC_{25} and RC_{50} (see Materials and methods – Stressing agents). Major responses were in the amides (W2), mixed (W3) and carbohydrates (W4) regions. More specifically, acetic acid (Fig. 1A) induced less mortality with less metabolomic alterations than formic acid (Fig. 1B), which caused a strong reaction at RC_{25} and RC_{50}. The metabolomic response to formic acid at higher concentrations was clearly due to the *post mortem* chemical reaction of cellular components, as indicated by the grey box. Both furfural and HMF induced a maximum of about 40% mortality, but different metabolomic alterations. Namely, cells actively responded to furfural at RC_{25} while HMF prompted the maximum alterations at RC_{50} and RC_{200} (Fig. 1C and D).

Metabolomic responses displayed by Fp84 strain were similar to those of Fm17, but of greater intensity (Fig. 1E–H). Acetic acid exposure determined the least metabolomic changes (Fig. 1E) while the other inhibitors caused a strong and similar response at RC_{25} and RC_{50}, three-fold than that displayed by Fm17 strain (Fig. 1F–H). Formic acid showed the highest biocidal efficacy by inducing 100% mortality already at RC_{100} (Fig. 1F). These data indicated that cells actively coped with low RCs of formic acid, furfural and HMF trying to counter the effect exerted by inhibitors. Conversely, at higher concentrations, the inhibitors rapidly killed the cells hampering any reaction.

Metabolomic analysis confirmed DSM70449 as the most sensitive of the three tested strains (Fig. 1I–L). Cells challenged by weak acids showed high mortality and metabolomic response, with all SIs curves increased until RC_{100}, following the mortality trend. The response to formic acid was moderately stronger than to acetic acid. After death, over RC_{100}, cells displayed similar chemical intracellular reactions (Fig. 1I and J). On the contrary, this strain did not actively react at low RCs of furans, although these inhibitors induced over 40% mortality already at RC_{25} (Fig. 1K and L).

In general, mortality values over 50% were observed for all strains challenged by weak acids. Interestingly, in all these experimental conditions, the Global Stress Index (GSI), represented by the orange line, reached values around or higher than 1.0 a.u. (arbitrary units), confirming an unhealthy cell state as previously observed with other stressing compounds.[27,33,34] Weak acids have been reported to contribute to ATP depletion, toxic anion accumulation and inhibition of aromatic amino acids uptake.[13,14,22] All these effects could be justified by the stoppage of catabolism with the reduction and then the extinction of all ATP-depending metabolic activities, such as the export of ions from the cell.[35] The high mortality induced in our experiments by formic acid in all strains, and by acetic acid mainly in DSM70449, confirmed this hypothesis. Furthermore, the mortality and the low metabolomic responses of Fm17 and Fp84 strains challenged by acetic acid suggested a decrease of the metabolic activity that cannot contrast the toxic effect exerted by this inhibitor.

Furans induced a lower mortality than weak acids and triggered the metabolomic response only at low RCs (GSI ranging around 1.0 a.u.), with the exception of the sensitive strain, unable to actively react. These compounds have been described to inhibit glycolysis acting specifically on alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALD).[13] Altogether, these effects suggested that catabolism was stopped or slowed, with a possible involvement of the carbohydrates region, as supported in our data by the prominence of the W4 SI.

Moreover, the comparison between metabolomic and mortality data at low inhibitors concentrations (RC_{25} and RC_{50}), enabled to define three different types of response, corresponding to the different tolerance levels of the strains tested. The yeast Fm17 disclosed low mortality values and relatively low metabolomic responses, the typical behavior of a resistant strain (Fig. 1A–D). On the contrary, in DSM70449, inhibitors exerted a strong and immediate action that prevented the metabolome reaction, as normally in a sensitive strain. Finally, *S. cerevisiae* Fp84 displayed a strong response and low mortality values, indicating an effort of the cells to produce endometabolites to contrast the inhibitors toxicity. The above observations corroborated that this FTIR bioassay allows to characterize the resistance of microbial strains to stressors, as already reported for other toxic agents.[36]

Mortality and spectral alterations due to quaternary mixtures. Inhibitors mixtures severely reduced cell viability with the exception of RC_{25}, a partially biocidal concentration causing 26, 44 and 64% mortality in Fm17, Fp84 and DSM70449, respectively (Fig. 2). The evolution of SIs indicated that the metabolomic response was proportional to the different strain resistance level, confirming what already discussed for single inhibitors and further supporting the original classification of these three strains as resistant, intermediate and sensitive, respectively.[6] More in detail, data reported in Fig. 2 for RC_{25} pointed out the similar strain specific pattern detected in the analysis of the metabolomic alterations induced by single inhibitors (Fig. 1). In fact, the sensitive DSM70449 strain was not able to contrast the high mortality rate inferred by inhibitors mixture (GSI = 0.6; 64% mortality), the tolerant Fm17 showed low metabolomic response (GSI: 0.9 a.u.) and low mortality (26%), while the intermediately tolerant Fp84 displayed a high metabolomic reaction (GSI: 2.4 a.u.) together with

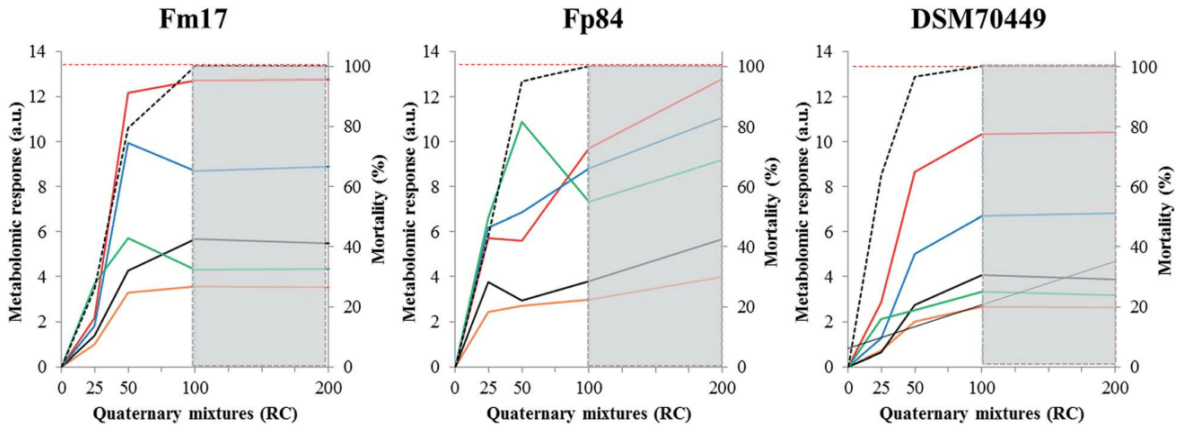

Fig. 2 Stress indexes of *S. cerevisiae* Fm17, Fp84 and DSM70449 cells subjected to increasing RCs of quaternary mixtures. Orange line represent the whole spectrum (GSI), black line W1 region, red line W2 region, blue line W3 region, green line W4 region, dashed line represents mortality. The degree of variability between replicas throughout the FTIR spectra ranged around 2.7×10^{-2}. *Post mortem* chemical reaction of cellular components is indicated by a grey box. a.u. stands for "arbitrary units".

a mortality of 44%. Conversely, all the metabolomic alterations detected at RC_{50}, RC_{100} and RC_{200} were attributable to a *post mortem* cells reaction, since, at these concentrations, the cell mortality ranged from 80% to 100%.

Analysis of the interactions between inhibitors

Mortality analysis. To evaluate whether positive (synergistic or additive) or negative (antagonistic) interactions occurred between inhibitors affecting yeast cell viability, the observed mortality (OM) of cells challenged with the inhibitors mixtures was compared with the expected mortality (EM), according to those caused by increasing concentrations of single inhibitory compounds. Since mortality induced by each inhibitor has hyperbolic curve (data not shown), EM was calculated according to the eqn (1) and reported, together with the OM values, in Fig. 3 for RC_{25} and RC_{50}. Data for RC_{100} and RC_{200} were not presented because the EM reached values over 100%.

Additive effect could be postulated with OM = EM, synergistic when OM > EM and antagonistic when OM < EM. ANOVA revealed that exposure to inhibitors mixtures resulted in statistically significant OM < EM values, indicating that some sort of mechanism induces antagonism among inhibitors. This phenomenon was evident mainly at RC_{25} where the most tolerant strain Fm17 exhibited the highest antagonistic effect, with an OM/EM ratio of about 0.51, meanwhile the intermediate yeast Fp84 and the sensitive strain DSM70449 showed lower antagonistic effects with 0.65 and 0.70 OM/EM ratios, respectively. At RC_{50}, antagonism was still detectable for *S. cerevisiae* Fm17 (OM/EM ratio = 0.79) and slightly observable for the other two strains (OE/EM ratio nearly 0.97).

Metabolomic absolute reduction indicator (ARI). Mortality data can be applied to evaluate the interactions between two or more chemicals only when the sum of the mortalities induced does not exceed 100%. This approach cannot be employed when the aggressors need to be tested at concentrations that saturate the cell mortality, such as for RC_{100} and RC_{200} of inhibitors

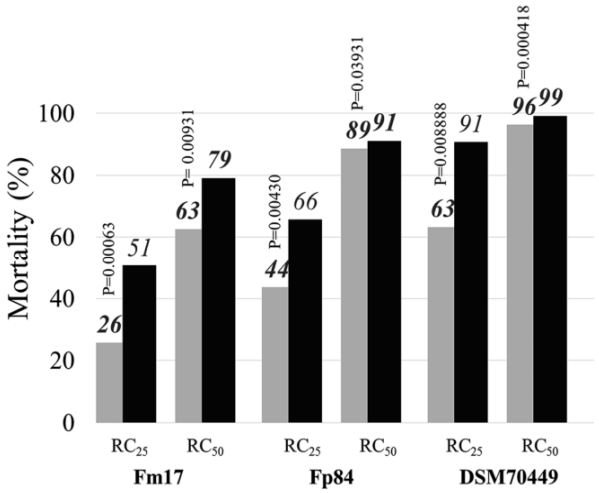

Fig. 3 Comparison of observed mortality values (grey bars) and expected (black bars) after exposure of the strains Fm17, Fp84 and DSM70449 to RC_{25} and RC_{50} mixtures. Observed and expected values of mortality are reported on each bar. Expected mortality values was estimated according to the fractional product method.[31] Data obtained were subjected to one-way analysis of variance (ANOVA), pair comparison was achieved by Tukey's procedure and probability values (*p*) are reported.

quaternary mixture. Conversely, FTIR analysis can bypass the analytical limits linked with the use of mortality data taking into account the metabolomic alterations induced by inhibitors both *pre-* and *post*-cell death.

Metabolomic data were analyzed yielding an Absolute Reduction Indicator (ARI), proposed as the difference between the sum of the metabolomic responses induced separately by each inhibitor and that induced by the mixture (Table 2). Positive ARI values indicate antagonism, negative synergism and figures close to 0 additivity. More specifically, considering that variation coefficients (*i.e.* the ratio between standard

Table 2 Distribution of the Absolute Reduction Indicator (ARI) in the spectroscopic regions[a]

	Strain	Mixture RC_{25}	Σ	Absolute reduction index (ARI)	Mixture RC_{50}	Σ	Absolute reduction index (ARI)	Mixture RC_{100}	Σ	Absolute reduction index (ARI)	Mixture RC_{200}	Σ	Absolute reduction index (ARI)
GSI	Fm17	0.97	4.52	3.55	3.31	4.59	1.28	3.56	3.78	0.22	3.51	4.73	1.22
	Fp84	2.43	11.53	9.10	2.71	10.08	7.38	2.99	4.58	1.60	4.00	6.77	2.77
	DSM70449	0.72	1.69	0.96	2.02	2.96	0.94	2.68	10.01	7.33	2.64	10.03	7.39
W1	Fm17	1.37	8.06	6.69	4.29	7.12	2.83	5.68	7.12	1.43	5.47	7.60	2.13
	Fp84	3.76	17.74	13.98	2.94	14.76	11.82	3.81	6.24	2.43	5.69	9.21	3.51
	DSM70449	0.62	2.06	1.44	2.74	4.62	1.88	4.06	16.57	12.51	3.88	18.66	14.78
W2	Fm17	2.16	11.09	8.92	12.16	13.40	1.24	12.73	7.79	−4.94	12.75	13.51	0.75
	Fp84	5.70	27.52	21.82	5.62	22.29	16.67	9.73	13.21	3.49	12.81	19.84	7.03
	DSM70449	2.86	4.30	1.44	8.66	8.57	−0.09	10.33	17.78	7.45	10.40	12.87	2.47
W3	Fm17	1.79	8.34	6.55	9.94	10.99	1.05	8.68	4.33	−4.36	8.90	8.39	−0.51
	Fp84	6.20	30.90	24.70	6.86	25.77	18.91	8.82	11.64	2.82	11.09	17.84	6.75
	DSM70449	1.25	2.90	1.66	5.03	5.21	0.18	6.69	20.09	13.39	6.82	11.35	4.53
W4	Fm17	3.68	10.43	6.74	5.72	9.19	3.47	4.30	7.60	3.30	4.36	10.56	6.20
	Fp84	6.60	29.03	22.42	10.87	29.78	18.91	7.31	12.27	4.96	9.18	18.00	8.82
	DSM70449	2.11	5.70	3.60	2.52	7.53	5.01	3.32	29.14	25.81	3.19	28.60	25.41

[a] SIs values indicate the metabolomic response of the whole spectrum (GSI) and the fatty acids (W1), amides (W2), mixed (W3) and carbohydrate (W4) regions. Positive ARI values indicate antagonism, negative synergism and figures close to 0 additivity.

deviation and the average of a measure) in metabolomic analyses range from 5 to 10%, ARI confidence limits for additivity should range from −0.20 to 0.20.

With very few exceptions, the analysis of the ARI values displayed an antagonistic effect among inhibitors, closely strain- and dose-specific. Fp84 showed the highest ARIs and DSM70449 the lowest at RC_{25} and RC_{50} while, at the highest relative concentrations, we observed an inversion. The spectral region mostly affected by the antagonism was the carbohydrates one, suggesting a possible involvement of these molecules in the phenomenon as well as in the reaction to the single compounds (Fig. 1). This hypothesis, together with the related mechanism(s), needs further studies to be deeply elucidated.

The present work was based on the postulate that FTIR approach could detect the interactions occurring between inhibitors in mixture through the analysis of the alterations that they induced on the cell metabolome. ARI values presented validated this assumption, highlighting that inhibitors mixtures exerted an antagonistic effect on the microbial metabolism, as already suggested by the analysis of the mortality data for RC_{25} and RC_{50} (Fig. 3). Moreover, metabolomic data analysis allowed to assess this phenomenon also at concentrations that saturate the cell mortality (RC_{100} and RC_{200}), similar to those usually present in the lignocellulosic ethanol processing. Finally, antagonism was found to be closely strain- and dose-specific, confirming that the resistance of a yeast strain to single inhibitors cannot be used to predict its behavior when exposed to inhibitory mixtures.

These results are not in accordance with the related literature, that mainly report an additive and synergistic effects of inhibitors on microbial metabolism.[13,22] So far, only two papers have described the antagonistic effects of two inhibitors, acetic acid and furfural, on yeast growth[37] and transcriptome.[38] This underlines the importance of the developing of new approaches, such as the one above proposed, to better understand the strain(s) behavior in real industrial conditions and to guide the selection of tolerant strains for the large scale production of lignocellulosic ethanol.

Inhibitors chemical interaction(s) and reaction(s) analysis outside the cells. Chemical analysis was performed to elucidate whether the antagonistic effects detected in this study would be due to chemical interaction(s) between inhibitors molecules. Depending on the reaction conditions glucose can be converted to HMF and/or levulinic acid, formic acid and different phenolics. Correspondingly, xylose can follow different reaction mechanisms resulting in the formation of furfural and/or acetic acid.[12] Furfural and HMF can undergo ring-opening or other chemical reactions accomplished by inorganic or organic acids in specific experimental conditions, such as high temperatures or in presence of inorganic catalysts.[39–43] Although different experimental settings have been employed in this study (25 °C, absence of catalysts), several experiments were performed in order to determine the occurrence of potential reactions between the chemical species outside the cells. Ultraviolet-visible (UV-vis) spectral analysis of furfural and HMF in presence of formic and acetic acids showed an almost complete overlap of the spectra of the molecules in all the spectral range

and at all the concentrations even with the use of a strong acid such as HCl and the increase of acid concentrations until pH = 1 (data not shown). This demonstrated that the presence of these acids did not determine a variation on the aromatic portions of the molecules, therefore their ring openings in our experimental conditions are not likely to occur. These data suggested that the different and lower biocidal and metabolomic effects of the inhibitors mixtures could not be ascribed to reactions between the molecules outside the cells.

Conclusions

To our knowledge, this study reports the first qualitative and quantitative evaluation of the antagonistic effects of inhibitors mixtures on *S. cerevisiae* metabolism. Remarkably, FTIR analysis was able to quantitatively assess the type of interactions among inhibitors, for all strains and RCs, overcoming the saturation effect obtained when the sum of observed mortality values is above 100%. The use of binary and ternary inhibitors mixtures will allow to deeply understand the mechanism sustaining this antagonism. Furthermore, this approach appears particularly promising for eco-toxicological settings, in which complex mixtures rather than single compounds are normally found.

Finally, in terms of strain tolerance characterization, this FTIR-based bioassay proved to be as effective as the measurement of relative growth rate in glucose-containing medium supplemented with inhibitors.[6,28] The ease and rapidity of the FTIR analysis indicate that this method could support future applications to assist the selection of highly inhibitors-resistant strains for the efficient industrial production of lignocellulosic ethanol.

Acknowledgements

This work was supported by the research projects entitled: "Engineering Consolidated Bioprocessing yeast for the one-step conversion of cellulosic substrates into bioethanol" (GRIC120EG8, University of Padova, Padova, Italy) and "Elucidating the metabolomic profiles related to industrial fitness in superior yeast for second generation bioethanol" funded by SIMTREA (Società Italiana di Microbiologia Agraria, Alimentare e Ambientale). TM was partly supported by a grant of the Umbria Region. RL was partly supported by a postdoctoral grant from the PRIN Project prot. 2010WZ2NJN. The authors declare no conflict of interest and are grateful to Nguyen Huu Minh (University of Padova) for valuable technical assistance.

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8. Acknowledgments

I want to thank my family for teaching me the importance of continuous learning and for placing my education ahead of any other priority in their lives.

Thank you to my supervisors and co-supervisor for guiding me through the development of my thesis project with precious and insightful advices. A special thank goes to Lorenzo, whose endless support was fundamental throughout this long journey. His continuous challenging me helped me to focus on improving every day.

I would like to thank our collaborators at the Microbiology Department (Stellenbosch University) and at Centro di Ricerca sulle Biomasse (University of Perugia) for providing technical and intellectual support to my project. I would also like to express my gratitude towards my colleagues for their technical support.

Thanks to my partner for always carefully bringing me back on track when I stumble upon problems I think are too big to overcome. Starting and concluding my PhD are just parts of it.