UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Facoltà di scienze Matematiche, Fisiche e Naturali

Dipartimento di Biotecnologie e Bioscienze

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XXIV ciclo

IMPROVING ROBUSTNESS AND METABOLIC PROFILE OF *Saccharomyces cerevisiae* FOR INDUSTRIAL BIOPROCESSES

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Il processo di una scoperta scientifica è, in effetti,
un continuo conflitto di meraviglie

Albert Einstein

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RIASSUNTO

RIASSUNTO

La diminuzione delle risorse energetiche di origine fossile ed i cambiamenti climatici causati dall'emissione di anidride carbonica (CO_2), hanno portato i paesi maggiormente industrializzati ad intraprendere politiche volte allo sviluppo ed all'utilizzo di fonti di energia rinnovabile. Tra le energie rinnovabili stanno assumendo un ruolo fondamentale le biomasse di origine vegetale, in quanto largamente disponibili e potenzialmente capaci di coprire fino al 200% dell'attuale richiesta mondiale di energia. Le biomasse vegetali possono essere sfruttate come principali materie prime per la produzione di composti chimici, biocarburanti ed energia, nel sempre più importante concetto di green economy basato sulla creazione di bioraffinerie. Sebbene le biomasse vegetali risultino ampiamente disponibili, l'incremento dei prezzi delle materie prime per uso alimentare come grano, mais e barbabietola da zucchero hanno posto un serio problema etico sull'utilizzo di queste risorse. Per evitare tale utilizzo di materie prime, lo sfruttamento di biomasse lignocellulosiche ha assunto un ruolo di primaria importanza a livello industriale. Tuttavia per un efficiente utilizzo delle biomasse lignocellulosiche è richiesto lo sviluppo di nuove tecnologie che trasformino la biomassa di partenza in molecole semplici, quali zuccheri pentosi ed esosi più facilmente utilizzabili da parte dei microrganismi, i quali avranno il compito di produrre sia *fine chemicals* che *bulk chemicals* in processi economicamente ed ecologicamente sostenibili. A questo proposito le biotecnologie industriali devono essere in grado di sviluppare nuovi microrganismi capaci di affrontare le difficili condizioni ambientali che si presentano durante un processo di produzione industriale. Per molte di tali produzioni si ricorre all'utilizzo del lievito *Saccharomyces cerevisiae*, che oltre alla naturale predisposizione alla produzione di grandi quantità di etanolo, risulta essere ampiamente conosciuto sia a livello genetico che metabolico, delineando un buon punto di partenza per lo sviluppo di ceppi produttori con elevata tolleranza agli stress da affrontare durante un processo industriale. In questo quadro si inserisce il progetto NEMO (Novel high performance enzymes and microrganisms for conversion of lignocellulosic biomass to ethanol), appartenente al settimo programma quadro dell'Unione Europea, nel quale diventa di primaria importanza sviluppare microrganismi,

specialmente *S.cerevisiae*, da una parte in grado di utilizzare efficacemente tutti gli zuccheri rilasciati dai pre-trattamenti delle biomasse lignocellulosiche, mentre dall'altra parte devono essere maggiormente tolleranti alle condizioni di processo, quali composti inibitori e stress ambientali, per la produzione di bioetanolo di seconda generazione.
Un punto di rilevante importanza è la capacità di utilizzare gli zuccheri pentosi, come il D-xilosio, rilasciato in grandi quantità dopo il pre-trattamento della lignocellulosa. Attualmente le ricerche a livello mondiale si sono focalizzate sullo sviluppo di ceppi di lievito, ingegnerizzati con pathway di degradazione dello xilosio che coinvolgono la via dei pentosi fosfati. Infatti la via fungina sfrutta la xilosio reduttasi e la xilitolo deidrogenasi e la via batterica sfrutta la xilosio isomerasi, ma ambedue degradano lo xilosio a xilulosio che entrerà nella via dei pentosi fosfati.
Oltre a queste due vie studiate dagli anni '80 del secolo scorso, si affiancano altre due vie descritte per la prima volta negli anni '70, ma rimaste poco conosciute, che producono alfa-chetoglutarato o piruvato e glicolaldeide mediante una degradazione ossidativa dello xilosio. Queste due vie, chiamate rispettivamente di Weimberg e di Dahms, dal nome dei due scopritori, appartengono alla famiglia tipicamente batterica dei pathway di Entner-Doudoroff. Queste due vie metaboliche sono composte da 5 reazioni enzimatiche per la via di Weimberg e 4 reazioni enzimatiche per la via di Dahms, ma le prime 3 reazioni risultano essere in comune tra i due pathway. Dopo analisi bioinformatiche è stato identificata la presenza del pathway di Weimberg nel batterio *Burkholderia xenovorans*, mentre la reazione che caratterizza il pathway di Dahms è stata identificata in *Escherichia coli*. I geni codificanti per tali attività enzimatiche sono stati così espressi in *S.cerevisiae* valutandone in seguito la capacità di crescere su xilosio come fonte di carbonio. La ricostruzione di queste due vie metaboliche conferisce al lievito una capacità di crescita limitata su terreni contenenti xilosio. Tale limitazione di crescita sembra essere legata a diversi fattori: la presenza di colli di bottiglia nella funzionalità degli enzimi coinvolti, come l'attività della xilonato deidratasi; la capacità da parte di lievito di internalizzare efficacemente lo xilosio; l'ottimizzazione dei geni coinvolti.
Come descritto in precedenza un altro aspetto importante risulta essere la capacità dei ceppi di lievito di affrontare e superare gli stress ambientali che vengono a crearsi durante un processo industriale. La

membrana citoplasmatica ricopre così un ruolo fondamentale nel garantire l'omeostasi cellulare, essendo l'interfaccia tra la cellula e l'ambiente esterno, reagendo ad eventuali modificazioni dell'ambiente stesso. È stato visto che in pianta la proteina di membrana TIL conferisce particolare resistenza alla cellula quando questa viene sottoposta a stress ambientali di rilevanza industriale, come la presenza di agenti ossidativi oppure durante variazioni di temperatura. TIL è stata così espressa in ceppi differenti di *S.cerevisiae* e si è visto che è in grado di aumentare la tolleranza dei ceppi di laboratorio quando questi vengono esposti a stress e shock ambientali, quali la presenza di agenti ossidativi, di acidi organici, le alte e le basse temperature. Tuttavia quando TIL viene espressa in ceppi industriali e/o di laboratorio ingegnerizzati per l'uso industriale, l'effetto protettivo all'esposizione a stress prolungati e a condizioni di processo vengono meno.

Infine un ulteriore aspetto di rilevante importanza durante un processo industriale è la capacità di *S.cerevisiae* di tollerare la presenza di composti inibitori della crescita presenti nei pre-trattati lignocellulosici. Infatti è stato ampiamente descritto come composti chimici come le aldeidi o gli acidi organici o ancora i composti fenolici, rilasciati durante il processo di pre-trattamento delle biomasse lignocellulosiche, siano tossici a determinate concentrazioni inibendo la crescita o addirittura provocando la morte di *S.cerevisiae*. Sono stati testati e valutati diversi ceppi di lievito *wild type* o ingegnerizzati su due diversi pre-trattati di abete rosso e canna gigante: in aggiunta gli stessi ceppi sono stati testati anche su terreni minimi formulati secondo la composizione del pre-trattato derivante da biomassa di abete rosso. I risultati hanno dimostrato come la combinazione di bassi pH e presenza di acidi organici, specialmente di acido acetico e acido formico, risultino drammaticamente deleteri per la crescita di ceppi di lievito sia industriali, quindi con maggior tolleranza naturale, sia ingegnerizzati, come per la produzione e il ricircolo di acido ascorbico. Tuttavia è risultato interessante il comportamento del ceppo di lievito ingegnerizzato per la produzione e il ricircolo di acido ascorbico in condizione di basso pH, in quanto ha mostrato una tolleranza maggiora rispetto agli altri ceppi in termini di velocità di crescita produzione e produttività di etanolo.

Nonostante i buoni risultati ottenuti ingegnerizzando microrganismi in laboratorio, *S.cerevisiae* su tutti, il loro utilizzo a livello industriale rimane limitato. Risulta perciò di estrema importanza la costruzione di

ceppi maggiormente robusti, capaci di resistere alle diverse condizioni ambientali a cui vengono sottoposti durante un intero processo industriale, cosa che ha una conseguente influenza anche su rese, produzioni e produttività. Per questi motivi la ricerca è volta a combinare questi aspetti per fornire all'industria il miglior microrganismo possibile per la relativa produzione.

ABSTRACT

ABSTRACT

The fossil energy resources decrease and climate changes, caused by carbon dioxide (CO_2) emissions, have led most industrialized countries to undertake policies aimed at the development and use of renewable energy sources. Among the renewable energies, vegetal biomasses play a key role because widely available and potentially able to cover up to 200% of the global energy demand. Vegetal biomasses can be used mainly as raw materials for the production of chemicals, biofuels and energy, in the increasingly important green economy concept based on biorefineries creation. Although the vegetal biomasses result widely available, rising costs of food raw material such as wheat, corn and sugar beet have raised a serious ethical problem using these resources. To avoid the use of such raw materials, the exploitation of lignocellulosic biomasses plays a fundamental role in the industry. However, for an efficient utilization of lignocellulosic biomasses, new technologies are required in order to transform the starting biomass into simple molecules, such as pentoses and hexoses sugars, more easily to use by the microrganism, which will have the task of producing both *fine chemicals* and *bulk chemicals* in an economically and environmentally sustainable processes. In this regard, industrial biotechnologies should be able to develop new microrganisms capable to face the harsh environmental conditions that occur during an industrial production process. For many of these productions the yeast *Saccharomyces cerevisiae* is largely used, not only because of its naturally ability to produce large ethanol amount, but also is widely known both at genetic and metabolic level, outlining a good starting point for the development of producers strains with high tolerance against different stresses occur during an industrial process. This is the view adopted by NEMO project (Novel high performance Enzymes and Microrganisms for conversion of lignocellulosic biomass to ethanol), belonging to the European Union seventh framework program, where it become of primary importance the development of microrganisms, especially *S.cerevisiae*, for the second generation ethanol production. Microrganisms must be, on the one hand able to efficiently utilize all the sugars released from lignocellulosic biomass pre-treatment, on the other hand should be more tolerant against process conditons, such as inhibitory compounds and environmental stresses.

A point of relevant importance is the ability to utilize pentose sugars, like D-xylose, released in large amount after lignocellulose pre-treatment. Currently, worldwide researches are focused on the development of yeast strains engineered with xylose degradation pathways involving the pentose phosphate pathway. In fact the fungal pathway exploits xylose reductase and the xylitol dehydrogenase while the bacterial pathway exploits xylose isomerase; both pathways degrade D-xylose into D-xylulose, which will enter into pentose phosphate pathway.

In addition to these two pathways studied since the '80s of the last century, there also two other poorly known metabolisms, described for the first time in the '70s, which produce alpha-ketoglutarate or pyruvate and glycolaldehyde through an oxidative xylose degradation. These pathways are composed of 5 enzymatic reactions by the Weimberg's pathway and of 4 enzymatic reactions by the Dahms' pathway, however they share the first 3 enzymatic reactions. After bioinformatics we were identified the presence of Weimberg's pathway into *Burkholderia xenovorans*, while the reaction that characterizes the Dahms' pathway has been identified in *Escherichia coli*. The encoding genes for these enzymatic activities were expressed in *S.cerevisiae*, and the capacity to grow on D-xylose as carbon source are evaluated. The reconstruction of these two pathways showed a poorly growth capacity on xylose. Such growth limitation seems to be related to several factors: the presence of bottlenecks associated to enzymes functionality, like D-xylonate dehydratase activity; the yeast ability to internalize xylose efficiently; the involved genes optimization.

Another important aspect is the yeast ability to face and overcome environmental stresses encountered during an industrial process. The cytoplasmic membrane plays a key role in cellular homeostasis, being at the interface between the cell and the external environment, and reacting at environmental changes. The plant membrane protein TIL gives particular strength to the yeast cells when these are subjected to environmental stresses of industrial relevance, such as the presence of oxidative agents or during temperature changes. However, when TIL is expressed in an industrial and/or in an engineered laboratory strains, for industrial use, the protective effect against prolonged stress exposure and process conditions disappear.

Finally, a further important aspect during an industrial process is the *S.cerevisiae* ability to tolerate the growth inhibitory compounds

presence into pre-treated lignocellulose. In fact has been largely described how chemical compounds like aldehydes, organic acids and phenolic compounds, released during lignocellulose pre-treatment process, are toxic at certain concentration, inhibiting *S.cerevisiae* growth or causing yeast death. The growth performance of different *wild type* or engineered yeast strains are evaluated on spruce and giant cane lignocellulose pre-treated: in addition the same strains were tested on minimal formulated medium according to the spruce pre-treated composition. The results showed that the combination of low pH and the presence of organic acids, especially acetic acid and formic acid, are dramatically harmful for growth of both industrial strain, naturally more tolerant, and engineered strain, for the production and recycle of L-ascorbic acid. However, the behavior of engineered strain for production and recycle of L-ascorbic acid is interesting at low pH, because showed higher tolerance than other strains in terms of growth rate and ethanol production and productivity.

Despite the positive results obtained by engineering microrganisms, especially *S.cerevisiae*, in laboratory, their industrial uses still remain limited. Therefore, appears extremely important the construction of more robustness strains, able to withstand different environmental conditions along an entire industrial process, with consequent influence on yields, production and productivity. For these reasons, the research is aimed to combine these aspects to provide the best microrganism possible to industry productions.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 A global overview

Since early years of last century, fossil fuels assumed a pivotal role as energy source in economic and industrial processes. In fact a broad percentage, about 80%, of worldwide primary energy supply (12150Mtoe) comes from fossil fuels like oil, coal and natural gas (fig.1A).

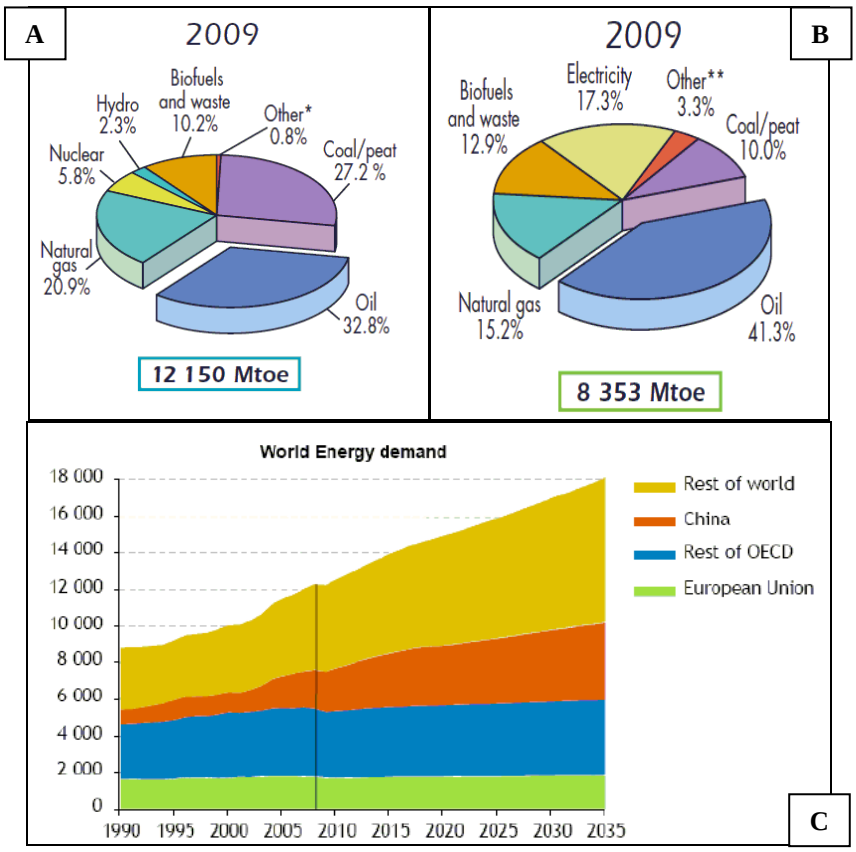

Fig.1 Energetic world demand snapshot and future perspective. Total primary energy supply shares by fuel in 2009 (A) and total final consumption energy shares by fuel in 2009 (B). Projections of world energy demand by region till 2035 (C), the energy amount are expressed in million tons of oil equivalent (Mtoe).
Source: IEA key world energy statistics 2011 and IEA world energy outlook 2010.

The total demand of primary energy will increase of 50% in 2035 due to an expansion of energy request in development countries like China and India (fig.1C). Moreover, the secondary energy consumption is composed in preponderant part of fossil fuels, where oil plays a key role with 41.3% of total energy consumption of 8353 Mtoe (fig.1B).
Oil is the most important fossil fuel and is primarily used as a transportation fuel (fig. 2): it represents 61.7% of the total consumption of oil; automobiles, freight trucks, diesel locomotives, jet planes and tanker ships all utilize the combustion of petroleum distillates for their energy.

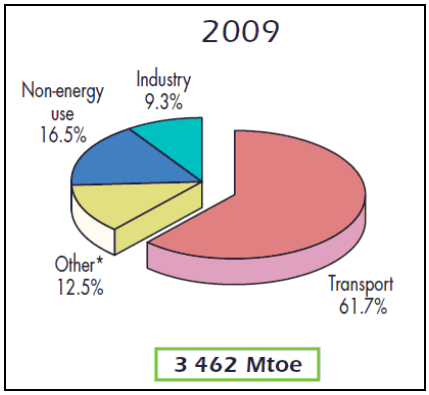

Fig.2 Total final oil consumption shares by sector.
Source: IEA key world energy statistics 2011

Although transportation is the most obvious use, oil is also used in petrochemicals industry for production of several kinds of goods such as soaps, detergents, plastics, cosmetics, clothings, carpetings and electronic devices. The abundance of this raw material in the 20th century has made these consumer goods affordable and widely available.
However availability of this resource is not infinite: oil is going to depletion because is essentially a non-renewable resource since it requires millions of years to form; furthermore it is estimated that no new big worldwide deposits will be available or, in case, that the costs and technologies to extract oil from those deposits, will require substantial investments.
Nowadays we are in the so called “peak oil”: this is a crucial point of the Hubbert theory, elaborated in the ’60s of past century, affirming that peak oil is the point at which the maximum production is reached,

afterward production begins to decline (fig. 3) and, consequently, the prices increase.

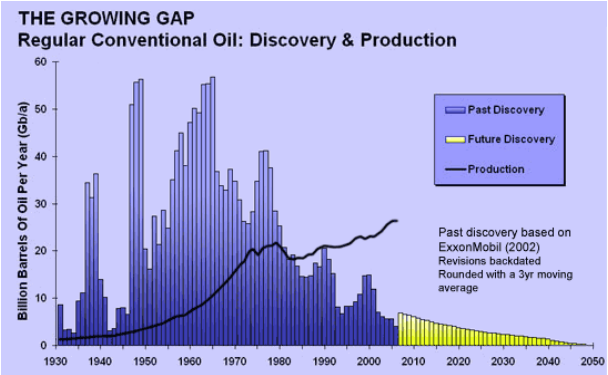

Fig. 3 The rate at which oil has been discovered is falling since 1981(blue bars), and now we use five barrels per every one being found. Unless the discovery increases greatly, the production (black line) will have to fall.
Source: Association for the study of peak oil & gas (ASPO)

In addition to this scenario, we have to remember that the combustion of fossil fuels, in any of its form, produce large amount of greenhouse gases (GHG), mostly carbon dioxide (CO_2) (fig.4A); is estimated that 65% of global emission of CO_2 derived from energy sector (IEA CO_2 emissions from fuel combustion Highlight 2011). Such emissions of GHG result to be the base of climate change and global warming: from the industrial revolution the continuous worldwide energy demand caused a dramatic increase of CO_2 emissions, which are likely to increase, in the next future, due to developing countries (fig.4B). In 1997 with the signature of "Kyoto protocol", the majority of the industrialized countries agree to decrease the GHG emission not less than 5% in 2008-2012 period, with a different percentage from country to country.
Finally, the depletion of fossil resources, the increase of oil price and the increase of GHG emission, lead politicians to develop new strategies to overcome fossil fuels dependence, and one solution for these problems are renewable resources.

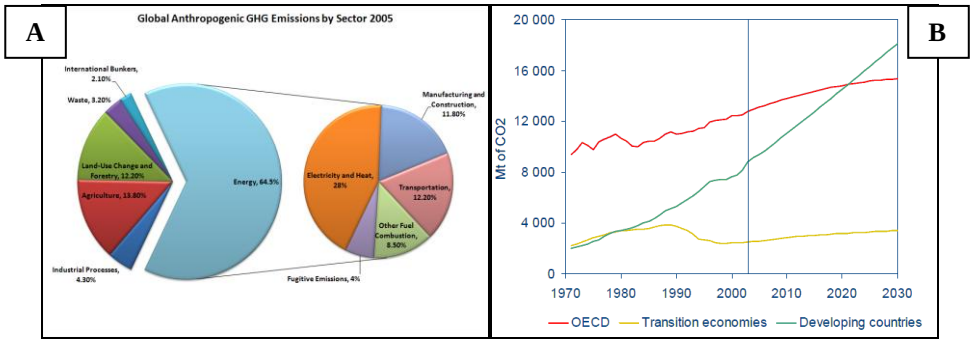

Fig.5 Current emission of GHG and in particular of carbon dioxide and relative projections until 2030. Global anthropogenic GHG emissions by sector (A). World energy related CO_2 emission (B).
Source: IEA world energy outlook, key challenge ahead of us 2006 and Climate analysis indicator tool, World resources institute.

1.2 Renewable resources and green economy

As seen before, the "peak oil" affects prices of production of human goods and commodities as well as climate change with increased GHG emissions; such problems are economically unsustainable. In the last years politicians all over the world agreed on a new sustainable model based on the use of economics, legislative and technological measures to reduce energy consumption, energy dependence, emission of GHG, waste of natural resources and global pollution in order to create a new green economy using renewable resources (UNEP 2010 annual report).
Renewable resources are any kind of natural sources that can be regenerated or not finite in the human time scale, without jeopardizing the natural resources for future generations; the main renewable resources are biomasses, solar radiation, tides, winds and geothermal. In 2009 renewable energy supplied an estimated 16% of global final energy consumption (fig. 5), and biomasses played a fundamental role as such resources.

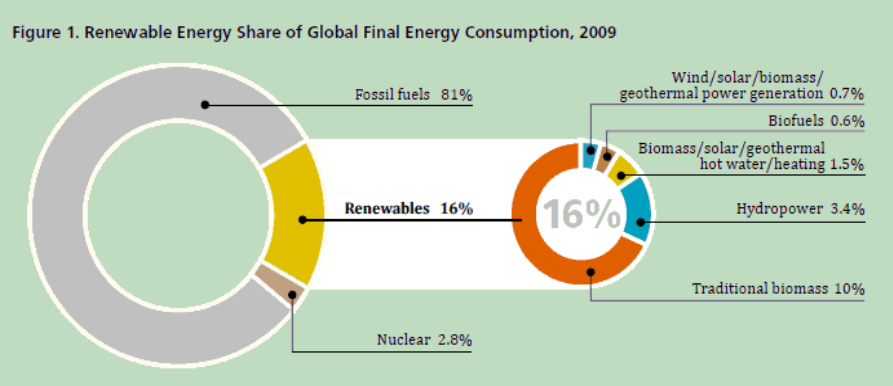

Fig. 5 In 2009 renewable energy supplied an estimated 16% of global final energy consumption; counting traditional biomass, hydropower, wind solar geothermal, modern biomass (used for industrial purposes) and biofuels. Biofuels, wind, solar and modern biomass which represent 2.8% are growing very fast in percentage both in developed and developing countries.
Source: REN21 Renewables 2011 global status report

1.3 Biomasses and biorefinery

Biomasses have been recognized as major world renewable energy: in fact, the global potentials range from 30% to over 200% of current total energy consumption (Industrial biotechnology and biomass utilisation, UNIDO 2007). Obviously this estimation does not take into account of biomasses production for food use: this is the main problem to overcome for an ethical use of these raw materials. Biomasses have great potential to provide feedstocks to make a wide range of chemicals and materials or bioproducts. Actually industries use the simplest and readily available biomass; however such feedstocks coincide with agricultural food crops like corn, wheat and sugar beet, raising prices of raw material for agro-food industries with final impact on prices of basic necessities.
As wrote before, this kind of bioeconomy is unsustainable and ethically incorrect. Lignocellulosic biomasses arise as principal raw materials for biorefineries because they do not compete with agricultural crops for food production, being very often constituted by the wastes from industrial and/or agricultural manufacturies or deriving from marginal lands cultivation; however lignocellulosic utilization requires new industrial processes and technologies. For example, to release sugars monomers from complex macromolecules which compose lignocellulose (see paragraph 1.4).

Biorefineries have the task of replacing the use of fossil molecules and of decreasing the emission of CO_2 and harmful compounds (fig. 6A) used or released during the processes, in order to produce bulk chemicals, like bioethanol, biopolymers, biodiesel and polyols and fine chemical, like vitamins, antibiotics, flavours, pharmaceuticals compounds and dyes (Octave and Thomas, 2009; Hatti-Kaul et al., 2006). To produce the wide range of compounds described before, biorefineries exploit the microrganisms capacities to convert simple or complex substrates, released from biomasses (after appropriate pre-treatments), in different molecules, which are involved or are final products of their metabolic processes (fig 6B) (Kamm and Kamm, 2007).

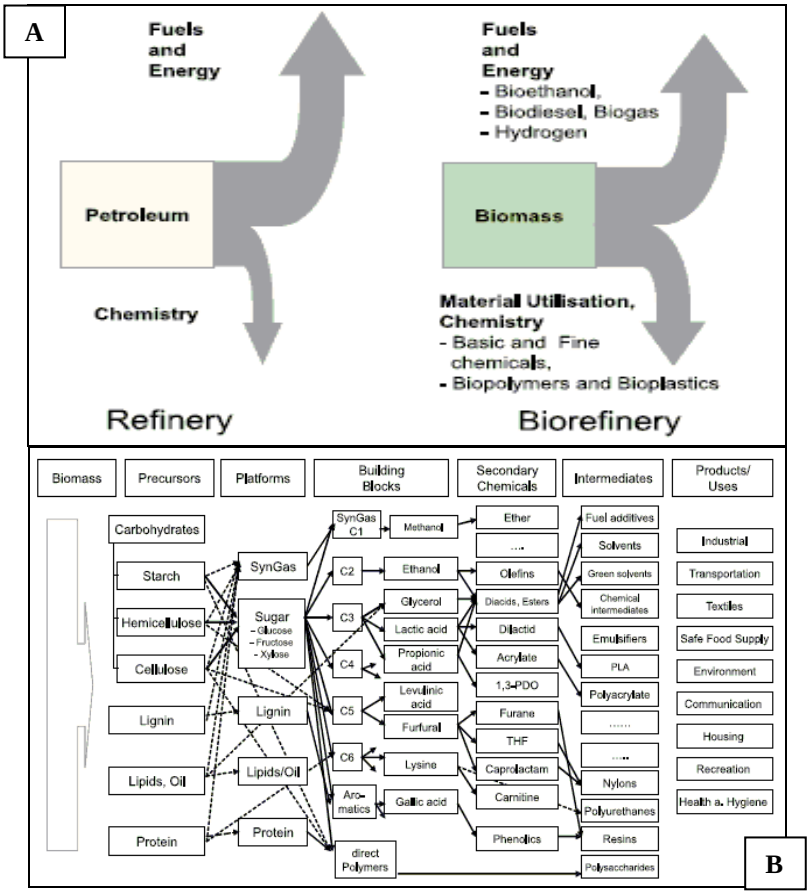

Fig. 6 Comparison of the basic-principles of petroleum refinery and biorefinery (A). Model of bio-based product flow-chart for biomass feedstock (B).
Source: kamm and kamm, 2007

1.4 Lignocellulosic biomasses

There are wide varieties of lignocellulosic biomasses, the nature and the availability of such feedstocks being dependent on climate, environmental factors and agricultural practice in different part of the world (Van Maris et al., 2006; Claaessen et al., 1999). Lignocellulose consists of an intermeshed and chemically bonded complex of three polymers: cellulose, hemicellulose, lignin and sometimes pectin (Perez et al., 2002). Cellulose is the most abundant polymers on the earth and is also the major component of lignocellulose in a percentage between 31% and 51% (fig. 7); it is composed of thousands of molecules of linear anhydrous glucose linked by β-(1,4)-glycosidic bonds and the basic repeated unit is cellobiose, which forms long chain linked together by hydrogen bonds and van der Walls forces (Zaldivar et al., 2001; Perez et al., 2002). Hemicellulose is a complex carbohydrate polymer and makes up 19%-34% of total lignocellulosic dry weight. It is a polysaccharide with a lower molecular weight than cellulose and it is composed by hexoses (D-glucose, D-mannose, L-galactose, L-rhamnose and L-fucose) pentoses (D-xylose and L-arabinose) and uronic acids (D-glucuronic acid and D-galacturonic acid). The principal component of hardwood hemicellulose is glucuronoxylan, whereas glucomannan is predominant in softwood; the main difference with cellulose is that hemicellulose has branches with short lateral chains consisting in different sugars. In contrast to cellulose, hemicellulose are easily hydrolyzable polymers, they don't form aggregates, even when co-crystalyzed with cellulose chain (Perez et al., 2002).

Lignin is present in the cellular cell wall, conferring structural support, impermeability and resistance against microbial attack and oxidative stress. Structurally lignin is an amorphous and non-water soluble heteropolymer, it consists of phenylpropane units joined together by different types of linkages. The principal structural units that compose lignin are coniferyl alcohol, coumaryl alcohol and sinapyl alcohol; all these alcohols belong to phenolic compound family (Zaldivar et al., 2001; Perez et al., 2002).

Finally pectin, a complex and heterogeneus polymer, is the minor fraction of lignocellulose that primarily act as hydrating and cementing agents for cellulosic matrix of plant cell wall (Van Maris et al., 2006). The principal unit of pectin is α-(1-4) linked galacturonic acid.

Fraction	Content in lignocellulose	Major monomers
Cellulose	33–51%	Glucose
Hemicellulose	19–34%	Glucose, mannose, galactose, xylose and arabinose
Lignin	20–30%	Aromatic alcohols
Pectin	2–20%	Galacturonic acid and rhamnose

Fig. 7 Polymer composition of lignocellulose.
Source: van Maris et al., 2006

1.5 Biomass pre-treatment

The main aim of biomass pre-treatment is to make the cellulose and hemicellulose accessible to enzymes without unnecessary degradation of sugars to unusable or inhibitory compounds for subsequent microrganism utilization (Tao et al., 2011). In fact the final purpose of pre-treatment is to remove lignin and hemicellulose, to reduce cellulose crystallinity and increase the porosity of materials (Sun and Cheng, 2001).
There are several kinds of biomass pre-treatments that include concentrated or diluted acid treatment, high pressure steam explosion, ammonia freeze explosion and wet oxidation, but these methods are often used in combination (Van Maris et al., 2006; Tao et al., 2011).
The most promising techniques currently used are: ammonia fibers explosion (AFEX), where biomass is exposed to ammonia at high temperature and pressure for a variable period of time; this treatment doesn't produce inhibitory compounds but it is not effective with all kind of biomasses. The dilute acid pre-treatment, where dilute sulphuric acid is added to biomasses at different temperature and at different time long; this treatment allows to convert high percentage of hemicellulose in monomers sugars, but it isn't so effective in converting cellulose to relative monomers. Lime pre-treatment utilizes alkali and allows to use low temperature and low pressure with good yield, especially with high lignin content biomass, but the reaction time is considerably long.
The last technique is steam explosion with SO_2: this is the most commonly pre-treatment used, and consists in treatment of biomass

with high pressure saturated steam for (relatively) short period of time and changing pressure; steam explosion allows to reach high yield of conversion of cellulose, hemicellulose and lignin in sugars monomer, on the other hand this technique produces several kind of inhibitory compound due to its heavy conditions (Mosier et al., 2005; Sun and Cheng, 2001; Tao et al., 2011; Galbe and Zacchi, 2007). Furthermore an enzymatic hydrolysis after pre-treatment is required to convert the remaining oligomers in monomeric sugar in order to achieve a yield as close as possible to 100% of total sugar inside the initial biomass. As previously underlined (paragraph 1.4), cellulose is harder to degrade than hemicellulose, therefore its complete enzymatic depolimerization is essential. Bioconversion of cellulose and hemicellulose in fermentable sugars is a biorefinering area that has invested enormous research efforts, to produce and to increase yield of conversion of the enzymes. Enzymatic hydrolysis is important because many microrganisms are not able to depolymerize macromolecules released from biomassess pre-treatment. Often in the enzymatic hydrolysis are used cocktail of enzymes, in order to depolymerize cellulose and hemicellulose as much as possible in the short possible time. Among cellulases are important endoglucaneses, cellobiohydrolases and β-glucosidases, instead among hemicellulase are important endo-1,4-β-xylanases, β-xylosidases, α-glucuronidases, α-L-arabinofuranosidases and acetylmannan esterases (Fig. 8) (Kumar et al., 2008).

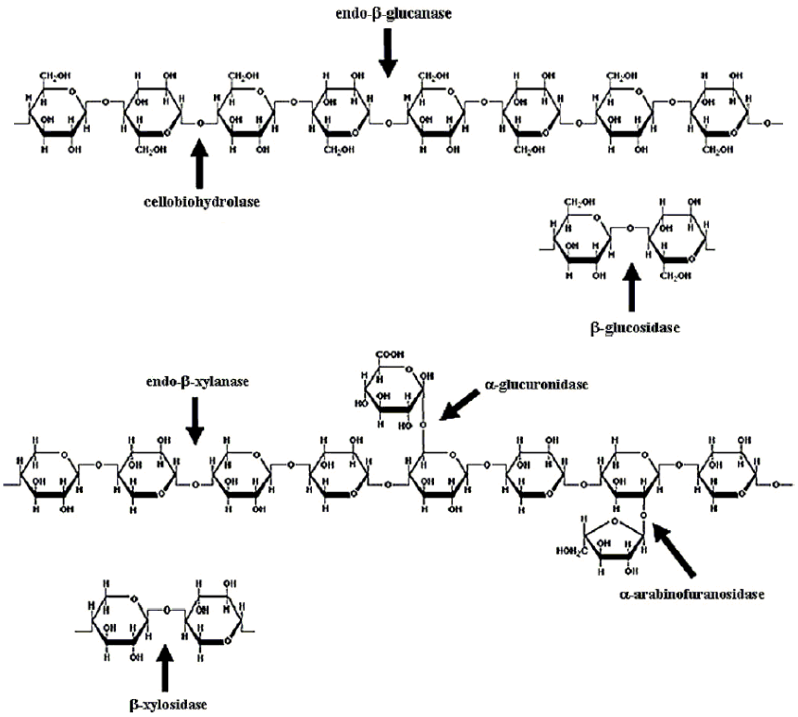

Fig.8 Molecular structure of cellulose (above) and hemicellulose (below) polymers and site of action of hydrolytic enzymes involved in degradation of both polymers. Source: Kumar et al., 2008

1.6 Hydrolysates inhibitors

The harsh conditions required by biomass pre-treatments, like high temperature and high pressure, cause not only depolymerization of cellulose, hemicellulose and lignin but also degradation reactions of monomers released with consequent release of inhibitory compounds derived from every single monomers.

The number and the identity of these toxic compounds vary according to the nature of the starting raw material and with undergoing treatment. The major categories of inhibitory compounds released during biomass pre-treatment are weak acids, furan derivatives and phenolic compounds (Palmqvist et al., 1999). Acetic acid, lactic acid, formic acid and levulinic acid are the most common weak acids released, being these acids derived from degradation of sugars and/or lignin. In their undissociated form those acids can diffuse across cell membrane and meet the higher pH of the cytosol, so dissociating and

acidifying cytosol, forcing membrane proton pump to use ATP to pump out H^+ ions. When cell is no longer able to pump out H^+, weak acids become toxic and cell meets death (Casal et al., 1996; Taherzadeh et al., 1997; Van Maris et al., 2006).

Furans come out from degradation of sugars, more precisely from dehydratation of pentoses and hexoses; furfural and 5-hydroxy-methyl-furfurl (HMF) are the most common furans derivatives inside lignocellulosic pre-treated. HMF derives from glucose degradation and furfural from xylose degradation (Almeida et al., 2009). In their presence growth rate decreases, because cells uses NAD(P)H to reduce HMF and furfural to less toxic alcohol derivatives by endogenous alcohol dehydrogenase (Almeida et al., 2008). High level of furfural and HMF cause an accumulation of reactive oxygen species (ROS), which could be the reason of DNA mutations, protein misfolding, membrane damage and cell death (Almeida et al., 2009).

Finally, various phenolic compounds derived from lignin degradation have a significant impact on microrganisms capabilities to grow on lignocellulosic hydrolysates. Among phenolic compounds there are vanillin, vanillic acid, cathecol and resorcinol; these compounds are toxic because they compromise the integrity of biological membranes (Van Maris et al., 2006).

1.7 Microrganisms and Metabolic Engineering

Industrial biotechnology has traditionally employed numerous bacterial and eukaryotic organisms as production platforms. Traditionally, the first criterion for choosing a certain host was its natural ability to produce the desired compound, be it a primary or secondary metabolite, an enzyme or a biopharmaceutical protein. Many microbial products have indeed been available in the industrial biotechnology market for a long time now (fig. 9) (Porro et al., 2011).

Fermentation product	Microorganism	Application
Ethanol (non-beverage)	*Saccharomyces cerevisiae*	Biofuel/Fine chemicals
2-Ketogluconic acid	*Pseudomonas* sp.	Intermediate for D-araboascorbic acid
Pectinase, protease	*Aspergillus niger, A. aureus*	Clarifying agents in fruit juice
Bacterial amylase	*Bacillus subtilis*	Modified starch
Bacterial protease	*B. subtilis*	Desizing fibres
Dextran	*Leuconostoc mesenteroides*	Food stabilizer
Sorbose	*Gluconobacter suboxydans*	Manufacturing of ascorbic acid
Vitamin B_{12}	*Streptomyces olivaceus*	Food supplements
Glutamic acid	*Brevibacterium* sp.	Food additive
Gluconic acid	*Aspergillus niger*	Pharmaceutical products
Lactic acid	*Rhizopus oryzae*	Foods and pharmaceuticals
Citric acid	*Aspergillus niger* or *A. wentii*	Food products, medicine
Acetone-butano	*Clostridium acetobutylicum*	Solvents, chemical intermediate
Culture starter	*Lactobacillus bulgaricus*	Cheese and yoghurt
Microbial protein (SCP)	*Candida utilis*	Food supplements
	Methylophilus methylotrophus	Food supplements
Penicillin	*Penicillium chrysogenum*	Antibiotics
Cephalosporins	*Cephalosparium acremonium*	Antibiotics
Erythromycin	*Streptomyces erythreus*	Antibiotics

Fig. 9 Examples of industrial products obtained by natural microrganisms. Source: Porro et al., 2011

Microrganism are widely used in Industrial biotechnology; they are advantageous hosts because of the high growth rates and common ease of genetic manipulation. In this respect, the dominance of *Escherichia coli* as host for the production of metabolites, heterologous protein, enzymes, aromatic compounds and aminoacids is clearly a reflection of the quantity and quality of information about it. Among the microbial eukaryotic systems, yeasts combines the advantages of the unicellular organisms, with the ability of the protein processing, typical of eukaryotic organisms, together with the absence of endotoxins as well as oncogenic or viral DNA (Branduardi et al., 2008); as for *E.coli*, this is a reflection of familiarity with deep knowledge about its genetics, biochemistry, physiology and fermentation technologies. *Saccharomyces cerevisiae* is the most important yeast because, in addition to what previously underlined, is also recognized by the american drugs and food administration (FDA) as an organism generally regarded as safe (GRAS). *S.cerevisiae* has been used for centuries in brewing and bakery productions, in fact it is the first eukaryote whose genome was completly sequenced (Goffeau et al., 1996).

However in some cases *S.cerevisiae* is not the optimal host for large-scale production: its metabolic traits require sophisticated equipment for fermentation and it is not highly resistant to severe stresses like pH, temperature, organic acids concentration and chemical inhibitors concentrations often imposed by the production process as could be the utilization of pre-treated biomasses described before (Branduardi et al., 2008). Infact for large-scale industrial productions, metabolic engineering is necessary to improve strains features in order to increase quality and quantity of final products, to decrease final purification costs, to increase yield of product and finally strains and process robustness (Porro et al., 2011; Sauer et al., 2008; Branduardi et al., 2008; Fischer et al., 2008).
Going back strictly to the role of *S.cerevisiae*, this host became extremely important in recent years for its capability to produce heterologous products such as flavonoids, isoprenoids, unsaturated fatty acids, fine chemicals, API, alkaloids, organic acids but over all for production of biofuels. Moreover, specific attention has been dedicated to make *S.cerevisiae* able to ferment every sugars released after lignocellulosic biomass pre-treatment, in special manner D-xylose and L-arabinose (Porro et al., 2011; Porro and Branduardi, 2009; Fischer et al., 2008).

1.8 Pentose metabolism

As described in previous paragraphs, lignocellulosic feedstocks are interesting for production of chemicals and materials in environmental friendly manner potentially at competitive costs. To use lignocellulosic biomass, the complete utilization of substrates is fundamental, and these substrates must be convert in final products by microrganisms. However the model host *S.cerevisiae* is not able to use and ferment pentose sugars like D-xylose and L-arabinose, for these purpose baker's yeast has been engineered to utilize C5 as carbon source.

1.8.1 D-xylose

D-xylose is the most abundant sugar after D-glucose in many pre-treated biomasses. However, as said before, *S.cerevisiae* is not able to ferment this sugar, but it is able to use an isomer of D-xylose such as D-xylulose. The obvious first step to allow D-xylose metabolism is to

introduce an heterologous pathway converting D-xylose into D-xylulose; over the years several approaches have been explored to express a pentose utilization pathway from naturally-utilizing bacteria and fungi in *S.cerevisiae* (Hahn-Hagerdal et al., 2007).
In naturally xylose utilizing anaerobic fungi, D-xylose is reduced to xylitol by a NAD(P)H dependent xylose reductase (XR), and xylitol is consequently oxidazed to D-xylulose by a NAD^+ dependent xylitol dehydrogenase (XDH) (fig. 10) (Rizzi et al., 1988; Rizzi et al., 1989). A similar pathway has been found in bacteria, but it involves a singular enzymatic reaction catalyzed only by xylose isomerase (XI) converting D-xylose into D-xylulose (fig. 10) (Harhangi et al., 2003). D-xylulose is then in both cases phosphorylated in D-xylulose-5-Phosphate, which is an intermediate of the Pentose Phosphate Pathway (PPP).

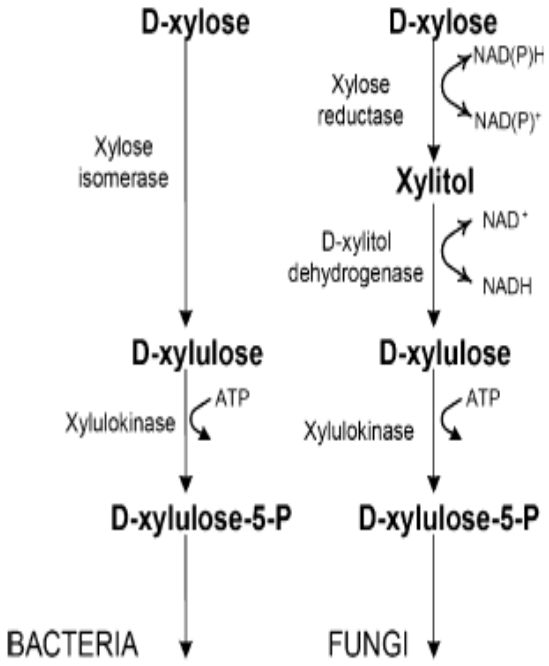

Fig.10 The initial D-xylose utilization pathways in bacteria and fungi.
Source: Hahn-Hagerdal et al., 2007

XI, or alternatively XR and XDH were introduced in *S.cerevisiae* but only when they are overexpressed in combination with the overexpression of the endogenous xilulokinase, the D-xylose

consumption becomes detectable. However, a direct evolution approach obtained in bioreactor is necessary to obtain a strain significantly consuming the C5 sugar and growing on that carbon source.

The first D-xylose utilizing strain of *S.cerevisiae* was generated by expressing *Pichia stipitis* (now *Sheffersomyces stipitis*) genes XYL1 and XYL2, encoding respectively for XR and XDH (Rizzi et al., 1988; Rizzi et al., 1989). *Pichia stipitis* was chosen because its ethanol production from D-xylose is close to the theoretically yield, while in other yeasts the production of byproduct, like xylitol, is predominant (McMillian and Boynton, 1994). Xylitol production is due to the inability of the cell to reduce cofactors in absence of oxygen. In fact *P.stipitis*, as well as *Pachysolen tannophilus* and *Candida shehatae* are able to use not only NADPH but also NADH as cofactor for the xylose reductase (Hahn-Hagerdal et al., 2007; Bruinenberg et al., 1984). However the first engineered *S.cerevisiae* strain expressing XR/XDH was not able to grow on D-xylose efficently, because XR has a very high affinity for NADPH and not for NADH creating a cofactors imbalance in anaerobic conditions. Kinetic modeling showed that the conversion of xylose to xylulose requires a ratio of 1:10 of the initial XR and XDH activities, necessary to overcome xylitol production (Hahn-Hagerdal et al., 2007; Rizzi et al., 1989b; Cornish-Bowden et al., 1995; Eliasson et al., 2001).

To overcome such a problem, a prokaryotic xylose isomerase (XI) catalyzing, the direct conversion of D-xylose into D-xylulose without cofactors was expressed. Xylose isomerase has been the subject of many researches because it is able also to isomerized D-glucose and D-fructose and it is abundant used in food industry (Van Maris et al., 2007). However for many years several attempts of expression of XI in *S.cerevisiae* failed (Briggs et al., 1984; Sarthy et al., 1987; Amore et al., 1989; Moes et al., 1996). The first XI correctly expressed was *Thermus thermophilis* ones in 1996 and it was seen that engineered *S.cerevisiae* cells consumed more D-xylose than control strain producing also ethanol and acetate at low level (Walfridsson et al., 1996). More recently several bacterial XI, were successfully expressed in yeast, all with high activity after a codon optimization strategy; however, the growth on D-xylose as unique carbon source remain slower than expected, mimicking the behaviour described for the XR/XDH pathway (Karhumaa et al., 2005; Kuyper et al., 2003; Winkler, 2006). Like for XR/XDH pathway also for XI pathway evolution in bioreactor is required to

improve xylose growth capacities and fermentation profiles of engineered *S.cerevisiae* strains.

1.8.2 L-arabinose

L-arabinose is the less abundant C5 in lignocellulosic pre-treated feedstock, and consequently only recently the improvements on arabinose metabolism in *S.cerevisiae* have been reached. In fact, similarly to what described for D-xylose utilization, also for L-arabinose there are two distinct pathways, from bacteria and fungi, which can convert L-arabinose into D-xylulose (fig. 11).

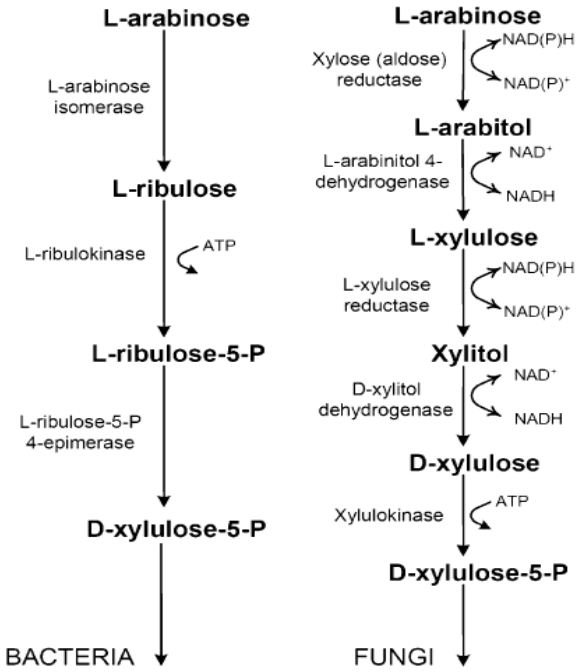

Fig.11 The initial L-arabinose utilization pathways in bacteria and fungi.
Source: Hanh-Hagerdal et al., 2007

In many bacteria L-arabinose is converted into D-xylulose-5-Phosphate (intermediate of the PPP) by the actions of three enzymes, often clustered in the bacterial operon *araBAD* (fig. 11), harbouring the genes encoding for: L-arabinose isomerase codified by *araA* gene, which converts L-arabinose to L-ribulose; L-ribulokinase codified by *araB*, which converts L-ribulose to L-ribulose-5-Phosphate; L-

ribulose-5-Phosphate-4-epimerase, codified by *araD*, which converts L-ribulose-5-Phosphate in D-xylulose-5-Phosphate (Schleif, 2000). The first attempt to introduce in *S.cerevisiae* the heterologous arabinose pathway from *E.coli* did not result in positive arabinose utilization (Sedlak and Ho, 2001). Only after the expression of more active L-arabinose isomerase from *Bacillus subtilis*, *S.cerevisiae* engineered strains became able to use L-arabinose (Becker and Boles, 2003), even if further improvement were necessary as for the D-xylose pathways.
The fungal L-arabinose pathway consists in four reduction-oxidation reactions (fig. 11), where L-arabinose is converted to L-arabitol by xylose (aldose) reductase; L-arabitol is converted to L-xylulose by L-arabitol-4-dehydrogenase; L-xylulose is converted in xylitol by L-xylulose reductase; xylitol is converted in D-xylulose by xylitol dehydrogenase like in D-xylose fungal pathway. As we can see, xylose and arabinose fungal pathways share the enzymes XR, XDH and XK, so we can infer that all xylose utilizing yeast can also use arabinose but not viceversa (Dien et al., 1996). Similar to fungal D-xylose pathway also in L-arabinose fungal pathway is required the regeneration of cofactor, which can occur in the presence of oxygen as external electron acceptor (Hahn-Hagerdal et al., 2007). Firsts attempts to improve *S.cerevisiae* L-arabinose metabolism with fungal pathway have seen the unfortunately expression of *P.stipitis* and *Trichoderma reesei* genes (Becker and Boles, 2003; Richard et al., 2003).

1.8.3 Improvements on pentose sugars metabolism

As described in previous paragraphs, it is evident that the simple expression of single genes in *S.cerevisiae* is not enough for a good fermentation of pentose sugars (Hahn-Hagerdal et al., 2007). Production of xylitol and arabitol has been rationalized with the difference in cofactor preferences. Protein engineering on XR for changing the cofactor preference was tested, but no significant improvements in fermentation were observed (Jeppsson et al., 2006; Saleh et al., 2006). In addition to counteract the cofactor imbalance, the overexpression of the PPP genes was demonstrated to be necessary to improve fermentation rate (Karhumaa et al., 2007; Johansson and Hahn-Hagerdal, 2002; Hahn-Hagerdal et al., 2007) together with the overexpression of XR/XDH/XK or XI acticvities (Karhumaa et al., 2005; Kuyper et al., 2004) or arabinose isomerase activity (Karhuma et al., 2006). Higher level of expression of the pentose pathways genes reduces xylitol or arabitol

excretion (Jin and Jeffries, 2003; Jeppsson et al., 2003; Karhumaa et al., 2007). Another important actor is the *S.cerevisiae* endogenous gene *GRE3*, which codifies for aldose reductase, converting D-xylose into xylitol, which in turn inhibits the XI activity (Kuhn et al., 1995). Consistently, the deletion of such gene improved xylose utilization (Traff et al., 2001), ethanol yield and decrease the xylitol formation (Kuyper et al., 2005; Lonn et al., 2003), however decrease *S.cerevisiae* growth by 30% (Kuhn et al., 1995; Lonn et al., 2003).

1.8.4 Pentose uptake

Despite the extensive and even successful efforts for improving xylose consumption, one major problem for lignocellulose-based production is C6-C5 co-consumption. Xylose uptake in *S.cerevisiae* is very poor because it doesn't have specific xylose transporter. The uptake of xylose is carried out by the hexose transporter belonging to HXT family and Gal2. The Km of membrane transporter of HXT family for xylose are 10-100 times higher than for glucose (Soloheimo et al., 2007; Rintala et al., 2008; Hamacher et al., 2002). Xylose uptake has little effect on the rate of xylose utilization when the expression levels of XR/XDH/XK or XI are limiting (van Maris et al., 2007; van Vleet and Jeffries, 2009). The glucose transporter encoded by SUT1-3 of xylose naturally using yeast *P.stipitis* were cloned and expressed in *S.cerevisiae*; the SUT1 expression enhanced xylose fermentation to ethanol (Katahira et al., 2008). Other xylose specific transporters were expressed in *S.cerevisiae,* for example GXF1 and GXS1 from *Candida intermedia*. However also these transporters are not specific, since they can improve xylose uptake only when xylose is the unique carbon source: even more, their expression is inhibited when glucose is also present in the medium (Leandro et al., 2006; Leandro et al., 2008; Runquist et al., 2009). *Arabidopsis thaliana* transporters At5g59250 and At5g17010 were also expressed in *S.cerevisiae* and the At5g59250 expressing strain showed 2.5 fold increase of xylose utilization in the presence of glucose, but rate of xylose consumption decrease of about 80% after glucose depletion (Hector et al., 2008). Finally an endogenous *S.cerevisiae* transporter for galactose Gal2, a *P.stipitis* AraT transporter and an *A.thaliana* Stp2 transporter were demonstrated to be able to increase arabinose uptake in *S.cerevisiae* with different affinities and capacities, being the first and the most promising one; however also in this case the expression Gal2 is repressed by glucose

(Subtil and Boles, 2011). At the moment there aren't any suitable transporter able to uptake pentose independently by glucose uptake. More efforts to explore and characterize sugar transporters should be expended (Jojima et al., 2010).

1.9 Conclusions

As underlined in this introduction, the crucial points, of a biobased economy focused on biorefinery concept are the ability of technologies to exploit the full potential of vegetal biomasses as renewable resource. The main challenges of industrial biotechnology are the complete utilization by microrganisms of chemical compounds released after lignocellulosic pre-treatment. Moreover microrganisms have to be able to face the severe industrial conditions and stresses like pH and oxygen fluctuations, inhibitors presence, temperature variations, osmotic pressure and accumulation of the final product.
In this thesis the research focused on the improvement in robustness and in metabolic profile of *S.cerevisiae* yeast for a better use of lignocellulosic pre-treated materials.
This goal was pursued following these strategies: improving the metabolic profile of *S.cerevisiae* by expressing novel D-xylose degrading pathways, (belonging to the bacterial Entner-Doudoroff pathways) and optimizing them for a better xylose consumption (chapter 2); improving yeast robustness to different process conditions by the expression of an *A.thaliana* lipocalin (chapter 3); studying the behaviour of a more tolerant engineered *S.cerevisiae* strains in medium based on lignocellulose pre-treated biomass and containing different amount of different inhibitory compounds (chapter 4).

CHAPTER 2

2. CONSTRUCTION OF BACTERIAL ENTNER-DOUDOROFF D-XYLOSE OXIDATIVE PATHWAYS IN *S.cerevisiae*

2.1 Introduction

Many microrganisms can utilize D-xylose as unique carbon source. There are generally two pathways for D-xylose metabolism, which have been extensively investigated; generally speaking, bacteria use D-xylose isomerase to convert D-xylose into xylulose, while fungi use xylose reductase and xylitol dehydrogenase to convert D-xylose in xylitol and consequently in xylulose (see paragraph 1.8). Two additional pathways, belonging to an Entner-Doudoroff pathways family, were described for the first time in '60s-70s of the last century. These pathways occur through an oxidative metabolism of D-xylose; the first named "Weimberg", converts D-xylose to a-ketoglutarate, while the second named "Dahms", converts D-xylose in pyruvate and glycolaldehyde. Both pathways were discovered after a complete characterization of the bacteria hexose (like glucose-gluconate) Entner-Doudoroff pathways, firstly in *Pseudomonas saccharophila* (Entner and Doudoroff, 1952). Hypothesizing the existence of several pathways, depending on the initial sugars substrate, in 1961 Weimberg discovered in *Pseudomonas fragi* similar Entner-Doudoroff metabolisms for pentoses like xylose and arabinose. He described the enzymatic activities of these pathways, demonstrating the formation of α-ketoglutarate as final metabolite (Weimberg, 1961). Similarly, Dahms and coworkers demonstrated the presence of an alternative bacterial oxidative pathway for xylose, which converts D-xylose into pyruvate and glycolaldehyde, in *Pseudomonas fragi* (Dahms and Russo, 1982; Dahms and Donald, 1982; Dahms and Donald, 1982b).

The Entner-Doudoroff pathways show the same reactions model for many sugar substrates, such as D-glucose, D-glucuronate, D-galacturonate, D-galactose, D-fucose, L-rhamnose, L-arabinose and D-xylose (Watanabe and Makino, 2009; Watanabe et al., 2006; Watanabe et al., 2007). These pathways, without phosphorylated intermediates, share three reactions in common, catalyzed by the following enzymes: dehydrogenase, lactonase and dehydratase. The resulting common intermediate, 2-keto-3-deoxysugar, can be further transformed into α-

ketoglutarate according to the Weimberg pathway by a dehydratase and a dehydrogenase; alternatively a unique reaction catalyzed by an aldolase, can cleave it in pyruvate and relative aldehyde (Watanabe and Makino, 2009). Weimberg's D-xylose metabolism belongs to the first type of ED pathways, since it converts D-xylose into α-ketoglutarate through five reactions, which catalyze the conversion of D-xylose in xylose-γ-lacton by D-xylose dehydrogenase; conversion of xylose-γ-lacton in D-xylonate by xylono-γ-lactonase; conversion of D-xylonate in 2-keto-3-deoxy-xylonate (KDX) by xylonate dehydratase; conversion of KDX in α-ketoglutaric semialdehyde by KDX dehydratase; and finally conversion of α-ketoglutaric semialdehyde in α-ketoglurate by α-ketoglutaric semialdehyde dehydrogenase (fig. 12). As final product, α-ketoglutarate is an important intermediate of TCA cycle and precursor for many aminoacids production.

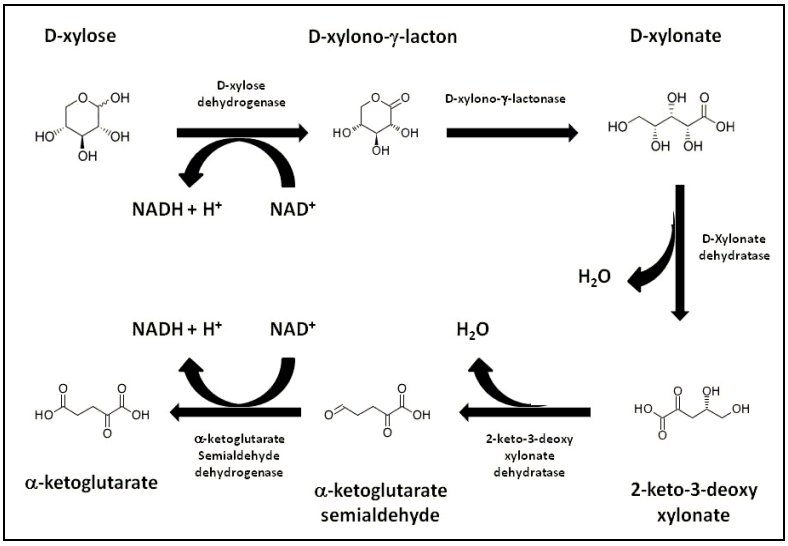

Fig. 12 Schematic representation of Weimberg's pathway.

The Dahms' pathways belongs to the second type of ED metabolisms, since it converts D-xylose in KDX similarly than in the Weimberg's pathway, but the last reaction cleaves KDX in pyruvate and glycolaldehyde by KDX aldolase (fig. 13). These final products are of particular interest from an industrial point of view, because they can be used as building block or precursor for other more complex molecules. Pyruvate is one of the most useful metabolite, and

glycoladehyde has assumed particularly importance as precursor of vitamin B6, glycolic acid and ethan-1,2-diol.

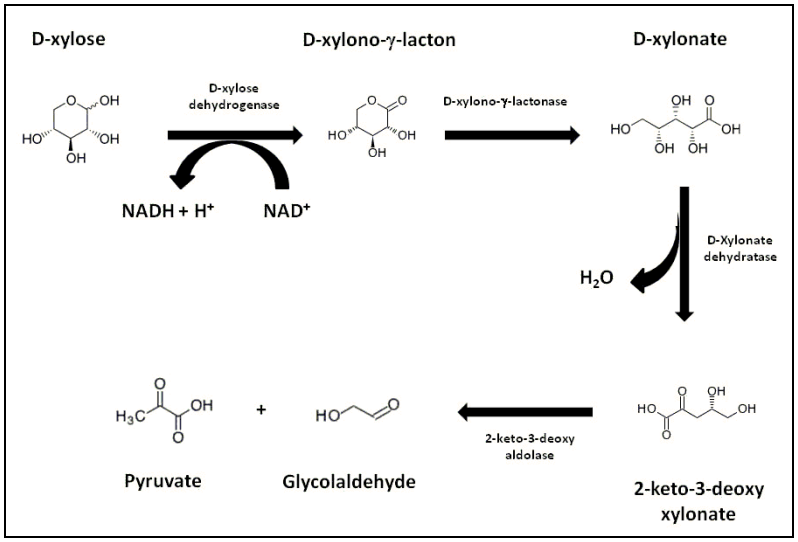

Fig. 13 Schematic representation of Dahms' pathway.

Although these pathways have been known for more than four decades, surprisingly the genes remained for a long time unidentified and only recently several studies focused their attention on these identifications and characterizations.

Stephens and coworkers discovered in the D-xylose natural utilizing bacteria *Caulobacter crescentus*, the presence of several genes clustered in an inducible operon *xylXABCD*; these genes are fundamental for growth on xylose: a mutation in *xylX* gene is sufficient to prevent growth on this sugar. Moreover the authors could demonstrate that the D-xylose dehydrogenase activity, encoded by the cloned *xylB* gene, is necessary and responsible to start the metabolism of D-xylose (Pointdexter, 1964; Stephens et al., 2007). The presence of genes codifying for D-xylose dehydrogenase have been discovered in several bacteria and archaea, like *Haloarcula morismortui* (Johnsen and Schoneit, 2004), *Haloferax volcanii* (Johnsen et al., 2009), *Sulfolobus solfataricus*, *Sulfulobus acidocaldarius* (Nunn et al., 2010), *Burkholderia xenovorans*, *Escherichia coli*, *Pseudomonas spp*. and *Chromohalobacter salaxigens* (Stephens et al., 2007). Some of these genes were already exploited in heterologous expression for the production of non-natural compounds. *E.coli*, has been engineered to

produce D-xylonic acid from D-xylose, as intermediate for production of rockets propellant 1,2,4-butantriol, with a yield of 70% using *P.fragi* and *C.crescentus* genes (Niu et al., 2003); *Pseudomonas putida*, which has been engineered with the whole Weimberg's pathways from *C.crescentus*, showed an efficiently growth on D-xylose as carbon source (Meijnen et al., 2009); *Saccharomyces cerevisiae*, has been engineered only with D-xylose dehydrogenase from *Trichoderma reseei* to produce D-xylonate; this engineered strain showed low production of D-xylonate despite further improvement (Toivari et al., 2010); *Kluyveromyces lactis*, which has been engineered with D-xylose dehydrogenase from *T.reseei* to produce D-xylonate, showed a yield of 60% from D-xylose (Nygard et al., 2011). However in all the reported examples, only the Weimberg's pathway was exploited, while the gene encoding for the KDX aldolase, belonging to the Dahms' pathway, is still unknown and its exploitation unexplored.
In this work we aimed at constructing two different recombinant *S.cerevisiae* strains harboring the genes of both ED D-xylose oxidative pathways. Furthermore, we also describe the efforts made to improve the engineered strains for a better use of D-xylose as carbon source, especially for industrial uses, in order to increase metabolic fluxes and to increase D-xylose uptake. Despite the results underline the need of a profound optimization, this work describes for the first time the construction of the whole Weimberg's and Dahms' pathways in yeast.

2.2 Results

2.2.1 Weimberg's and Dahms' genes bioinformatic analysis in bacteria

The genes responsible for D-xylose oxidative Weimberg's pathway have been characterized in the operon *xylXABCD* of *C.crescentus*. Bioinformatics analysis was firstly carried out in order to detect if there were similar operons in other microrganisms with annotated genomes. Using Kyoto Encyclopedia of Genes and Genomes (KEGG) database, we found operons corresponding to the Weimberg's pathway in three microrganisms: *Burkholderia spp.*, *Phenylobacterium zucineum* and *Asticaccaulis excentricus* (fig. 14).

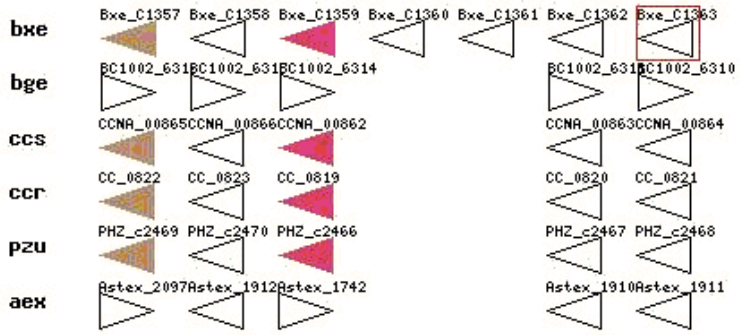

Fig. 14 KEGG alignment of different microrganisms operons containing genes that codify for enzymes of Weimberg's pathways based on *C.crescentus* operon. bxe: *Burkholderia xenovorans*; bge: *Burkholderia sp.* CCGE1002; ccs: *Caulobacter crescentus* NA1000; ccr: *Caulobacter crescentus* CB15; pzu: *Phenylobacterium zucineum*; aex: *Asticaccaulis excentricus*. D-xylose dehydrogenase codifying gene, in red frame, has been used to align other operons.

These bacterial genes have got high similarity percentage with genes of *C.crescentus* belonging to *xylXABCD* operon described by Stephens and coworkers (Stephens et al., 2007).

In the patent describing 1,2,4-butantriol production, deposited by Frost and collaborators (WO2008091288A2), D-xylonate dehydratase of *P.fragi*, the bacterium in which Dahms and Weimberg discovered their pathways, has been characterized and sequenced. Such gene is 100% identical to the *B.xenovorans bxe_C1359* gene and to *E.coli yagF* and *yjhG* genes; moreover, in the same patent Frost described the *E.coli yagE* and *yjhH* genes as putative KDX aldolase genes, which seem to be involved in the last reaction of Dahms' pathway (WO200891288A2). For these reasons we chose as a source for the genes of interest four bacterial strains, *B.xenovorans* LB400, *C.crescentus* CB2, *P.fragi* and *E.coli* K12-W3110 which have been described in literature to have the ability to grow on D-xylose using either Weimberg's and Dahms' pathways. We performed kinetics of growth of the above mentioned microrganisms in minimal medium (M9) with 20 g/L of D-xylose as carbon source. Figure 15 shows that *B.xenovorans* can growth better than all the other microrganisms on xylose, and similarly on glucose as sole carbon source (20 g/L). *P.fragi*, the microrganism where the oxidative pathways have been discovered, and *C.crescentus*, in which the genes of the Weimberg's pathways were characterized for the first time, grow at the same rate

but 2.5 fold less than *B.xenovorans*. Finally *E.coli* seems unable to growth on xylose, accordingly with the cryptic role of genes for xylose utilization (WO200891288A2).

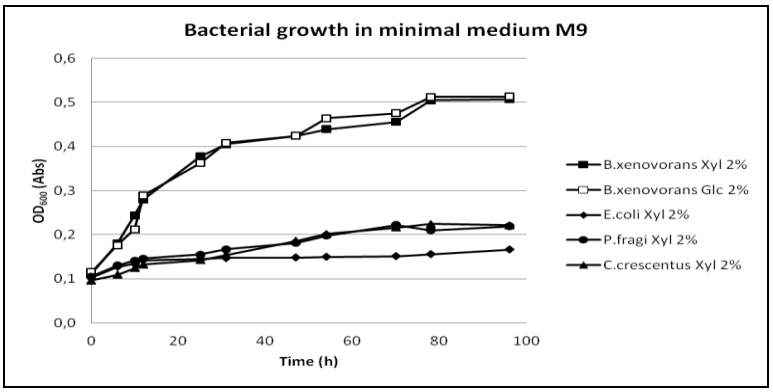

Fig. 15 Kinetics of growth of different bacterial strains in minimal medium in presence of xylose (20 g/L) or glucose (20 g/L) as sole carbon source of different bacterial strains carrying genes for oxidative metabolisms of D-xylose. The graphic represent the mean value of three independent experiments.

B.xenovorans and *E.coli* were chosen for amplifying the genes necessary to engineer *S.cerevisiae* with Weimberg's and Dahms' D-xylose metabolisms.

2.2.2 Construction of engineered *S.cerevisiae* strains and measurement of the heterologous activities

The genes of the two pathways were cloned into integrative plasmids (table 1, see methods for a complete description), used to transform *S.cerevisiae* CEN.PK strain obtaining recombinant CEN.PK Dahms, CEN.PK Weimberg and CEN.PKc control strains. The activities of the heterologous enzymes were measured in cell extracts of engineered strains. More precisely, the activity of D-xylose dehydrogenase, D-xylonate dehydratase, KDX aldolase for Dahms' pathway and α-ketoglutarate semialdehyde dehydrogenase for Weimberg's pathway were measured; moreover, for the last cited enzyme the activity can be measured only with an indirect reaction using D-xylonate as substrate since its direct substrate, α-ketoglutarate semialdehyde is not commercially availaible. Xylono-γ-lactonase and KDX dehydratase activities were not determined, because substrates are unstable. Figure

16 shows D-xylose dehydrogenase activity, higher in both engineered strains if compared to the control. The absolute values of activities are 4.27±0.94 U/mg for CEN.PK Weimberg strain, 3.34±0.74 U/mg for CEN.PK Dahms strain and 0.09±0.16 U/mg for control strain. The results show that the higher substrate specificity of D-xylose dehydrogenase is for D-xylose; with D-glucose and similarly with L-arabinose the activity drops drastically to values near zero for all strains. These results demonstrate that this enzyme results specific for D-xylose.

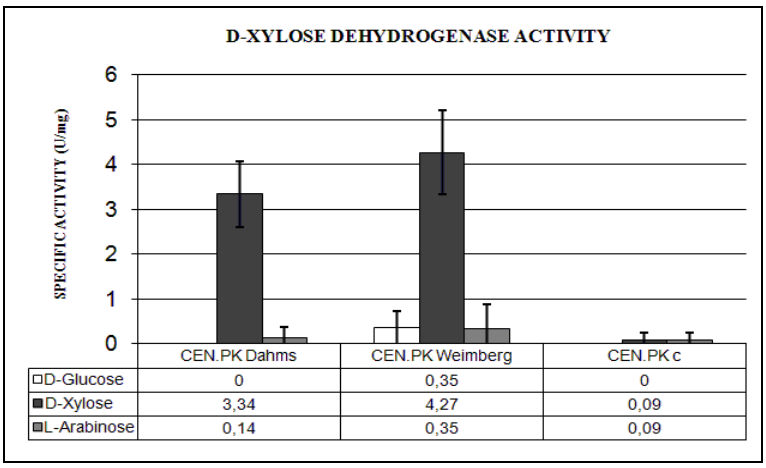

Fig. 16 Enzymatic activities of D-xylose dehydrogenase in *S.cerevisiae* strains with Weimberg's and Dahms' pathways compared to control strain. Data represent the mean value of three independent experiments and the specific activity is expressed as Unit per milligrams of proteins (U/mg), where the Unit definition is the amount of enzyme that convert 1μmol of substrate to product per minute.

The KDX aldolase activity was measured using the retro-condensation ability of the enzyme to form KDX from pyruvate and glycolaldehyde. As shown in figure 17 the activities have absolute values of 4.28±2.66 mU/mg for yeast engineered with Dahms' pathway and 1.66±0.89 mU/mg for control strain. However the low activity and the high variability of data render the reliability of the results quite dubious, although the numbers for transformed strains were always higher than controls.

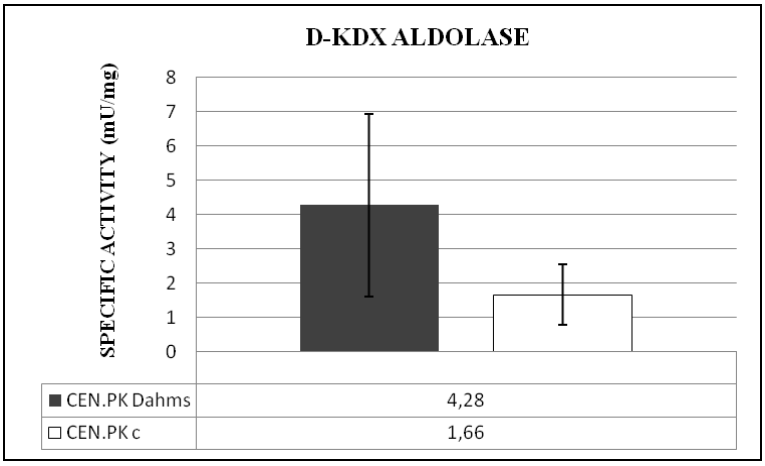

Fig. 17 Enzymatic activities of KDX aldolase in *S.cerevisiae* strains engineered with Dahms' pathways compared to control strain. Data represent the mean value of three independent experiments and the specific activity is expressed as milliUnit per milligrams of proteins (mU/mg), where the Unit definition is the amount of enzyme that convert 1μmol of substrate to product per minute.

The activities of D-xylonate dehydratase and of α-ketoglutaric semialdehyde dehydrogenase resulted undetectable or impossible to measure with the used assays.

2.2.3 Kinetics of growth of engineered *S.cerevisiae* strains and determination of D-xylose consumption

In parallel to enzymatic assays we performed kinetics of growth initially in rich medium (YP) added or not with D-xylose as carbon source. As shown in figure 18 the biomass accumulation of the engineered strains grown on YPX is slightly but reproducibly higher if compared to the control strain, being the difference clearly evident starting from 48 hours from the inoculum (right panel). Significantly, the growth of all the strains in YP is comparable (left panel). This result suggests that the engineered strains with Weimberg's and Dahms' metabolisms seem to be able to use D-xylose to grow, even if poorly.

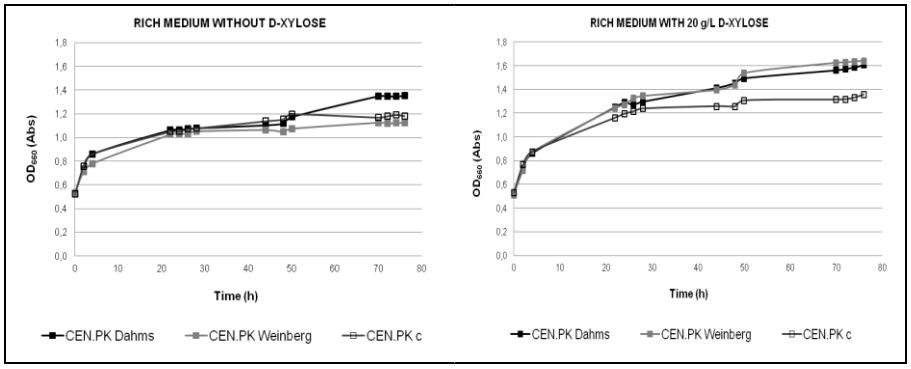

Fig. 18 Kinetics of growth in rich medium (YP) with or without D-xylose as carbon source. Weimberg's and Dahms' engineered strains: full square; control strain: empty square. Cellular grow has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of three independent experiments.

When cells were grown in minimal medium with D-xylose as sole carbon source (fig. 19), all the strains are almost unable to grow, but the difference in biomass accumulation of the engineered strains started to be evident at 24h from the inoculum (left panel). If we try to quantify this advantage in terms of percentage, this corresponds to 19% and 16% for the Weimberg and the Dahms strain respectively, compared to the control strain (fig. 19, right panel).

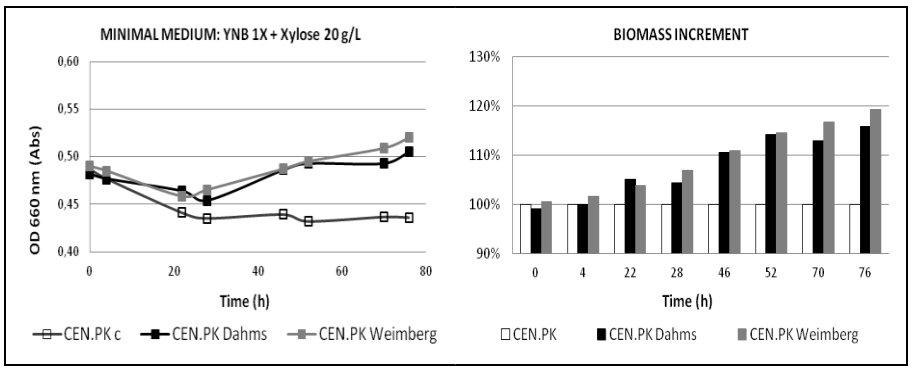

Fig. 19 Kinetics of growth in minimal medium with 20 g/L of D-xylose as carbon source. Weimberg's and Dahms' engineered strains: full square; control strain: empty square. Cellular growth has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of three independent experiments. The biomass increment has been calculated setting the control value at 100% and the engineered strains values are related to control.

Unfortunately the HPLC analysis could not further support the described findings since D-xylose consumption and/or D-xylonate accumulation were not detectable, likely because of the very slow and inefficient metabolism. The lack of D-xylose consumption, the very poor growth and the low activity of some enzymes suggested that one possibility to improve the results could be obtained by expressing the heterologous genes in high copy number.

2.2.4 Construction of strains expressing heterologous genes in multicopy plasmids and relative enzymatic activities

We have transformed *S.cerevisiae* CEN.PK strain with multicopy plasmids containing the four genes of Dahms' pathway, obtaining a recombinant mCEN.PK Dahms and its control mCEN.PKc strains (see table 1 in methods).
We measured again the activities of D-xylose deydrogenase, D-xylonate dehydratase and KDX aldolase. Figure 20 shows a higher activity of D-xylose dehydrogenase in mCEN.PK Dahms if compared with control and CEN.PK Dahms. The absolute values of mCEN.PK Dahms D-xylose dehydrogenase is 21.73±1.41 U/mg, while the activity in the control strain is 0.38±0.59 U/mg.

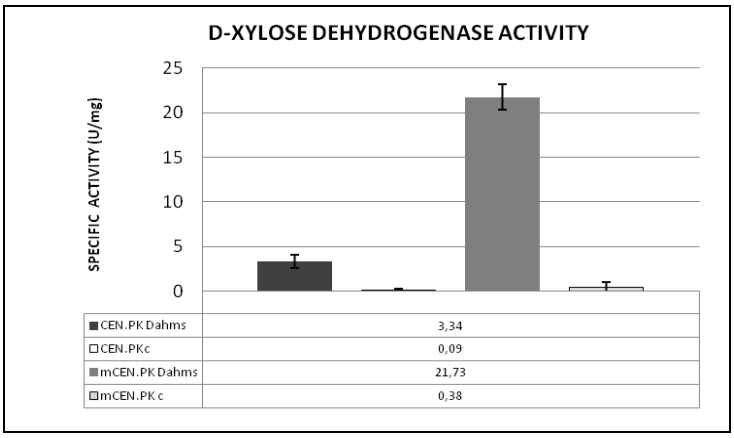

Fig.20 Enzymatic activities of D-xylose dehydrogenase in *S.cerevisiae* engineered with Dahms' pathway genes expressed in multicopy (grey bar), compared with the strain with Dahms' pathway genes expressed in integrative plasmids (dark grey bar) and their relative control (white and light grey bordered bars). Data represent the mean value of three independent experiments and the specific activity is expressed

as Unit per milligrams of proteins (U/mg), where the Unit definition is the amount of enzyme that convert 1μmol of substrate to product per minute.

Figure 21 shows the D-xylonate dehydratase activity in mCEN.PK Dahms strain. The absolute values were 1.33±0.26 mU/mg, while the control presented an activity of 0.05±0.06 mU/mg. The results showed very low but reproducible activity of D-xylonate dehydratase.

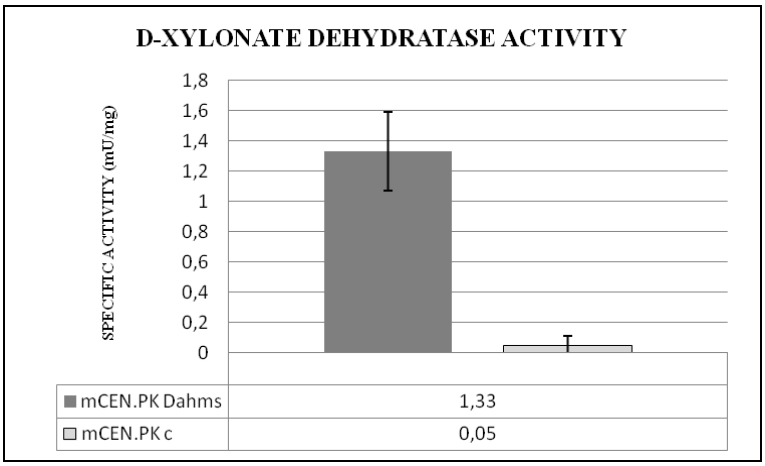

Fig. 21 Enzymatic activities of D-xylose dehydratase in *S.cerevisiae* engineered with Dahms' pathway genes expressed in multicopy compared with its control strain. Data represent the mean value of three independent experiments and the specific activity is expressed as milliUnit per milligrams of proteins (mU/mg), where the Unit definition is the amount of enzyme that convert 1μmol of substrate to product per minute.

Finally we measured the activity of KDX aldolase (fig. 22) in mCEN.PK Dahms strain and it was 9.54±2.72 mU/mg while in the mCEN.PKc was 2.83±0.79 mU/mg. The activity of KDX aldolase in mCEN.PK Dahms was higher and reproducible if compared with CEN.PK Dahms and controls, but still remained low.

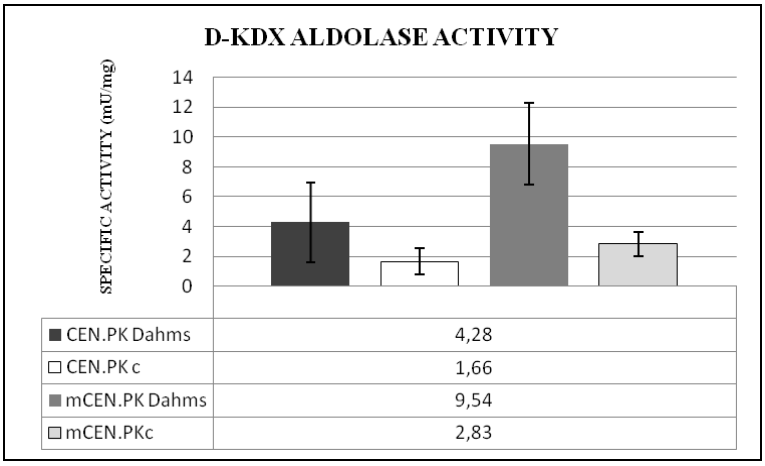

Fig. 22 Enzymatic activities of KDX aldolase in *S.cerevisiae* engineered with Dahms' pathway genes expressed in multicopy (grey bar), compared with the strain with Dahms' pathway genes expressed in integrative plasmids (dark grey bar) and their relative control (white and light grey bordered bars). Data represent the mean value of three independent experiments and the specific activity is expressed as Unit per milligrams of proteins (U/mg), where the Unit definition is the amount of enzyme that convert 1μmol of substrate to product per minute.

2.2.5 Kinetics of growth of *S.cerevisiae* strains engineered with multicopy plasmids and determination of D-xylose consumption

Increasing the copy number of genes we supposed to see a better growth on D-xylose and a detectable consumption of it. However, as shown in figure 23, this did not occured. In any case we have continued to see a poor but reproducible growth difference between the engineered strain with Dahms' pathway and its control. Also in this case we haven't seen any detectable D-xylose consumption with HPLC analyses.

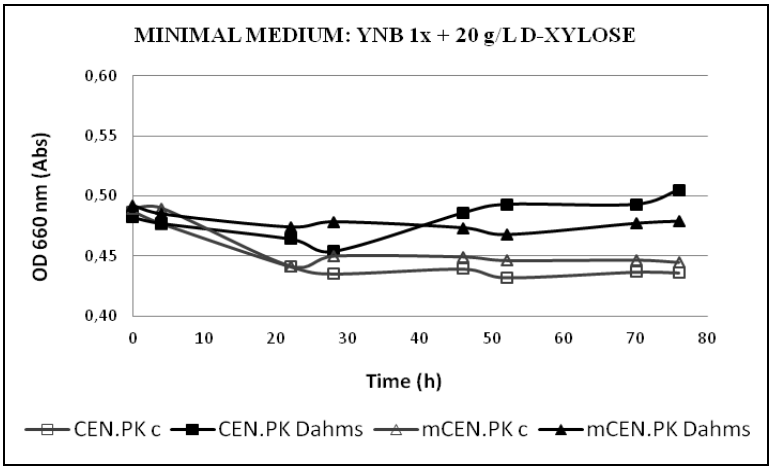

Fig. 23 Kinetics of growth in minimal medium with 20 g/L of D-xylose as carbon source. Dahms' engineered strain with integrative plasmid: full squares; Dahms' engineered strain with multicopy plasmids: full triangles; control strains: empty squares and empty triangles. Cellular grow has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of at least three independent experiments.

2.2.6 D-xylonate dehydratase bottleneck improvement

The low enzymatic activity of D-xylonate dehydratase could be the cause of growth and D-xylose consumption failure. We have therefore overexpressed *B.xenovorans* gene *bxe_C1359* in a multicopy plasmid, but with no positive results, as shown in figure 24. Furthermore, we have tested the strain with the firsts two enzymatic activities of the pathways: CEN.PK [bxe_C1363][bxe_C1362], and the same strain with the overexpression of *bxe_C1359* gene, CEN.PK [bxe_C1363][bxe_C1362] m[bxe_C1359]; the results showed that such strains had the same growth profile of the engineered strains with the whole pathways (fig. 24). In addition we haven't seen any D-xylose consumption and/or accumulation of D-xylonate by HPLC analysis of medium.

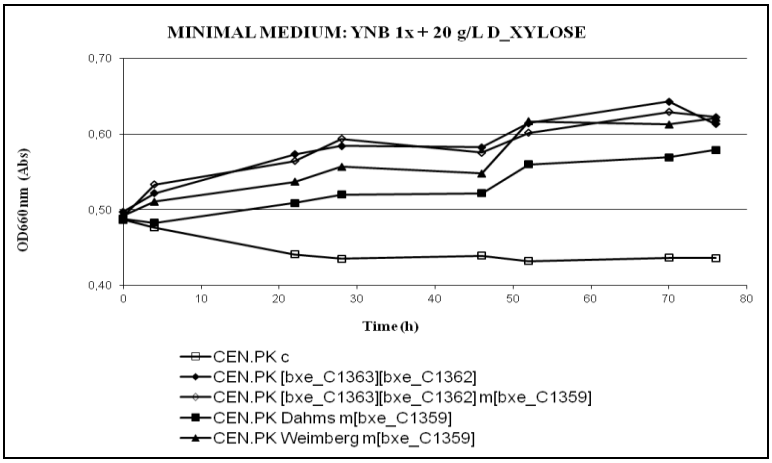

Fig. 24 Kinetics of growth in minimal medium with 20 g/L of D-xylose as carbon source. The control strain: empty square; strains with firsts two enzymes with or without overexpression of *bxe_C1359*:full and empty diamonds; strains with the whole pathways and overexpression of *bxe_C1359*: full square and full triangle. Cellular grow has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of at least three independent experiments

We hypothesized that a high copy number of dehydratase activities could be improved the growth and D-xylose consumption of engineered strains. In literature it was reported that an endogenous gene of *S.cerevisiae* encoding for a dehydratase activity could catalyze the dehydratation reaction of dihydroxy acids into the relative keto-deoxy-acids. This activity was dihydroxy acid dehydratase codified by *ILV3* gene (Velasco et al., 1993). Since D-xylonate was a dihydroxy acid, it could be used by dihydroxy acid dehydratase as substrate. However as shown in figure 25, also the overexpression of *ILV3* gene didn't improve the growth of both CEN.PK Dahms (right panel) and CEN.PK Weimberg strains (left panel).

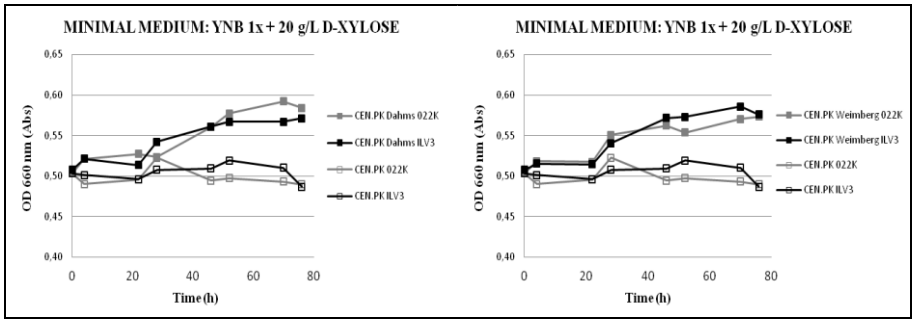

Fig. 25 Kinetics of growth in minimal medium with 20 g/L of D-xylose as carbon source. The *ILV3* expressing strains are in black lines while the control strains are in grey lines. Cellular grow has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of at least three independent experiments.

Furthermore, we haven't seen any D-xylose consumption after HPLC analysis. These results suggested that the D-xylonate dehydratase still remains one of the principal bottleneck to overcome for an efficient use of D-xylose by the engineered strains.

2.2.7 D-xylose uptake improvement

As described in the paragraph 1.8.4, the uptake of D-xylose was fundamental in *S.cerevisiae*. We have added 0.1 g/L of D-glucose in minimal medium with 20 g/L of D-xylose in order to facilitate the uptake of D-xylose into the cell by Hxt transporters family. More precisely, a low glucose concentration triggers the expression of *HXT7*, encoding for a glucose transporter which can also transport xylose, even if with very low efficiency. The data of biomass accumulation positively confirmed our speculations (fig. 26, left panel), but still xylose consumption was insufficient to be detectable by HPLC analyses. A further improvement in biomass accumulation is visible if D-glucose 0.1 g/L is again added after 24h (fig. 26, right panel).

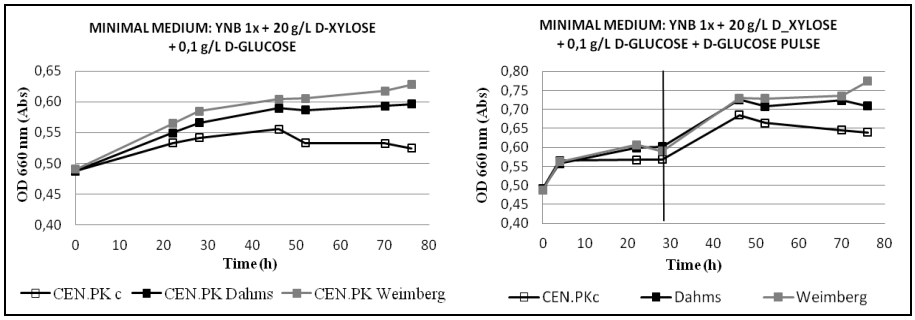

Fig. 26 Kinetics of growth in minimal medium with 20 g/L of D-xylose and 0.1 g/L of D-glucose as carbon source. In the right panel we have added a pulse of 0.1 g/L of D-glucose after 24h (black vertical line). Dahms strain: black squares; Weimberg strain: grey squares; control strain; empty squares. Cellular growth has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of at least three independent experiments.

Because of these data, we have then overexpressed the *HXT7* gene in the engineered strain, but growth was not positively affected, neither in minimal medium with D-xylose as only carbon source (fig. 27, left panel), nor in minimal medium with D-xylose and D-glucose as carbon source (fig. 27, right panel). These results suggested that D-xylose could enter, despite poorly, into the cells helped by unspecific transporter, but very likely said transporter is not encoded by the *HXT7* gene.

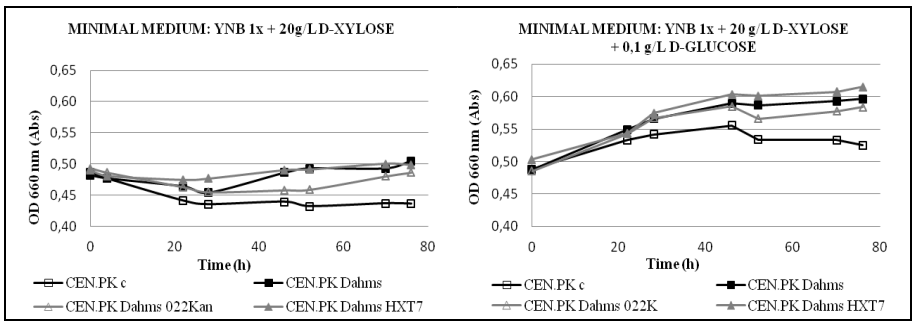

Fig. 27 Kinetics of growth in minimal medium with 20 g/L of D-xylose and with the addition of 0.1 g/L of D-glucose as carbon sources. HXT7 expressing strain and its control: grey lines with full and empty triangles respectively; CEN.PK Dahms and its control: black lines with full and empty squares respectively. Cellular growth has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of at least three independent experiments.

In literature it was reported that the expression in *S.cerevisiae* of *P.stipitis* gene *SUT1*, encoding for a D-glucose specific transporter, seems to improve the D-xylose consumption of 15% (Katahira et al., 2008). It was also reported that another gene of *P.stipitis*, *SUT2*, encoding for another D-glucose specific transporter, showed higher affinity for D-xylose than Sut1 transporter (Jojima et al., 2010; Weierstall et al., 1999). We have expressed the *SUT1* and *SUT2* genes in CEN.PK Dahms and CEN.PK Weimberg strains, but also in this case no improvement was observed (fig. 28).
In conclusion these results confirmed that D-xylose uptake in *S.cerevisiae* is another key drawback that we have to overcome to improve strains in order to reach a good growth on D-xylose as carbon source.

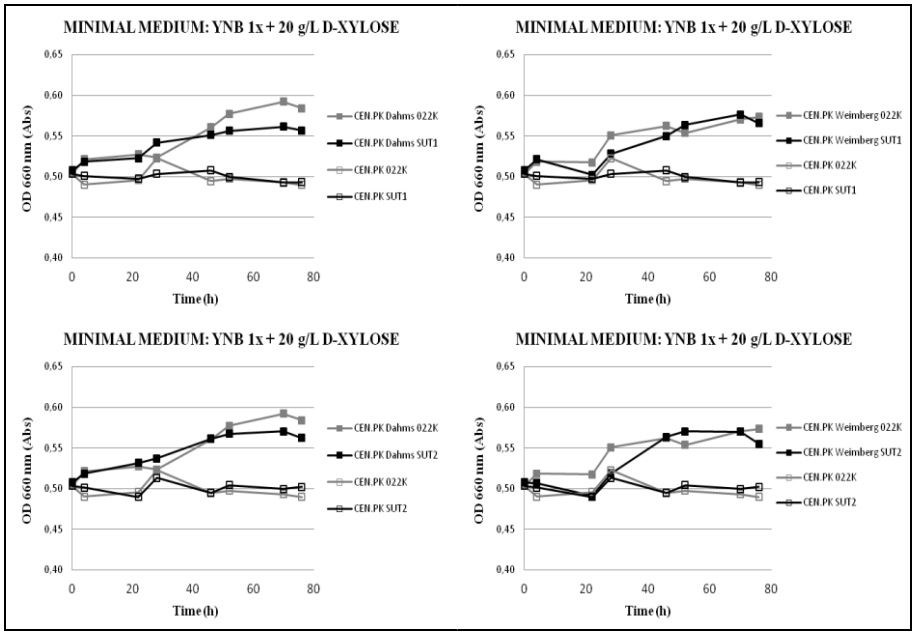

Fig. 28 Kinetics of growth in minimal medium with 20 g/L of D-xylose as carbon source. The *SUT1* and *SUT2* expressing strains are represented in the different panels in black lines; control strains are in grey lines. Cellular growth has been measured as optical density at 660 nm with 0.5 of initial OD_{660}. Data represent the mean value of at least three independent experiments.

2.2.8 Shake flasks evolution of *S.cerevisiae* engineered with Dahms' and Weimberg's pathways

In chapter 1, we underlined that for a better use of D-xylose by *S.cerevisiae* cells engineered with XR/XDH or XI pathways, a strain evolution step was required (Hahn-Hagerdal et al., 2007). Likewise, we have started an evolution experiment of our strains in shake flasks. Strains were initially inoculated in minimal medium with 20 g/L of D-xylose adding low amount of D-glucose, 1 g/L at the beginning and 0.1 g/L successively, to help the strains in the initial steps of growth and for reinvigorate the strains after medium changes. After 3 or 4 weeks of growth the strains were re-inoculated in fresh medium, initially transferring all the cells, and subsequently transferring the cells in order to have an initial optical density at 660 nm of 0.1. The experiment required long time, in fact in figure 29 is reported the growth profile of the strains at the beginning and after eight months of evolution. The strains are CEN.PKc, CEN.PK Dahms, CEN.PK Weimberg and CEN.PK [bxe_C1363][bxe_C1362]. Initially, the growth after 35 days is very low, strictly dependent on D-glucose presence in the medium (fig. 29, left panel). After 238 days, about 8 months later, the growth after 28 days (266th day) was significant and strictly related to D-xylose consumption. Surprisingly, we have obtained an improved growth on D-xylose not only for the strains engineered with Dahms and Weimberg pathways, but also in the strain harboring only the first two enzymatic activities of the pathway(s) and, even more surprisingly, also in the control strain (fig. 29, right panel).

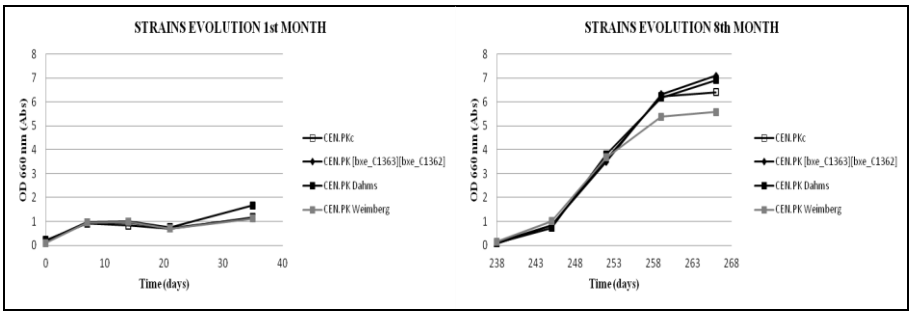

Fig. 29 Shake flasks evolution growth profile after 1 month and after 8 months of growth in minimal medium with 20 g/L of D-xylose and 1 g/L or 0.1 g/L of D-glucose. Cellular growth has been measured as optical density at 660 nm.

The substrates consumption profile (fig. 30) indicated that D-xylose was not consumed along the first month. Instead, when the analyses was repeated after 8 months of evolution, a clear D-xylose consumption is visible in all the strains (5 g/L per week), and this consumption correlated with biomass increase; furthermore the strains produced a little amount of xylitol (0.25 g/L per week). These results showed an acquired ability of all the tested strains to grow on D-xylose, regardless of their genetic background.

	Only medium	CEN.PKc			CEN.PK[*bxe_C1363*][*bxe_C1362*]			CEN.PK Dahms			CEN.PK Weimberg		
Days	D-xylose	D-Glucose	D-Xylose	Xylitol	D-Glucose	D-Xylose	Xylitol	D-Glucose	D-Xylose	Xylitol	D-Glucose	D-Xylose	Xylitol
0	22,58	1,14	22,58	0	1,14	22,58	0	1,14	22,58	0	1,14	22,58	0
7	24	0	24,42	0	0	23,95	0	0	24,04	0	0	24,06	0
14	25,5	0	25,3	0	0	24,18	0	0	23,67	0	0	25,05	0
21	27,12	0	27,73	0	0	26,93	0	0	28,71	0	0	27,92	0
35	30,15	0	31,71	0	0	30,36	0	0	32,33	0	0	31,32	0
238	19,32	0,09	19,32	0	0,09	19,32	0	0,09	19,32	0	0,09	19,32	0
245	20,75	0	20,58	0	0	19,34	0	0	19,34	0	0	19,44	0
252	22	0	18,52	0,25	0	18,02	0,29	0	18,47	0,35	0	16,85	0,45
259	23,56	0	12,28	0,36	0	12,31	0,5	0	13,44	0,49	0	12,31	0,71
266	26,97	0	12,97	0,4	0	12,48	0,6	0	13,64	0,59	0	13,79	0,84

Fig. 30 Shake flasks evolution consumptions profile. In the "only medium" column is reported the amount of D-xylose in the medium considering the evaporation; this column should be compared with the D-xylose column of each strain. The amount are expressed in g/L.

2.3 Discussion

In this chapter we have described for the first time the whole expression of two D-xylose oxidative pathways, belonging to the Entner-Doudoroff family, in *S.cerevisiae*. We have demonstrated that the expression of Weimberg and Dahms pathways in *S.cerevisiae* produced a very poor but reproducible biomass accumulation in defined medium with D-xylose as carbon source. However, we were not able to demonstrate a corresponding D-xylose consumption even when we expressed heterologous genes in multicopy plasmids. Furthermore, we have seen how the activities of D-xylonate dehydratase, in special manner, and of KDX aldolase appeared to be very stringent bottlenecks for a successful growth on D-xylose. The evolution of the engineered yeast strains with Dahms' and Weimberg's pathways showed how difficult was to express a novel metabolic pathway, without facing inner regulation systems evolved

in thousand years. Another important aspect is the D-xylose uptake into the yeast cells: in fact nowadays there isn't stable and functional specific transporter for D-xylose that could be expressed in *S.cerevisiae*. In conclusion, we need a lot of work to implement the D-xylose Entner-Doudoroff pathways in *S.cerevisiae*, especially because it will be necessary to fit a novel sugar metabolism in an eukaryotic microrganism optimized by centuries of natural evolution. However these results are promising because also for XR/XDH and XI pathways, a poor D-xylose consumption was initially seen, and only after optimization of heterologous genes and evolution, a D-xylose efficient metabolism was observed (Hahn-hagerdal et al., 2007). In fact one of the major problems to express Dahms and Weimberg pathways in *S.cerevisiae* is correlated to the optimization of the expression of *Burkholderia xenovorans* and *Escherichia coli* genes in yeast, since they have a codon adaptation index (CAI) very low (0.03 where the maximum efficiently expression have CAI value of 1).

2.4 Materials and methods

2.4.1 Strains and media

Bacterial strains are: *Caulobacter crescentus* CB2 DSM 4727; *Burkholderia xenovorans* LB400 DSM 17367; *Escherichia coli* K12-W3110 DSM 5911; *Pseudomonas fragi* DSM 3456; Yeast strains are: *Saccharomyces cerevisiae* CEN.PK 102-5B (MATa, ura3-52, leu2-3/112, TRP1, MAL2-8c, SUC2 – Dr. P.Kotter, Institute of microbiology, Johan Wolfgang Goethe university, Frankfurt, Germany (van Dijken et al., 2000); *Pichia Stipitis* (now *Sheffersomyces stipitis*) CBS 6054. The bacterial strains were grown at 37°C and agitation at 160rpm in mineral medium M9 (Sigma aldrich) supplemented with 20 g/L of D-xylose or 20 g/L of D-glucose. The engineered yeast strains obtained (Table 1) were grown in shake flasks at 30°C and agitation at 160 rpm in rich medium with yeast extract (Biolife) 1% and bacterial tryptone (Biolife) 2% (YP), in YPX medium we added 20 g/L of D-xylose to YP; the minimum medium were formulated with 0.67% of YNB (yeast nitrogen base – DIFCO) with 20 g/L of D-xylose and/or 1 g/L and/or 0.1 g/L of D-glucose. Where requested we added the G418 antibiotics (BIOSPA) with final concentration of 500 μg/mL.

Yeast strains	Plasmids used
CEN.PKc	pYX012 - pYX042 - pYX022
CEN.PK Dahms	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[yjhH]
CEN.PK Weimberg	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[bxe_C1357-bTPI-bxe_C1358]
mCEN.PK Dahms	pYX212[bxe_C1363-bTPI-bxe_C1362] – pYX242[bxe_C1359] – p427TEF[yjhH]
mCEN.PKc	pYX212 – pYX242 – p427TEF
CEN.PK [bxe_C1363][bxeC_1362]	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042 – pYX022
CEN.PK [bxe_C1363][bxe_C1362] m[bxe_C1359]	pYX012[bxe_C1363-bTPI-bxe_C1362] - pYX242[bxe_C1359] – pYX022
CEN.PK Dahms m[bxeC_1359]	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX242[bxe_C1359] – pYX022[yjhH]
CEN.PK Weimberg m[bxeC_1359]	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX242[bxe_C1359] – pYX022[bxe_C1357-bTPI-bxe_C1358]
CEN.PK 022K	pYX012 - pYX042 - pYX022 – pYX022Kan
CEN.PK Dahms 022K	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[yjhH] – pYX022Kan
CEN.PK Weimberg 022K	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[bxe_C1357-bTPI-bxe_C1358] – pYX022Kan
CEN.PK Dahms HXT7	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[yjhH] –

	pYX022Kan[HXT7]
CEN.PK ILV3	pYX012 - pYX042 - pYX022 – pYX022Kan[ILV3]
CEN.PK SUT1	pYX012 - pYX042 - pYX022 – pYX022Kan[SUT1]
CEN.PK SUT2	pYX012 - pYX042 - pYX022 – pYX022Kan[SUT2]
CEN.PK Dahms ILV3	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[yjhH] – pYX022Kan[ILV3]
CEN.PK Dahms SUT1	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[yjhH] – pYX022Kan[SUT1]
CEN.PK Dahms SUT2	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[yjhH] – pYX022Kan[SUT2]
CEN.PK Weimberg ILV3	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[bxe_C1357-bTPI-bxe_C1358] – pYX022Kan[ILV3]
CEN.PK Weimberg SUT1	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[bxe_C1357-bTPI-bxe_C1358] – pYX022Kan[SUT1]
CEN.PK Weimberg SUT2	pYX012[bxe_C1363-bTPI-bxe_C1362] – pYX042[bxe_C1359] – pYX022[bxe_C1357-bTPI-bxe_C1358] – pYX022Kan[SUT2]

Table 1 List of engineered strains used in this work

2.4.2 Genetic techniques and plasmids

Desired genes (table 2) were amplified using as template the genomic DNA extracted from *B.xenovorans* LB400, *E.coli* K-12 W3110, *P.stipitis* and *S.cerevisiae* CEN.PK 102-5B. Primers and plasmids are listed in table 3. Pwo DNA polymerase (Roche) was used on a GeneAmp PCR system 9700 (PE applied biosystem). DNA manipulation was carried out according to standard techniques (Sambrook and Russel). After amplification genes are subcloned in pSTBLUE-1 cloning vector (Novagen) checked and sequenced. The

coding sequences are cloned into *S.cerevisiae* expression vectors as described: *bxe_C1363, bxe_C1357, yjhH, ILV3, SUT1* and *SUT2* were cut in EcoRI site and ligated in pYX012, pYX212, pYX022, pYX022Kan and p427TEF plasmids cut with the same enzymes; *bxe_C1359* were cut in HindIII and MluI sites and ligated in pYX042 and pYX242 cut with the same endonucleases; *bxe_C1362* and *bxe_C1358* were cut into XbaI site and ligated into MCS of bTPI promoter of pYX012bTPI, pYX212bTPI and pYX022bTPI plasmids cut with the same enzymes; *HXT7* were cloned into the pYX022Kan by Rossi (Rossi et al., 2010). The double expression vectors pYX012bTPI, pYX212bTPI and pYX022bTPI were obtained cutting the plasmids pYX012, pYX212 and pYX022 in KpnI site and ligated with the bTPI construct, carrying triose phosphate isomerase promoter of *Zygosaccharomyces bailii* with a multiple cloning site and polyA terminator, cut in KpnI.

Enzymes of pathways	**Weimberg's genes**	**Dahms' genes**
D-xylose dehydrogenase	Bx *bxe_C1363*	Bx *bxe_C1363*
Xylono-γ-lactonase	Bx *bxe_C1362*	Bx *bxe_C1362*
D-xylonate dehydratase	Bx *bxe_C1359*	Bx *bxe_C1359*
KDX dehydratase	Bx *bxe_C1358*	
α-semiglutarate dehydrogenase	Bx *bxe_C1357*	
KDX aldolase		Ec *yjhH*
Proteins or Enzymes	**Genes**	
HXT7 transporter	Sc *HXT7*	
Dihydroxyacids dehydratase	Sc *ILV3*	
SUT1 transporter	Ps *SUT1*	
SUT2 transporter	Ps *SUT2*	

Table 2 List of genes used in this work

Plasmids	Features
pYX012	Integrative vector by R&D system with URA marker and TPI promoter
pYX012bTPI	Integrative double expression vectors derived from pYX012 with bTPI as second promoter
pYX042	Integrative vector by R&D system with LEU marker and TPI promoter
pYX022	Integrative vector by R&D system with HIS marker and TPI promoter
pYX022bTPI	Integrative double expression vectors derived from pYX022 with bTPI as second promoter
pYX212	Multicopy vector by R&D system with URA marker and TPI promoter
pYX242	Multicopy vector by R&D system with LEU marker and TPI promoter
pYX022Kan	Integrative vector derived from pYX022 with G418 marker and TPI promoter
p427TEF	Multicopy plasmid by Multisystem biotech with G418 marker and TEF promoter
pSTBLUE-1	Cloning vector by Novagen with ampicillin marker
Primers	**Sequences**
bxe_C1363 FW	5'- ACGCCATGTATTTGTTGTCATACC -3'
bxe_C1363 RW	5'- AATTTTCATTCTCCGTACCACCC -3'
bxe_C1362 FW	5' – TGGTATCTAGAATGAAAATTCATCCC – 3'
bxe_C1362 RW	5' – TGTCTTATTGCCACCTTATTGCG – 3'
bxe_C1359 FW	5' – ACAGGCACATGTCAGCATCCA – 3'
bxe_C1359 RW	5' – AACCTGCGGTTCAGTGCGAAT – 3'
bxe_C1358 FW	5' – AGAAATCTAGACTCCATGTCC – 3'
bxe_C1358 RW	5' – ATGTTCGAGCCTCTCAGG – 3'

bxe_C1357 FW	5’ – ACCGGAGAAAGAACATGAGC – 3’
bxe_C1357 RW	5’ – TATTGCCTCGATCTCAAACCG – 3’
yjhH FW	5’ – AGGAAACTGAGGTAATGAAAAAATTCA – 3’
yjhH RW	5’ – ATGAGTTTCTCCTCAGACTGGT – 3’
ILV3 FW	5’ – AAGACCTAGGTAGAGATGGGCTTGTTAA – 3’
ILV3 RW	5’ – TGGGGCCTATAATGCATCAAGCATC – 3’
SUT1 FW	5’ – ATAAGTTAAATAACTAA TTAAAATGTCTTCTCAAG – 3’
SUT1 RW	5’ – ATAAGACATTAGGGATTTAAACATGTTCG – 3’
SUT2 FW	5’ – ACACATTATTCAATACAATGTCCTCACA – 3’
SUT2 RW	5’ – AGAAAAACATTAATCGTAAAGCTAAACTTG -3’

Table 3 List of plasmids and primers used in this work

2.4.3 Yeast transformation and strain construction

Transformation of all *S.cerevisiae* strains was performed using lithium acetate methods (Gietz and Wood, 2002). For yeast transformation, the integrative plasmids obtained were linearized as described: pYX012 with PstI; pYX022Kan with BssHII. After the transformation every strain were controlled with a PCR to verify the presence of the right genes.

2.4.4 Enzymatic assays

Engineered *S.cerevisiae* strains were cultivated overnight in YPD medium at 30°C and agitation at 160 rpm. The cell extracts were prepared harvesting cell culture and washing them with deionized water. The cells were resuspended in Tris-HCl buffer 100 mM at pH 8 and disrupted with Fast Prep (Savant) for three periods of 20 seconds and 1 minute in ice, with an equal amount of glass beads. Extracts were centrifuged and the total proteins were measured with the Bradford method. The D-xylose dehydrogenase assay were carried out measuring the formation of NADH spectrophotometrically at 340 nm; the cell extracts was added to a mixture containing Tris-HCl buffer 100 mM at pH 8, $MgCl_2$ 10 mM, NAD^+ 1 mM and D-xylose 10 mM, the molar extinction coefficient for NAD^+ was 6.22 $mM^{-1}cm^{-1}$. The α-ketoglutaric semialdehyde dehydrogenase was carried following the formation of NADH spectrophotometrically at 340 nm; the cell extract was added to a mixture containing: Tris-HCl 100mM pH 8, $MgCl_2$ 10 mM, NAD^+ 1 mM and D-xylonate 10 mM. The D-xylonate dehydratase activity was measured with thiobarbituric acid assay

(TBA), following the formation of relative keto-deoxyacid spectrophotometrically at 549 nm; the cell extract was added to a mixture containing: Tris-HCl 100 mM pH 8, $MnCl_2$ 0.2 M, D-xylonate 10 mM; after cell extract addition the reaction was incubated for 15 minutes at 30°C, after incubation 100 μL of TCA 12% solution was added to 100 μL of enzymatic reaction, after centrifuged 50 μL of supernatant was added to 125 μL of a solution 25 mM of periodic acid in 0.25M of sulphuric acid and incubated for 20 minutes at room temperature; after incubation was added 250 μL of a 2% solution of sodium arsenite in 0.5 M of chloridic acid, then was added 1 mL of TBA 0.3% solution and incubated for 10 minutes at 99°C; after incubation 500 μL of solution was added to 500 μL DMSO and the formation of a colorful complex was measured at the spectrophotometer; the molar extinction coefficient for TBA was $6.78*10^4$ $mM^{-1}cm^{-1}$. Finally the KDX aldolase activity was measured with the TBA assay described before, following the reaction of formation of keto-deoxyacid; cell extract was added to a mixture containing Tris-HCl 50 mM pH8, $MnCl_2$ 0.2 M, pyruvate 0.5 M, glycolaldehyde 250 mM; initially the reaction was incubated for 15 minutes at 30°C before the TBA assay procedures.

2.4.5 Metabolites detection

Metabolites analyses were performed on samples harvested at different time of a kinetics of growth. The samples were centrifuged and 500 μL of surnatant were analyzed at HPLC. D-glucose, D-xylose, xylitol were measured with an Aminex HPX-87H column (Biorad), while D-xylonate was measured with the addition of Fast column (Biorad) linked to the other. The mobile phase was sulphuric acid 5 mM.

CHAPTER 3

3. EXPRESSION OF THE *Arabidopsis thaliana* LIPOCALIN TIL IN *Saccharomyces cerevisiae* AND ITS EFFECT ON STRESSES TOLERANCE

3.1 Introduction

Industrial biotechnology uses renewable raw materials as starting products and microrganisms to make useful compounds and biomaterials (Branduardi et al., 2008). This application lead to production of bulk chemicals, like bioethanol as biofuel, and fine chemicals, like vitamins, therapeutic proteins, additives, cosmeceuticals, nutraceuticals etc.
In this optic, the choice of microrganisms is extremely important for the success of an industrial bioprocess. Why the choice of microrganisms is so important? Because, beyond their ability to produce efficiently high added value products, they have to endure challenging environments during the production processess (Texeira et al., 2010). *Saccharomyces cerevisiae* is one of the most important industrial microrganisms, because of a long history of domestication, a deep knowledge of its genome, a well optimized tools for its manipulation and a good natural robustness.
Inside the european NEMO project (Novel high performance Enzymes and Microrganisms for conversion of lignocellulosic biomass to ethanol, seventh framework program) for production of bioethanol of second generation, one of the objectives is the development of *S.cerevisiae* strain resistant to multiple stresses imposed by the process. There are two major stress response pathways in yeast, one is the heat shock response (HSR), mediated by heat shock transcription factors, and the general stress response (GSR), activated by several environmental stresses like oxygen, pH, heat, osmotic pressure, cold and ethanol (Gibson et al., 2007). Heat increases the fluidity of the membrane, which interferes with energy generation and trasduction, pH mantainance and caused protein aggregation and denaturation; it triggers the expression of heat shock proteins and threalose overproduction, both increasing thermotolerance (Fischer et al., 2008). Oxidative stress is caused by the cellular respiration producing

reactive oxygen species (ROS). They trigger membrane lipid peroxidation, protein inactivation and nucleic acids damages; over production of antioxidant molecules, like D-erythroascorbic acid, flavohaemoglobin, glutathione, metallothioneins, polyamines, ubiquinol, threalose and ergosterol or overexpression of enzymes like catalase, cytochrome c peroxidase, superoxide dismutase, glutaredoxin, glutathione peroxidase, glutathione reductase, thioredoxin, thioredoxin peroxidase and thioredoxin reductase can improve oxidative tolerance (Gibson et al., 2007). Osmotic stress is caused by an imbalance between intracellular and extracellular osmolarities; osmotolerance is promoted by the abundance of osmoprotective macromolecules that stabilize cellular membranes, enzymes other proteins and nucleic acids (Hernandez-Saavedra et al., 1995). Another important stress is caused by intracellular variation of pH, generally a quickly drop down, caused by accumulation of H^+ iones in the cytoplasm; there are important proton pump membrane proteins, which counteract the accumulation of H^+ in cytoplasm pump it out avoiding reduction in viability and membranes default (Gibson et al., 2007). Cold stress is caused by a repentine downshift of temperature under 20°C, causing a decrease of membrane fluidity, influencing the fatty acids chains of membrane phospholipids; overexpression of cold shock genes, an overproduction of threalose and antioxidant molecules can improve cold tolerance (Gibson et al., 2007). Ethanol stress causes membrane permeability, mithocondrial redox imbalance, enzymes and lipids modification; overexpression of proton pump membrane proteins, over production of threalose and antioxidant molecules which can improve ethanol tolerance (Gibson et al., 2007). As already mentioned, the integrity of cellular membrane plays a key role to counteract environmental stress conditions that can occur in an industrial process (Morel et al., 2006). For a good survival, microbial cells have to be able to change the plasma membrane properties in relation to environmental conditions (Russell et al., 1995).

We have previously developed a Saccharomyces cerevisiae laboratory strain expressing, for the first time, the *Arabidopsis thaliana* temperature induced lipocalin (TIL). The recombinant strain resulted more tolerant to several stresses such as freezing, heating, oxidative, organic acids shocks (Codazzi pH.D. thesis, 2010). This small membrane demonstrated to have a key role in temperature variation response, but its mechanism of action is still unknown (Charron et al., 2008; Chi et al., 2009). In this chapter we further investigated the recombinant yeast

strain to better clarify that the increased robustness was due to a direct effect of *TIL* expression and not an indirect consequence. Furthermore, we tested recombinant *S.cerevisiae* industrial and laboratory strains under limiting conditions relevant for the process of bioethanol production.

3.2 Results

3.2.1 Does TIL expression have a direct or an undirect effect on tolerance?

It was previously demonstrated by immunocytofluorescence that in *S.cerevisiae TIL* localizes not only at the plasma membrane, as it does in plant, but also in the cytoplasm of the yeast cells. The signal was intense, appearing in some cases more concentrated like in spots (fig. 31).

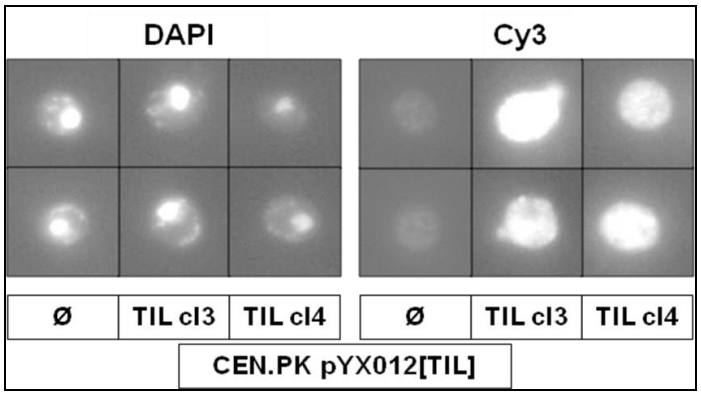

Fig. 31 TIL localization in yeast cells analyzed by immunofluorescence microscopy. Cover slips were incubated with anti-HA monoclonal antibody and then with a secondary antibody Cy3-conjugated (right panel). In the left panel DAPI staining of nuclear DNA (Codazzi Ph.D thesis, 2010).

One possible explanation of this could be the abundance of the protein determined by the constitutive gene expression (the heterologous construct is under the control of the *Sc*TPI promoter). However, we could not exclude that the protein was partially misfolded, and for that reason not properly localized. In these cases cells trigger the Unfolded Protein Response (UPR), which could partially explain the increased

robustness of transformants. To elucidate if the acquired tolerance of TIL expressing cells was a direct or an indirect effect, the presence of mRNA of the gene *HAC1*, which is the final effector of UPR response, was monitored by RT-PCR. In fact, when UPR response is activated by an unfolded protein in the cell cytoplasm, the protein Ire1, with a mechanism still unknown, triggers the splicing of 251 base pairs (bp) of the *Hac1* gene mRNA, activating the expression of UPR genes codifying for ER-resident cahaperons (Shamu et al., 1997). If the UPR is not active, *Hac1* mRNA full lenght is produced (969 bp) but it is rapidly degraded (Shamu et al., 1997).
RT-PCR was performed on indipendent transformants bearing or not the *TIL* gene treated or not with dithiotreitol (DTT), which triggers UPR. Figure 32 reports the result of a representative RT-PCR experiment performed on a single transformant compared to the control strain: the band of about 700 bp size, expected when UPR is induced, was amplified only when samples where DTT treated. The dimension of this band is compatible with the dimension of the spliced *Hac1* mRNA. These results seem to exclude an activation of the UPR response in yeast expressing *A.thaliana* TIL lipocalin, suggesting its direct contribution in improving cell robustness.

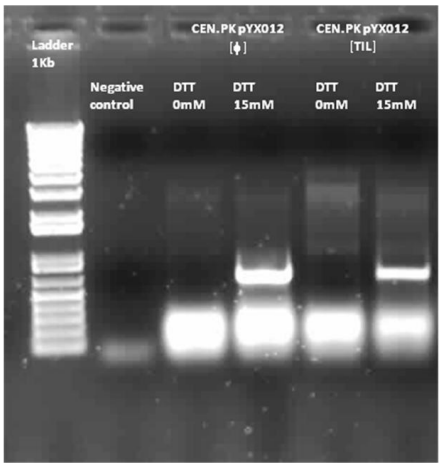

Fig. 32 RT-PCR for HAC1 mRNA. The figure is representative of the results of three repeated experiments on *S.cerevisiae TIL* expressing independent strains and their relative control, both treated and untreated with DTT.

3.2.2 TIL Expression in laboratory *S.cerevisiae* strain engineered for industrial processes

TIL expression can help *A.thaliana* to survive after temperature stresses, acting as peroxidated lipids scavenger and helping to restore membrane integrity (Charron et al., 2008).
We previously demonstrated how the expression of TIL in yeast strains can increase tolerance, especially against oxidative shocks. From an industrial point of view, the challenge is to obtain strains able to overcome oxidative stress throughout the process, also for long period. For example, the production of ethanol requires strains able to face the ROS formation caused by increasing ethanol presence during fermentations. For this purpose we have transformed an engineered laboratory *S.cerevisiae* strain ready for industrial processes, provided by NEMO project, with *At*TIL gene and we have tested the strains in kinetics of growth with the addition of limiting concentration of H_2O_2 (fig. 33). Surprisingly, in this genetic background there is no positive effect caused by TIL expression.

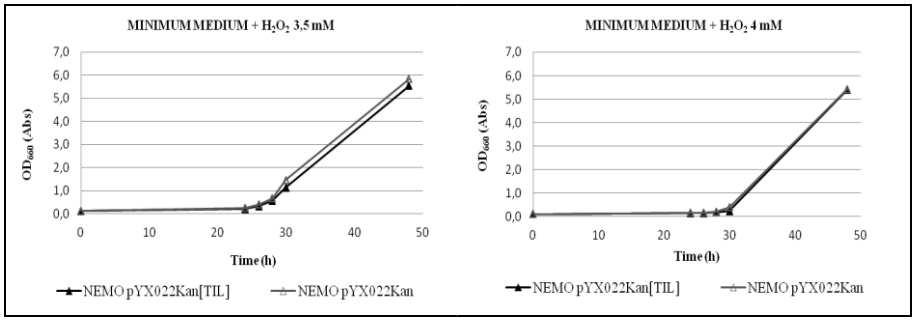

Fig. 33 Kinetics of growth in presence of oxidative stress of yeast expressing TIL (black line and full triangles) and its relative control (grey line and empty triangles). The growth was measured as optical density at 660 nm with an initial OD of 0.1. The data represent the mean value of three independent experiments.

These results suggest that in an engineered, and very likely for that reason more robust, strain the expression of TIL does not further improve stress tolerance.

3.2.3 Expression of TIL in an industrial *S.cerevisiae* strain

Another important industrial stressful condition is the continuous temperature changing, especially if we consider that during the fermentation process the metabolism of the cells causes a temperature

increase, which can interfere with the optimal working process temperature. So another challenge for an industrial application, is to develop a cell factory able to overcome this stress.
In plant *TIL* expression is induced to facing temperature changes, resulting in higher *A.thaliana* robustness (Charron et al., 2008). Previous experiments performed in our laboratory demonstrated similar findings for transformed yeast cells: transformed *S.cerevisiae* laboratory and in industrial strain could grow until 42°C and 55°C, respectively (Codazzi Ph.D thesis, 2010). Because of these positive results, the engineered Ethanol red strain was tested in batch fermentation in process condition for ethanol production. Using Verduyn medium (Verduyn et al., 1992) with 160 g/L of D-glucose as carbon source we have analyzed the fermentation metabolites profile at 35°C and 40°C; the higher temperature is desirable in second generation productions since it better match the parameters needed for an optimal enzymatic hydrolysis of pre-treated biomasses. Figure 34 unfortunately shows that also in this case the presence of the plant gene does not improve the microbial traits. It can be even said that the control strain performs better than the recombinant one, since the second accumulates more glycerol and less biomass than the control. This difference is explained by the close connection between glycerol and biomass anaerobic productions, to mantain the redox power balance, mediated by NAD^+/NADH couple.
The result suggest that TIL expression seems not able to improve robustness of a yeast industrial strain, like Ethanol Red, against prolonged heating stress.
It has to be mentioned that in this case the experiment is obviously conducted in anaerobic condition, to favor the sugar fermentation to ethanol: we have not enough evidence to say if this can influence *TIL* effects and in which condition.

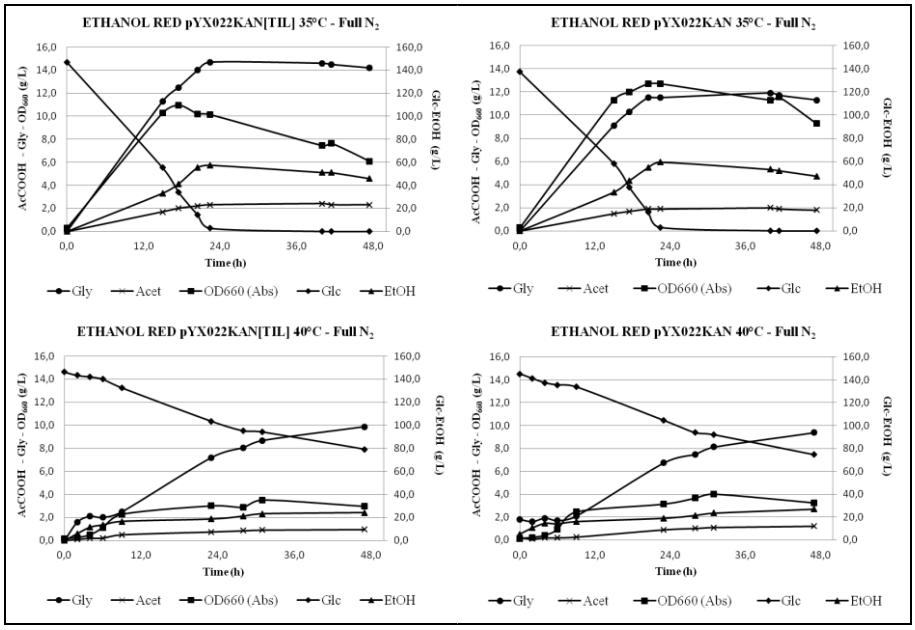

Fig. 34 Batch fermentations at 35°C and 40°C of industrial *S.cerevisiae* Ethanol red strain exspressing TIL and its relative control. Black squares: biomass accumulation measured with optical density at 660 nm. The metabolites are: Glycerol in black circles, Acetate in black crosses, Glucose in black diamonds and Ethanol in black triangles.

3.3 Discussion

It has been previously demonstrated that the expression of plant temperature induced lipocalin TIL in *S.cerevisiae* could increase the robustness of laboratory CEN.PK strain against environmental shocks and stresses (Codazzi Ph.D thesis, 2010). In this chapter we have learnt something more about increased robustness in laboratory strains: TIL expression gives a direct contribution to increased robustness, also if the mechanism remains unclear, avoiding the indirect effect due to activation of UPR system for an incorrect protein folding. However when TIL is expressed in a laboratory strain engineered for industrial processes, the beneficial traits against prolonged stress are lost, also if it maintains higher tolerance against shock conditions. Furthermore, when we move in an industrial strain background, like Ethanol Red, TIL expression doesn't improve tolerance against heating stress, tested in process conditions for ethanol production. These results can

be explained with a possible TIL cellular protection mechanism; in fact TIL seems able to modulate the membrane in order to counteracting sudden and brief shocks, but this does not help when the stresses are prolonged in time and the membrane is more exposed. Probably because industrial strains and recombinant strains have an innate higher tolerance or because their membrane become more tolerant after activation of other factors, in response to prolonged stresses, the TIL expression effect gets lost. Moreover, these hypotheses can further explain why the TIL positive effect was more evident for shocks conditions instead than for prolonged stress conditions, also when expressed in laboratory strain (Codazzi Ph.D thesis, 2010).

3.4 Materials and methods

3.4.1 Strains and media

S.cerevisiae strains used are: CEN.PK 102-5B (MATa, ura3-52, leu2-3/112, TRP1, MAL2-8c, SUC2 – Dr. P.Kotter, Institute of microbiology, Johan Wolfgang Goethe university, Frankfurt, Germany (van Dijken et al., 2000); yeast strain provided by NEMO project with a CEN.PK background; industrial strain Ethanol Red provided by NEMO project. The engineered yeast strains obtained and plasmids used are listed in table 4. Microrganisms were grown in shake flasks at 30°C and agitation at 160 rpm in rich YPD medium within yeast extract (Biolife) 1% and bacterial tryptone (Biolife) 2% and 20 g/L of D-glucose; the minimal medium were formulated with 0.67% or 1.34% of YNB (yeast nitrogen base – DIFCO) with 20 g/L or 50 g/L of D-glucose and where used 50 mg/L of aminoacids histidine (H), uracil (U), leucine (L), lysine (K), isoleucine (I) and valine (V). In kinetics of growth under oxidative stress was added hydrogen peroxyde (Sigma) at final concentration of 3.5 mM and 4 mM. Where requested was added G418 antibiotics (BIOSPA) with final concentration of 500 μg/mL. Fermentations were carried out in Biostat A bioreactor (Sartorius stedim) in a final volume of 1.5 L and the medium used was Verduyn medium (Verduyn et al., 1992) with 160 g/L of D-glucose as carbon source in accord with NEMO project standard operation procedure.

Strains	Plasmids
CEN.PK pYX012[TIL]	pYX012[TIL] (Codazzi Ph.D thesis, 2010)
CEN.PK pX012	pYX012 (R&D system)
NEMO pYX022Kan[TIL]	pYX022Kan[TIL] (Codazzi Ph.D thesis, 2010)
NEMO pYX022Kan	pYX022Kan (derived from pYX022 R&D system, with the addition of G418 marker)
Ethanol red pYX022Kan[TIL]	pYX022Kan[TIL] (Codazzi Ph.D thesis, 2010)
Ethanol red pYX022Kan	pYX022Kan (derived from pYX022 R&D system, with the addition of G418 marker)

Table 4 List of engineered strains and plasmids used in this work

3.4.2 Yeast transformation and strain construction

Transformation of all *S.cerevisiae* strains was performed using lithium acetate methods (Gietz and Wood, 2002). For yeast transformation, the integrative plasmids were linearized as described: pYX012 with PstI and pYX022Kan with BssHII. After the transformation every strain were controlled with a PCR to verify the presence of the right genes

3.4.3 RT-PCR and RNA extraction

RNA extraction was carried out using TRI-REAGENT solution (Sigma aldrich) following the protocol provided by Sigma aldrich. The retro transcription was carried out using ImProm-II reverse transcriptase system kit (Promega) following the protocol provided by Promega. The PCR were made using 5' prime PCR extender system (Eppendorf) following the protocol provided by 5'prime. DNA

manipulation was carried out according to standard techniques (Sambrook and Russel).
Hac1 amplification was carried out using primers listed in table 5.

Primers	Sequences
HAC1 FW	5' – ATGGAAATGACTGATTTTGAACTAACTAG – 3'
HAC1 RW	5' – TCATGAAGTGATGAAGAAATCATTCAATTC – 3'

Table 5 List of primers used in this work

CHAPTER 4

4. FERMENTATION PERFORMANCE OF *WILD TYPE* AND ENGINEERED *S.cerevisiae* STRAINS ON LIGNOCELLULOSIC PRE-TREATED

3.1 Introduction

Energy and bioproducts obtained from vegetal biomasses represent the best eligible raw materials in alternative to petroleum in meeting increasing global sustainable growth and food demand (Keating et al., 2005). Lignocellulose derived feedstock are abundant, poorly utilized, worldwide accessible and have got a high carbohydrate content around 70%. Despite these good features, the close association of carbohydrate with lignin in the plants cell wall makes pre-treatment necessary to release them for subsequent enzymatic hydrolysis and fermentation. The bio-processing of lignocellulose inevitably involves different form of chemical treatments. The two most common methods are diluted acids hydrolysis and acid catalyzed steam explosion (Garrote et al., 1999; Keating et al., 2006). The effects of these pre-treatments lead to generate and release several lignocellulosic degradation products that can have inhibitory effects on subsequent *S.cerevisiae* fermentation (Klinke et al., 2004). In order to obtain an economic and feasible conversion process, one of the biggest challenge to face by industrial biotechnology is to reduce the inhibitory compounds concentration or, alternatively, to increase the robustness of the cell factories.

But why these compounds turn to be toxic for the cells? The contribution of organic compounds, such as furans, weak acids and phenolic derivates, produced by lignocellulose degradation is significant, and the proportion of these inhibitors depend on the lignocellulose starting composition. As example softwoods contain more lignin than hardwoods, where however is more acetylated (Keating et al., 2006; van Maris et al., 2006; Palmqvist and Hahn-Hagerdal 2000; Palmqvist and Hahn-Hagerdal, 2000b; Sun and Cheng, 2002; Galbe and Zacchi, 2007). Consequently the degradation products depend on the starting biomass and treatment conditions used. Among these inhibitory compounds the most important are: aldehydes, like furfural and 5'-

hydroxy-methyl-furfural (HMF), which result from sugars degradation at high temperature. Furfural and HMF can interact with cellular structures, generate oxygen reactive species (ROS) and inhibit the enzymes of central carbon metabolism, causing thus reduction of fermentation rate, growth arrest, lag-phase prolongation, and reduction of ethanol yield and/or productivity (Almeida et al., 2011). At permissive levels *S.cerevisiae* can slowly reduce furfural and HMF in less toxic compound like furfuryl alcohol and HMF alcohol through alcohol dehydrogenases pool; however, these enzymes use the redox potential of NAD^+/NADH couples, kidnapping it to regular cellular functions (Taherzadeh et al., 2000; Almeida et al., 2008; Almeida et al., 2011). Other important lignocellulosic inhibitory compounds are weak acids, like acetic acids, formic acid, lactic acid and levulinic acid deriving from degradation of acetylated hemicellulose and breakdown of furfural and HMF molecules (Palmqvist and Hahn-Hagerdal, 2000; Almeida et al., 2011). Acetic acid and lactic acid are known as lag-phase prolonger and growth and ethanol production inhibitor; acetic acid (6 g/L) is generally speaking more inhibitory than lactic acid (25 g/L) (Narendranath et al., 2001), and normally present at higher concentrations in pre-treated materials. The effects of these acids are strongly dependent on pH: in fact, at pH below pKa value the acids remain in undissociated form and can easily diffuse into the cell, where they encounter a higher pH value and consequently dissociate. The release of H^+ ions decrease cellular pH: to counteract this detrimental effect cells need to pump out said protons, but the specific membrane pumps are ATP-dependent. As consequence, the weak acid exposure at low pH negatively affects cell growth and, in turn, ethanol production (Almeida et al., 2011; Bellissimi et al., 2009).

In this chapter we describe the fermentation performance of *S.cerevisiae* strains able to produce L-ascorbic acid (L-AA) and able to recycle the oxidized form of L-AA through the expression of *A.thaliana DHAR* gene codifying for dehydroascorbate reductase; these strains previously engineered in our laboratory showed higher tolerance to oxidative stress, accumulating less ROS than wild type strains. Furthermore these strains also turn to be more tolerant against acetic acid and formic acid than wild type strains (Branduardi et al., 2007; Fossati et al., 2010).

Inside the european NEMO project, belonging to seventh framework program, for production of second generation ethanol, we tested our L-AA producer and L-AA recycling strains in comparison with wild

type and industrial strains, in two different lignocellulosic pre-treated hydrolysates of spruce and giant cane, provided by NEMO consortium members.

3.2 Results

3.2.1 Kinetics of growth of different kind of *S.cerevisiae* strains on lignocellulosic pre-treated biomasses

One of the major goals of the European seventh framework program NEMO project is to find new strain(s) of *S.cerevisiae* able to ferment pre-treated biomass hydrolysates into ethanol. To do that the yeast strains not only have to efficiently co-ferment C5 and C6 sugars, but they also have to tolerate the inhibitory compounds released from biomass pre-treatment. NEMO consortium has provided us with two kind pre-treated hydrolysates. One derives from spruce and has been treated in harsh conditions with SO_2 as catalyst and steam explosion. The other hydrolysate derives from giant cane and has been treated by steam explosion in harsh conditions.
We have used these two hydrolysates for kinetics of growth of two *S.cerevisiae* laboratory strains, one was a common CEN.PK laboratory strain, called CEN.PKc (see methods), and the other was a CEN.PK strain engineered for L-ascorbic acid production on CEN.PK background, called L-AA (Branduardi et al., 2007). We have made a kinetics of growth in both biomasses pre-treated, using spruce hydrolysate as provided, and using giant cane hydrolysate at 50% w/v, after 30 minutes of infusion in 60°C water. The initial pH values for was 2.5 for spruce pre-treated and 3.8 for giant cane pre-treated. As shown in figure 35, the growth rate of the L-AA producing strain is not higher than control, independently from the medium. In giant cane pretreated materials cells accumulates a bit more biomass, but this very likely correlates with the higher initial pH value. The addition of YNB as nitrogen source (grey lines) does not modify the picture.

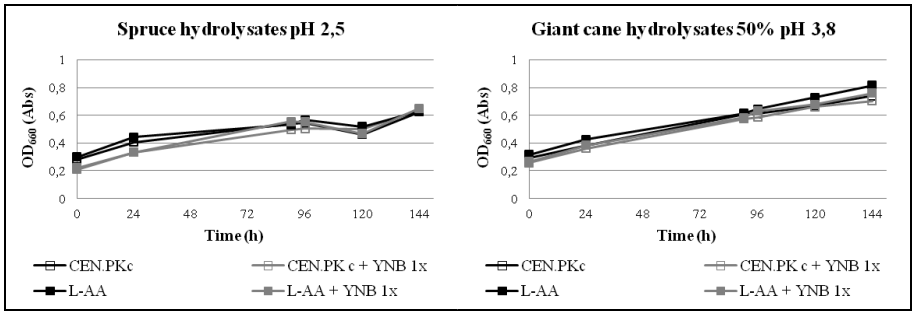

Fig. 35 Shake flask kinetics of growth with spruce and giant cane pre-treated hydrolysates. The CEN.PKc and L-AA producing strains are represented with black line and respectively empty and full squares; the same strains grown in medium added with YNB are represented with grey lines and respectively empty and full squares. The growth was measured as optical density at 660 nm.

Winery strains are naturally more efficient in ethanol production from grape must, that is one of the most ancient biomass hydrolysates. For that reason, said strains were also tested in spruce and giant cane hydrolysate. Furthermore these winery strains were isolated from wine production process as wild type strains, hence they results naturally more robustness than laboratory strains also because they are diploid (in some cases also polyploid) and not only haploid. However when we grew the winery strains called VIN13, AP, BL and SV (see methods and fig. 36), the picture we got was really similar to what seen for laboratory strains, despite an initial higher inoculum was chosen to favour the growth. These results suggests that these hydrolysates with their original pH and compositions represent an hard rock to overcome also for natural wild type winery *S.cerevisiae* strains.

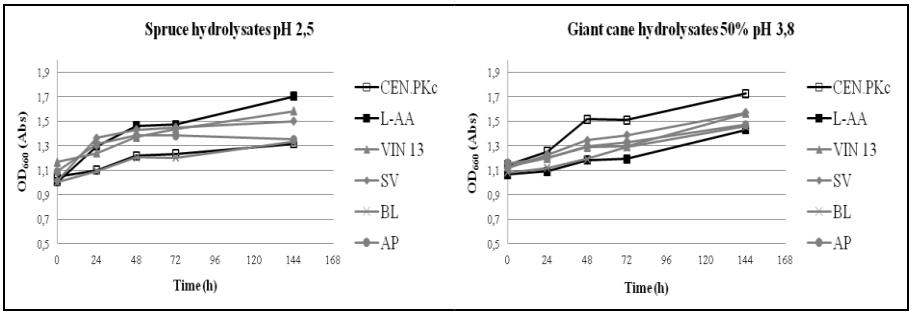

Fig. 36 Shake flasks kinetics of growth with spruce and giant cane pre-treated hydrolysates. The CEN.PKc and L-AA strains are represented with black line and empty and full squares respectively, wild type winery strains are represented in grey lines and full symbols: triangles to VIN13; diamonds to SV; crosses to BL; circles to AP. The growth was measured as optical density at 660 nm

Finally, we tested on provided hydrolysates, the industrial Ethanol Red strain, which was isolated from ethanol production industry. Figure 37 shows Ethanol Red incapacity to grow on both hydrolysates adjusted to pH value of 2.5. These results confirm that the composition and the pH of pre-treated biomass are the limiting factor for a better *S.cereisiae* growth.

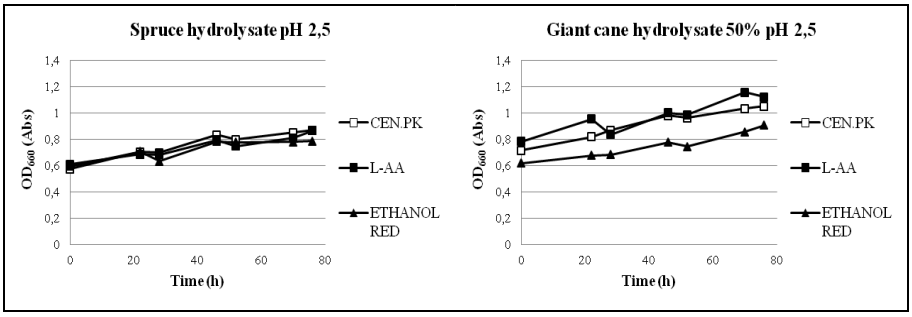

Fig. 37 Shake flasks kinetics of growth with spruce and giant cane pre-treated hydrolysates adjusted to pH 2.5. The CEN.PKc are represented with black line and empty squares; L-AA strain is represented by black line and full squares; Ethanol red is represented with black line and full triangles. The growth was measured as optical density at 660 nm

3.2.2 Kinetics of growth in formulated medium mimicking the spruce hydrolysates composition

After growths on original hydrolysates, we investigated if the inability to grow on hydrolysates is correlated with pH and/or chemical composition. We tested CEN.PKc and L-AA strains on minimal medium mimicking the composition of spruce hydrolysate. We chose this pre-treated materials because show a composition ready to fermentation, while giant cane pre-treated revealed the necessity of an enzymatic hydrolysis to release the sugar monomers (fig. 38).

Spruce pre-treated composition		**Giant cane pre-treated composition**		
Molecules	Amount (g/L)	Molecules		Percentage w/w
D-Mannose	23.01	Glucan	Insoluble fraction	35.71
D-Glucose	14.45	Xylan		3.21
D-Galactose	4.50	Acetyl group		3.26
D-Xylose	10.61	Lignin		26.70
L-Arabinose	3.72	Ash		5.61
Xylitol	0.68	Glucan	Soluble fraction	1.14
Lactic acid	2.67	Xylan		8.21
HMF	1.52	Acetic acid		4.20
Furfural	0.54	Furfural		0.29
Acetic acid	4.07	HMF		0.08
Formic acid	1.34	Formic acid		0.14
		Extractives		8.58

Fig. 38 Composition of spruce and giant cane pre-treated hydrolysates.

Figure 39 shows, the CEN.PKc and L-AA producing strains growth, in minimal medium at pH 2.5 (upper panels) formulated with the spruce hydrolysate sugars concentration (SY) and with or without addition of inhibitory molecules one by one. CEN.PKc and L-AA producing strains growth in similar manner; in fact they grew well on medium with, not only, sugars mix but also in medium added with xylitol, HMF, furfural and lactic acid, but when acetic acid or formic acid were added to sugars medium, the growth was inhibited for both

strains. However when we made kinetics of growth with pH 5 (fig. 39, lower panels), both CEN.PKc and L-AA producing strains were able to grow in every medium.

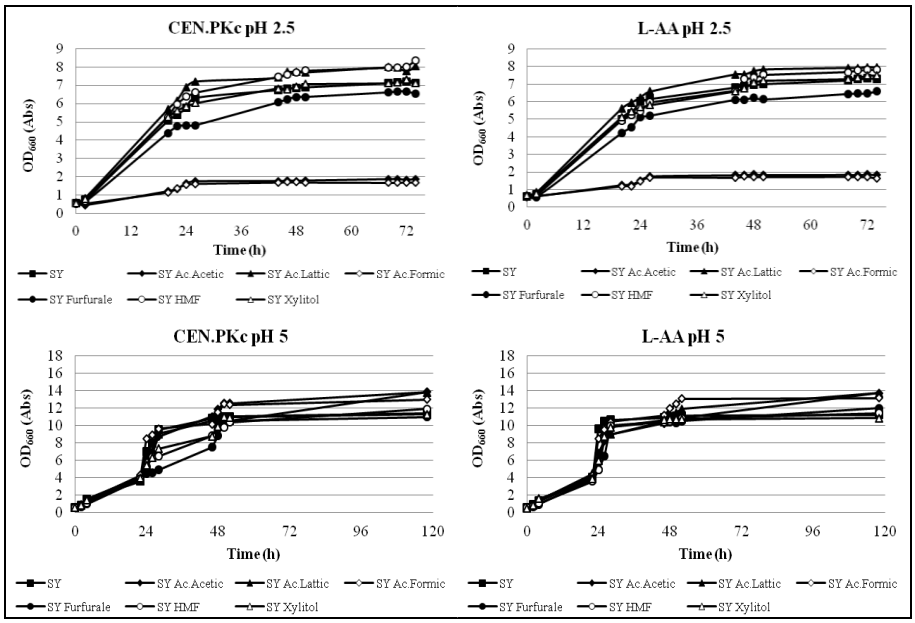

Fig. 39 Shake flasks kinetics of growth in minimal medium mimicking spruce hydrolysate at pH 2.5 and 5. The medium SY contains spruce hydrolysate sugars mix (full squares). Other medium contains SY and: acetic acid (full diamonds); lactic acid (full triangles); formic acid (empty diamonds); furfural (full circles); HMF (empty circles) and xylitol (empty triangles). The growth was measured as optical density at 660 nm.

These results suggest that the interaction between low pH and the singular presence of Acetic acid (4 g/L) and Formic acid (1.3 g/L) is harmful for CEN.PKc and L-AA producing strains growth.
To investigate the role of acetic acid and formic acid we have made kinetics of growth in minimal medium mimicking spruce hydrolysate concentrations. The medium SY this time was added with the permissive inhibitor molecules like lactic acid, xylitol, HMF and furfural (SY INIB medium). Moreover at SY and SY INIB mediums we added acetic acid and/or formic acid. At pH 5 the CEN.PKc and L-AA producing strain growth results not inhibited at all (fig. 40, lower panels). At pH values of 2.5 the CEN.PKc and L-AA producing strain growth are similar, but in this case we have seen a synergistic effect of

permissive inhibitors; in fact the growth decrease of about 50% in both strains (fig. 40, upper panels). However the worst effect occurred when we added acetic acid and/or formic acid, eliminating the both strains growth.
These results suggest one more time the connection between pH and inhibitory compounds; in fact it results dramatic for growth, the presence of weak acids such as acetic acid or formic acid. Furthermore results clear that there is a cumulative inhibitory effect on growth by permissive inhibitory molecules at low pH.

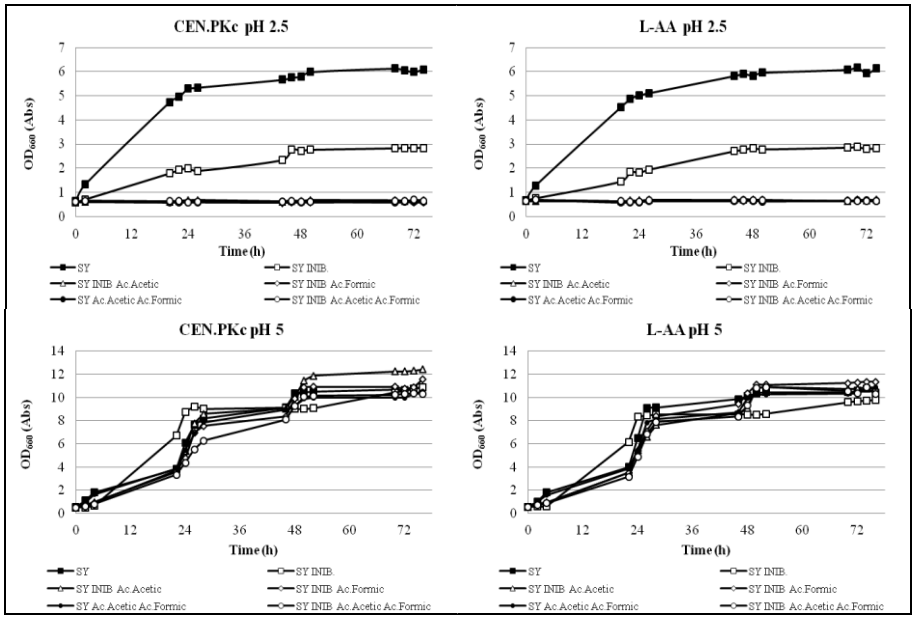

Fig. 40 Shake flasks kinetics of growth in minimal medium mimicking spruce hydrolysate at pH 2.5 and 5. For both strains the medium SY contains spruce hydrolysate sugars mix (full squares); the medium SY INIB contain: HMF, furfural, lactic acid and xylitol (empty squares); other medium contains SY or SY INIB medium with: acetic acid (empty triangles); formic acid (empty diamonds); acetic acid and formic acid (full and empty circles). The growth was measured as optical density at 660 nm.

3.2.3 Kinetics of growth and metabolite profiles of different *S.cerevisiae* strains on formulated medium mimicking the spruce hydrolysate at different pH.

We tested CEN.PKc, L-AA, Ethanol red and an engineered CEN.PK for production and recycle of L-ascorbic acid (L-AAR) strains. L-AAR strain was built previously by our laboratory (Fossati et al., 2010) and show more robustness against ROS and organic acids stresses. We have carried out the kinetics of growth in three different medium mimicking spruce hydrolysate composition: SY medium, made with the sugar mix at concentration reported in figure 38; the SY INIB medium, made adding the permissive inhibitor HMF, furfural, lactic acid and xylitol into the SY medium; the SY FULL medium made with adding acetic acid and formic acid into the SY INIB medium. The three mediums were buffered at pH values 2.5 and 5. As showed in figure 41, at pH 2.5 CEN.PKc and L-AA strains have got the same behavior in the SY and SY INIB mediums, while Ethanol red strain growth a little bit more than other strains. Surprisingly the strain L-AAR grow better than other strains both in SY and SY INIB mediums. However when all the strains were grown in SY FULL medium we haven't seen any growth.

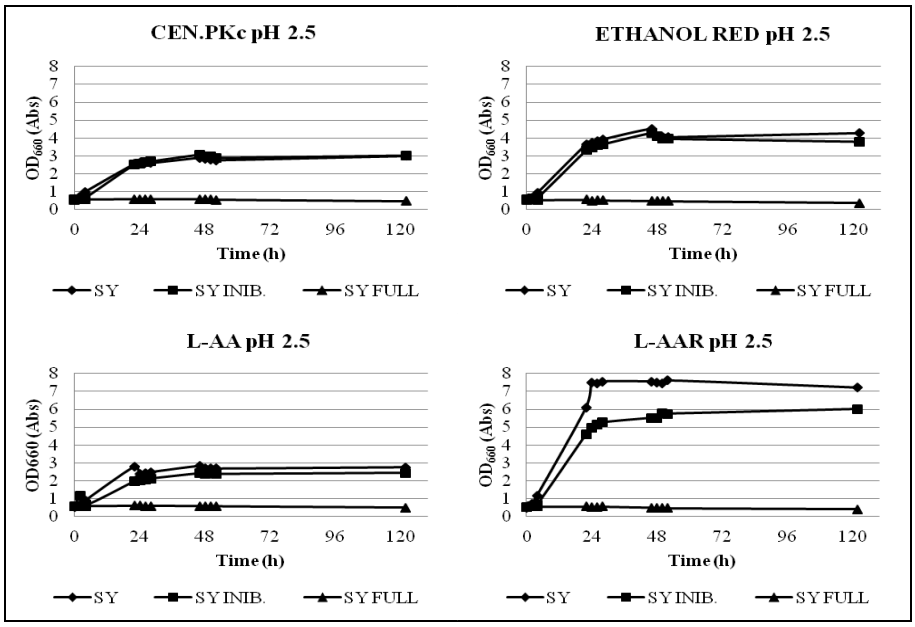

Fig. 41 Kinetics of growth in three different minimal medium mimicking spruce hydrolysate at pH 2.5. The SY mediums are in black diamonds; the SY INIB mediums are in black squares; the SY FULL mediums are in black triangles. The growth was measured as optical density at 660 nm.

When grew the four strains in the same mediums but at pH 5, we have seen the same growth behavior for all strains on the same medium. The only exception was for L-AAR growth in SY medium, where strain growth better than other three yeast strains (fig. 42). When the medium was SY FULL the growth results slower for all strains.

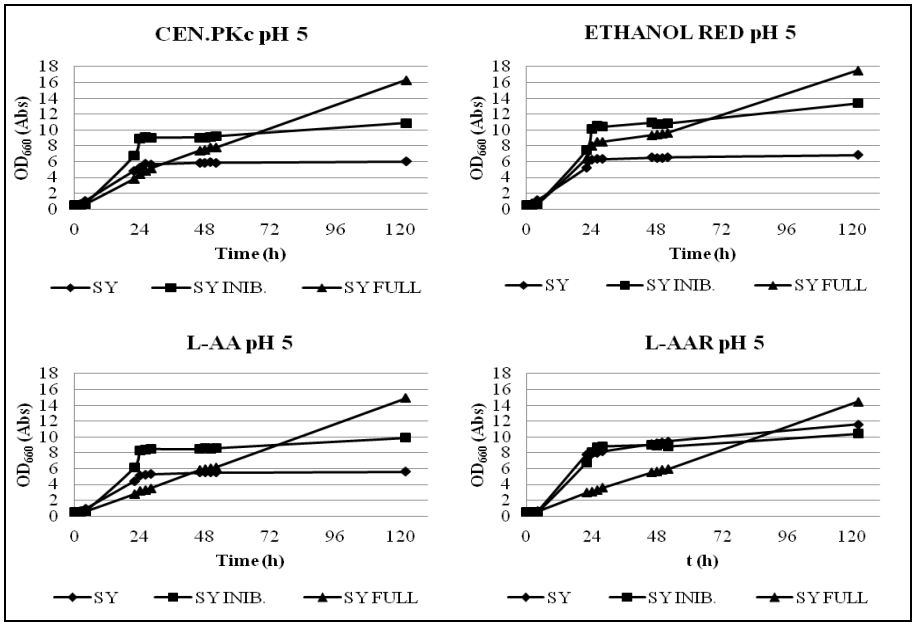

Fig. 42 Kinetics of growth in three different mediums mimicking spruce hydrolysate at pH 5. The SY mediums are in black diamonds; the SY INIB mediums are in black squares; the SY FULL mediums are in black triangles. The growth was measured as optical density at 660 nm.

The ethanol production was higher in Ethanol Red and L-AAR strains than in CEN.PKc and L-AA strains at pH 2.5 (fig. 43). Ethanol Red and L-AAR showed the same production level, but the ethanol productivity were much higher in L-AAR than Ethanol Red in SY and SY INIB mediums. Otherwise at pH 5 we have different production profiles, infact in SY medium Ethanol Red and L-AAR strains shown higher production, yields and productivity than other two strains. In SY INIB medium, the production and yields were quite the same for all strains, but the productivity decrease dramatically for L-AAR strain. In SY FULL medium we have seen an higher ethanol production, yield and productivity by Ethanol Red than other strains (fig. 43).

For Glycerol at pH 2.5 in SY and SY INIB medium Ethanol Red strain shown a higher production and productivity levels than other strains, however, we have seen different yields strain by strain. At pH 5, in SY medium we observed a higher glycerol production, yield and productivity by Ethanol Red than other strains, but in the other mediums the productions, yields and productivities were the same for all strains (fig. 44).
Figures 45 and 46 show sugars consumption in the different situations tested. At pH 5 in every medium D-glucose was always consumed after 24h, while L-arabinose wasn't consumed at all. The sugars D-mannose, D-xylose and D-galactose were measured together because they have got the same retention time when analyzed with HPLC. D-xylose was never consumed and D-mannose and D-galactose resulted fundamental to explain different ethanol and glycerol yield profiles. In fact Ethanol Red and L-AAR strains consumed entirely this sugars, after 24h, in SY medium, while CEN.PKc and L-AA didn't consume them fully. In SY INIB medium the sugars consumption have got the same consumption profile for all strains; in fact they consumed D-mannose and D-galactose entirely after 24h. In SY FULL medium, only Ethanol Red strain was able to consume entirely D-mannose and D-galactose after 24h, while other strains consumed them after at least 48h.
At pH 2.5 (fig. 47) Ethanol Red and L-AAR strains were able to consumed entirely D-mannose and D-galactose after 24h in both SY and SY INIB medium, while CEN.PKc and L-AA consumed only one third of them. The sugars consumption profile explain how at pH 2.5 L-AAR and Ethanol Red shown better growth and productions profiles.

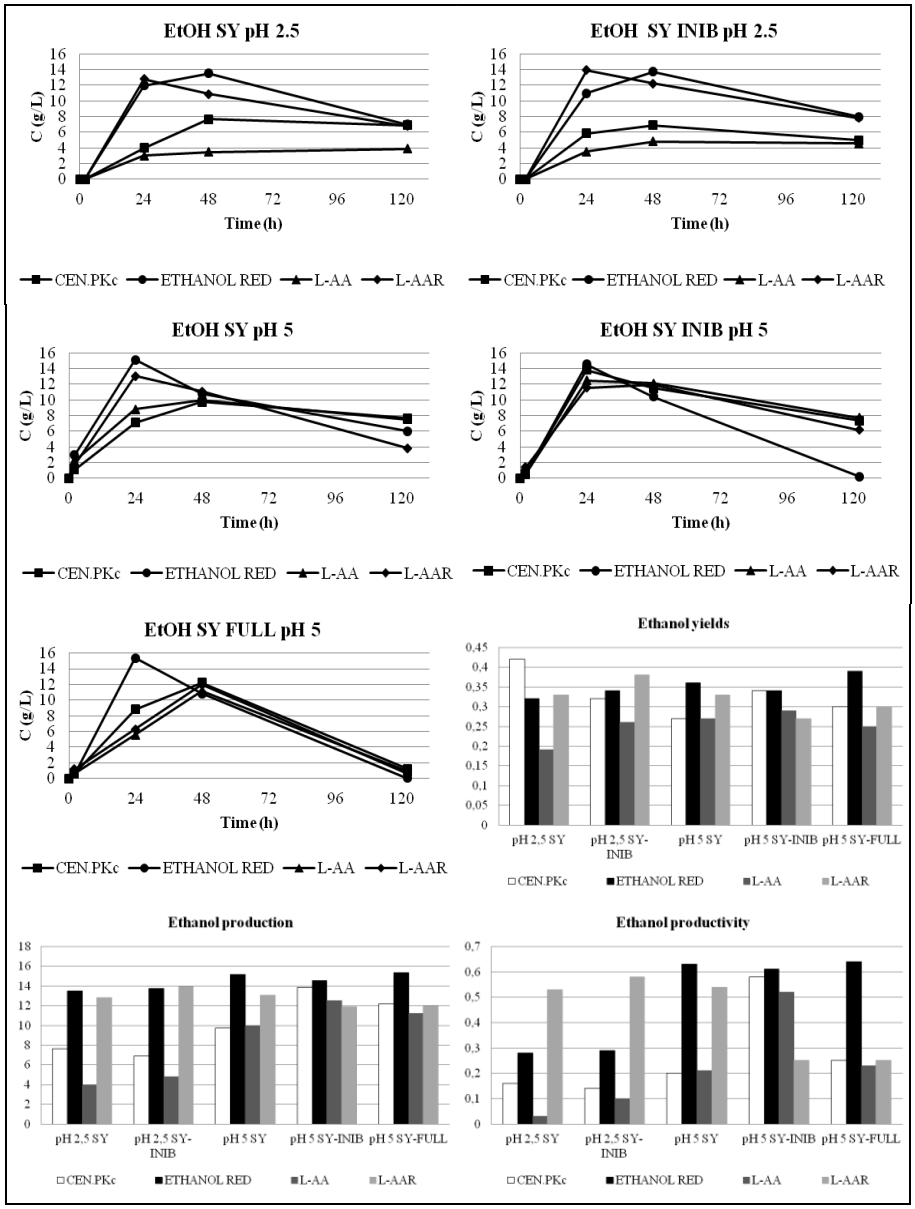

Fig. 43 Ethanol yields, productions and productivities of CEN.PKc, Ethanol Red, L-AA and L-AAR strains in two or three different medium mimicking spruce hydrolysate at pH 2.5 and 5. The ethanol production were measured as g/L. Ethanol yield were measured as g_{EtOH}/g_{sugars}. Ethanol productivity were measured as g/L*h.

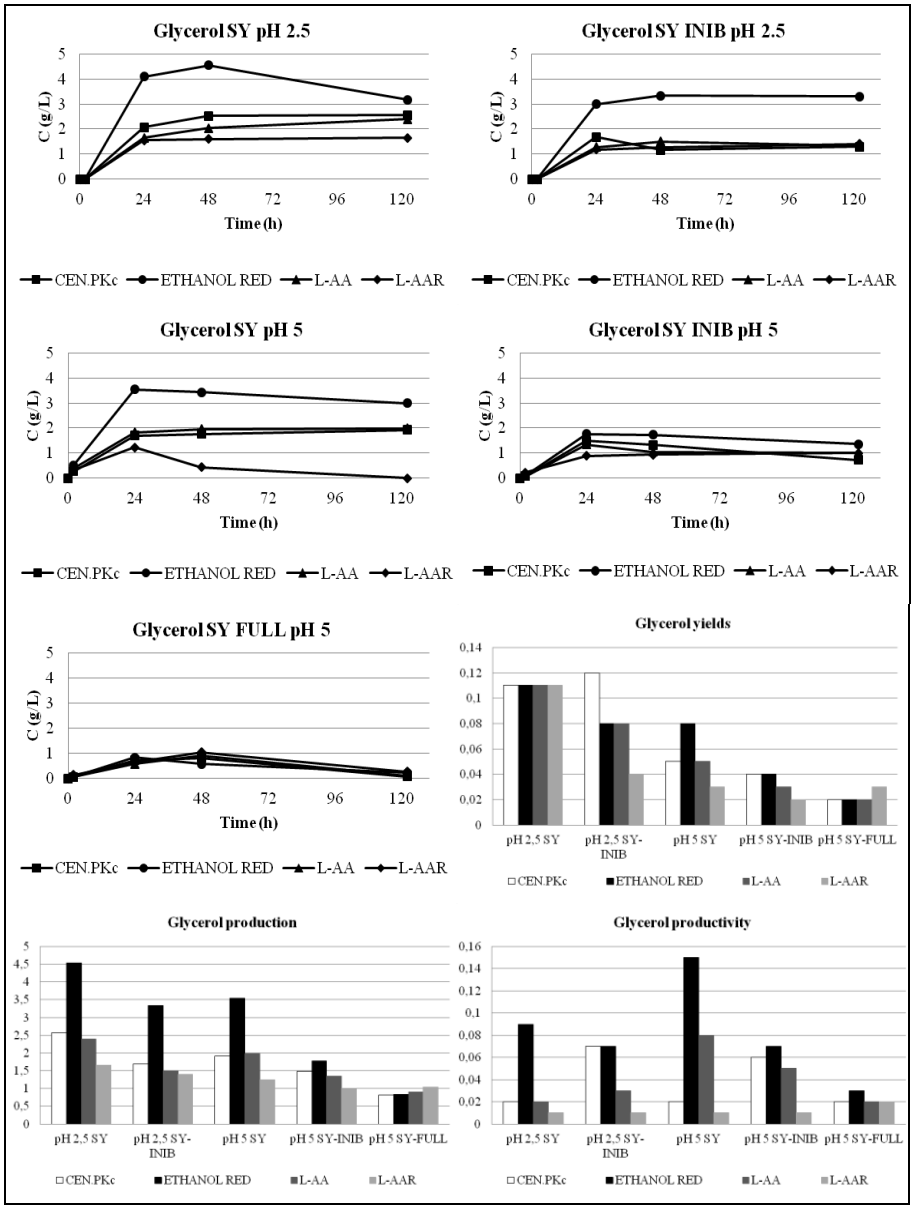

Fig. 44 Glycerol yields, productions and productivities of CEN.PKc, Ethanol Red, L-AA and L-AAR strains in two or three different medium mimicking spruce hydrolysate at pH 2.5 and 5. The glycerol production were measured as g/L. Glycerol yield were measured as g_{Gly}/g_{sugars}. Glycerol productivity were measured as g/L*h.

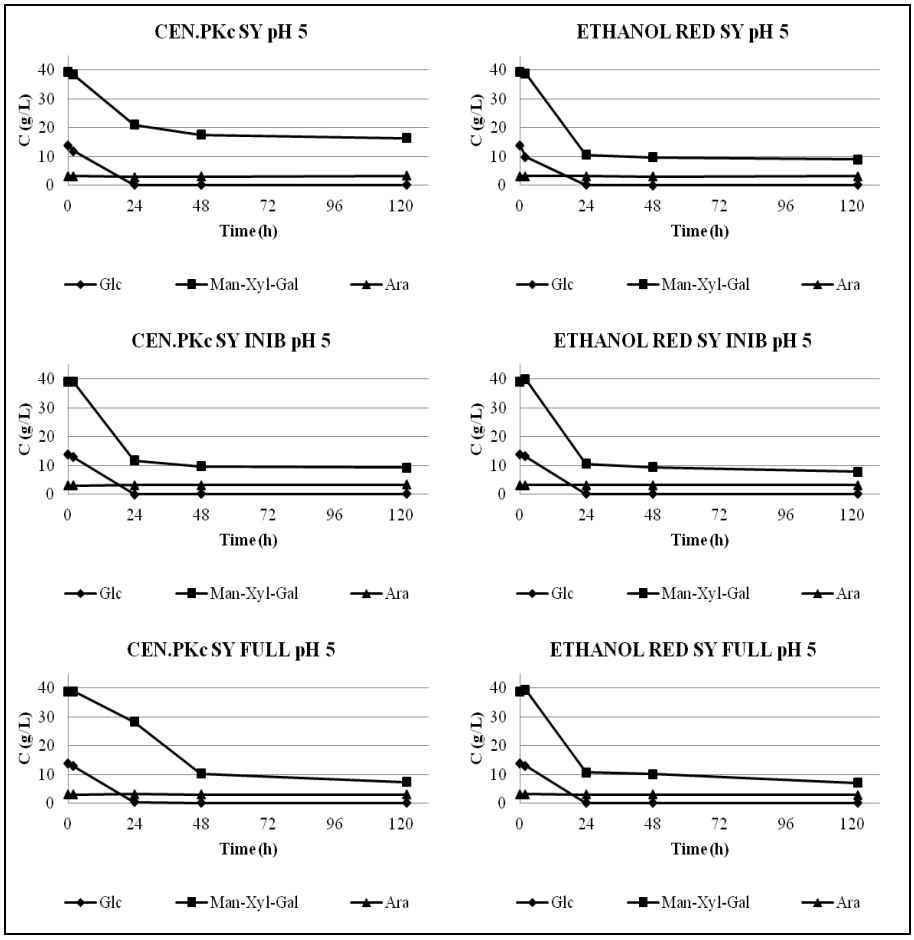

Fig. 45 Sugars consumption profiles of CEN.PKc and Ethanol Red strains in three different mediums mimicking spruce hydrolysate at pH 5. D-glucose are represented with diamonds; D-mannose, D-xylose and D-galactose are represented with squares; L-arabinose are represented with triangles. The concentration are measured as g/L.

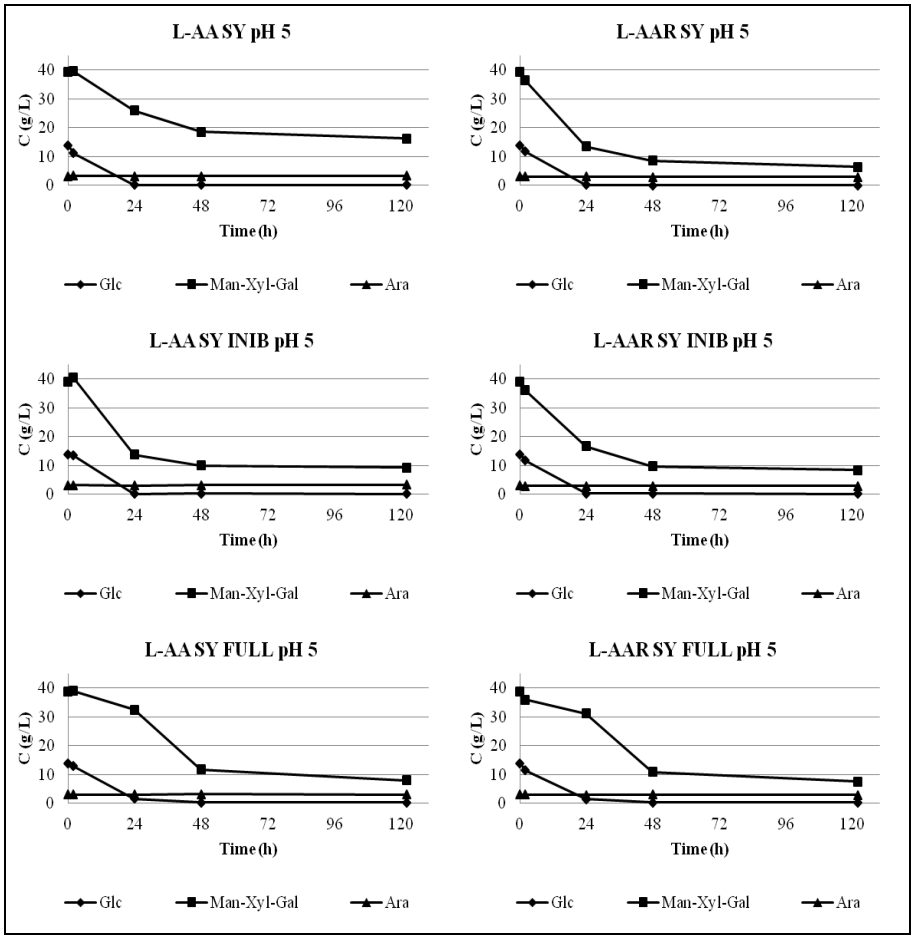

Fig. 46 Sugars consumption profiles of L-AA and L-AAR strains in three different mediums mimicking spruce hydrolysate at pH 5. D-glucose are represented with diamonds; D-mannose, D-xylose and D-galactose are represented with squares; L-arabinose are represented with triangles. The concentration are measured as g/L.

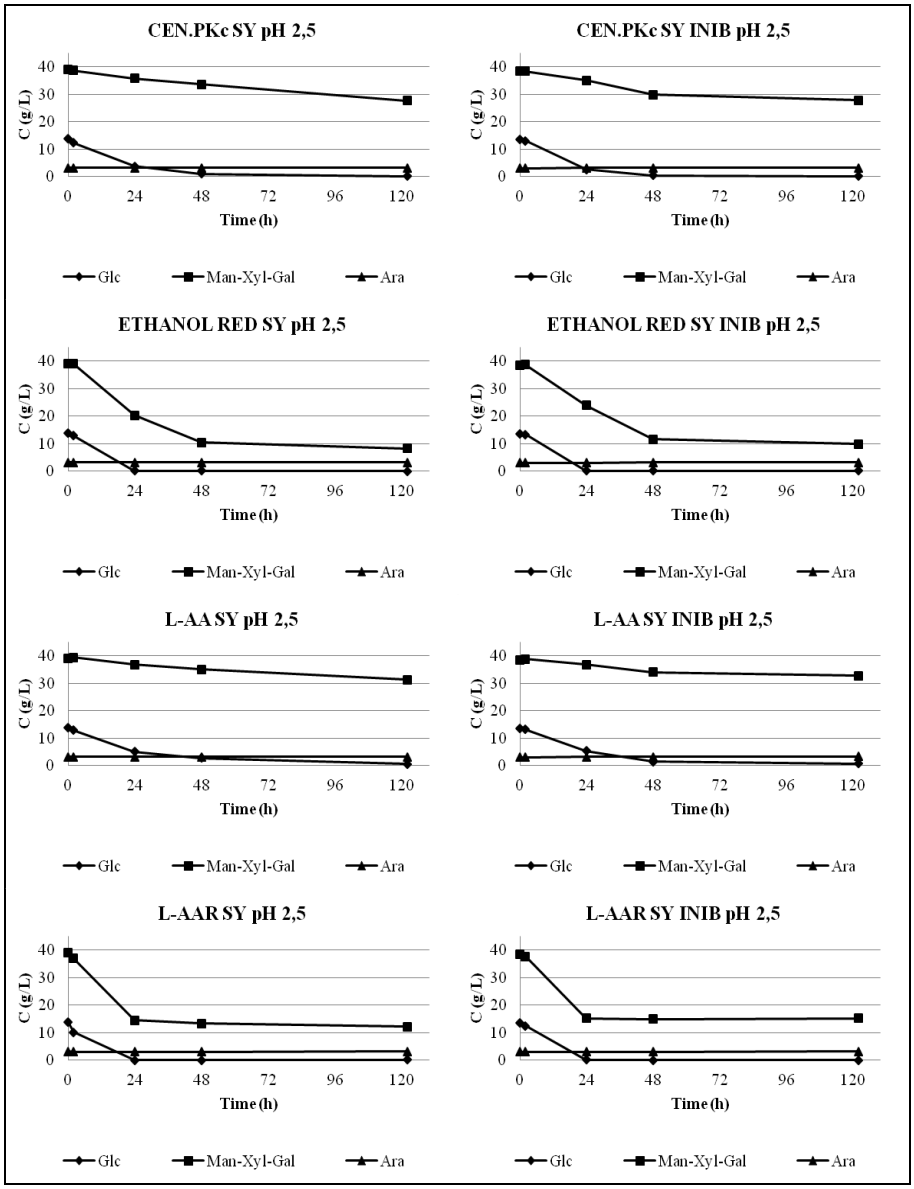

Fig. 47 Sugars consumption profiles of CEN.PKc, Ethanol Red, L-AA and L-AAR strains in two different mediums mimicking spruce hydrolysate at pH 2.5. D-glucose are represented with diamonds; D-mannose, D-xylose and D-galactose are represented with squares; L-arabinose are represented with triangles. The concentration are measured as g/L.

At this point it was interesting to see how these strains detoxified the mediums. At pH 2.5 (fig. 48) all strains were able to detoxify HMF and furfural in 24h, while the amount of xylitol and lactic acid remained the same as the initial concentration. The detoxification occurred only in SY INIB medium, because in SY FULL medium at pH 2.5 the presence of acetic acid and formic acid inhibited the growth of all strains. At pH 5, all strains detoxified all inhibitory permissive compounds after 24h, both in SY INIB and SY FULL medium (fig. 49). Finally the consumption of acetic acid and formic acid in SY FULL medium at pH 5 were different: formic acid were not consumed at all, while acetic acid were slightly consumed 50% (fig.50).

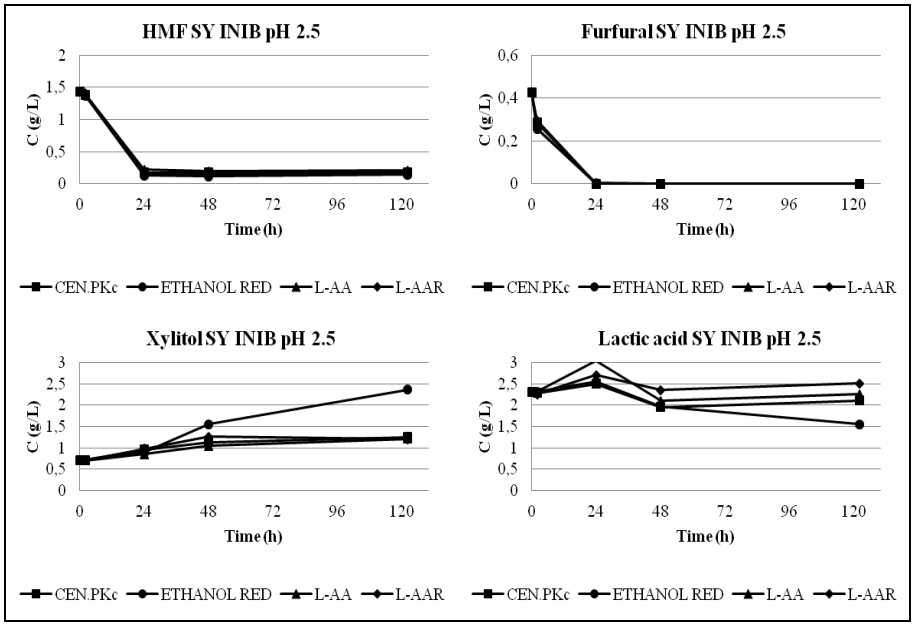

Fig. 48 Inhibitory compounds consumption profiles in SY INIB medium at pH 2.5. HMF, furfural, xylitol and lactic acid consumption were measured for all strains, which were represented as: CEN.PKc squares; Ethanol Red circles; L-AA triangles; L-AAR diamonds. The concentration are measured as g/L.

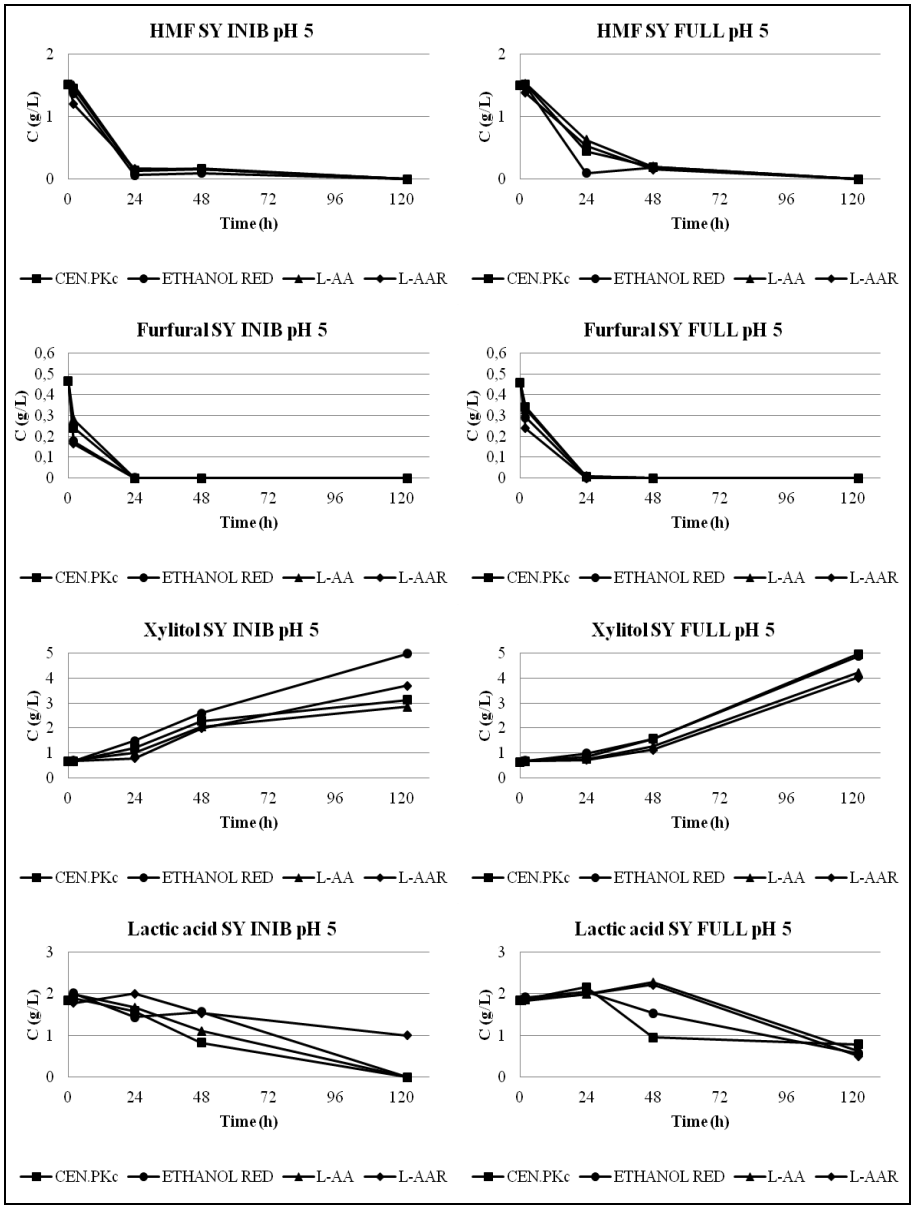

Fig. 49 Inhibitory compounds consumption profiles in SY INIB and SY FULL mediums at pH 5. HMF, furfural, xylitol and lactic acid consumption were measured for all strains, which were represented as: CEN.PKc squares; Ethanol Red circles; L-AA triangles; L-AAR diamonds. The concentration are measured as g/L.

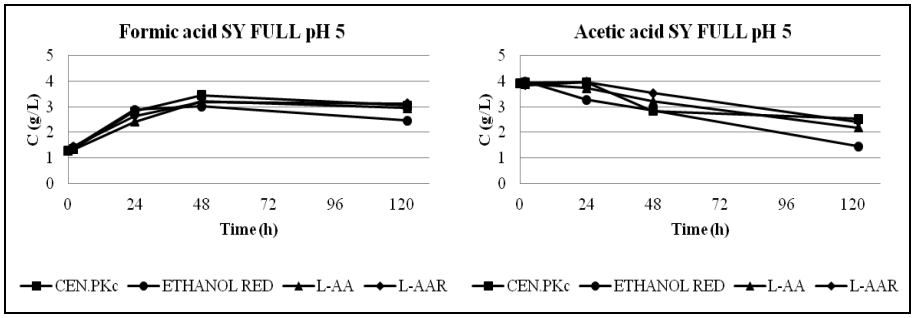

Fig. 50 Formic acid and acetic acid consumption profiles in SY FULL medium at pH 5. Consumption were measured for all strains, which were represented as: CEN.PKc squares; Ethanol Red circles; L-AA triangles; L-AAR diamonds. The concentration are measured as g/L.

These results suggests that L-AAR strain is more robustness than its parental L-AA strain and have got the same growth ability of Ethanol Red strain when grew at pH 2.5 in formulated medium mimicking spruce hydrolysates without strong inhibitors as acetic acid and formic acid. Moreover Ethanol Red results the best strain for ethanol production at pH 5 in the same formulated mediums.

3.3 Discussion

In this work we have reported the growth performance on lignocellulose pre-treated hydrolysates of different kind of *S.cerevisiae* strains, ranging from industrial strain, to winery strains, to laboratory strains, engineered or wild type yeasts. In any case growth performances are preponderantly affected in negative manner by composition of pre-treated, which is dependent on physical conditions applied when the pre-treatment was carried out. We have seen dramatic effects on *S.cerevisiae* strains tested growth in two different lignocellulosic hydrolysates at low pH. But when the inhibitory compounds are taken in singular manner, we have seen that yeast strains are able to growth well, at least that there are acetic acid and/or formic acid. Moreover at low pH is important the additive and synergistic effect of permissive inhibitor like HMF, furfural, xylitol and lactic acid, which decrease the growth. Paradoxically at pH 5, the presence of permissive inhibitor enhance and don't inhibit the growth of yeast strains.

However it is important for an industrial application, like ethanol production, the ability of *S.cerevisiae* strains to overcome the inhibitory effects of these organic compounds at low pH, in order to decrease costs linked to pH correction.
The strain of *S.cerevisiae* engineered for production and recycle of L-ascorbic acid (L-AAR) shows more robustness and higher ethanol production and productivity at low pH likewise to Ethanol Red strain, which remain the best industrial ethanol producer and the reference strains to match, in order to develop new ethanologenic industrial yeast strains.
Finally for a better improvement of *S.cerevisiae* growth on lignocellulose pre-treated hydrolysates, the strains have to be able to overcome the synergistic effect of pH and inhibitory compounds, especially organic acids like formic and acetic acid, that affect negatively the growth on these kind of raw materials (Klinke et al., 2004).

3.4 Materials and methods

3.4.1 Strains and media

S.cerevisiae L-AA, L-AAR and CEN.PKc strains background were: CEN.PK 102-5B (MATa, ura3-52, leu2-3/112, TRP1, MAL2-8c, SUC2 – Dr. P.Kotter, Institute of microbiology, Johan Wolfgang Goethe university, Frankfurt, Germany (van Dijken et al., 2000). The yeast strains are listed in table 6. Yeasts were grown in shake flasks at 30°C and agitation at 160 rpm in rich YPD medium within yeast extract (Biolife) 1% and bacterial tryptone (Biolife) 2% and 20 g/L of D-glucose; the minimal medium mimicking spruce hydrolysate were formulated with 0.67% of YNB (yeast nitrogen base – DIFCO) and: SY medium with D-glucose 14.4 g/L, D-mannose 23 g/L, D-xylose 10.6 g/L, D-galactose 4.5 g/L, L-arabinose 3.7 g/L; SY INIB medium with SY and HMF 1.5 g/L, furfural 0.5 g/L, xylitol 0.7 g/L, lactic acid 2.6 g/L; SY FULL medium with SY INIB and acetic acid 4 g/L, formic acid 1.3 g/L.

Strains	Features
CEN.PKc	CEN.PK 102-5B pYX012 – pYX042 – pYX022
L-AA	Branduardi et al., 2007
Ethanol red	Wild type yeast provided by NEMO project
L-AAR	Fossati et al., 2010
VIN13	Wild type winery yeast from Stellenbosch university (South Africa)
AP	Fermol arome plus wild type yeast (AEB SpA)
BL	Fermol blanc wild type yeast (AEB SpA)
SV	Fermol sauvignon wild type yeast (AEB SpA)

Table 4 List of engineered strains and plasmids used in this work

3.4.2 Metabolites detection

Metabolites analysis were performed on samples harvested at different time of kinetics of growth. The samples were centrifuged and 500 μL of surnatant were analyzed at HPLC. All metabolites were measured with an Aminex HPX-87H column (Biorad).

CHAPTER 5

5. CONCLUSIONS AND PERSPECTIVES

The requirement of new energetic sources, the decrease of fossil fuels and the increase of greenhouse effect, lead the major industrialized country to wage politics in order to develop and utilize new renewable sources. In this global context of fossil energy dependence linked to oil and gas prices, there is now an alternative way which involves the use of vegetal biomasses in order to produce biobased products. Production and development of these new products is the cornerstone of the biorefinery concept. Inside this new industrial concept, the exploitation of lignocellulosic biomasses as raw materials and the development of new microrganims for efficient fermentation of such feedstocks are becoming more and more important for the production of bulk and fine chemicals. However, we still have to do much work to develop better microrganisms: the implementation of metabolic profiles needs to be matched with cellular robustness to be successfully and efficiently applied in lignocellulosic-based industrial processes. Among microrganisms, many biological engineering efforts have been and are still done to develop *S.cerevisiae* strains able to use all the sugars contained in the lignocellulosic biomass, including pentose sugars, and to overcome growth and production despite the inhibition determined by the organic compounds released by lignocellulosic pre-treatments.

In this work we have focused on three major crucial point for a better utilization of lignocellulosic pre-treated hydrolysates by *S.cerevisiae* strains. These three points are: the development of yeast strains able to use D-xylose as carbon source; the development of yeast strains able to face the adverse environmental stress conditions that occur in an industrial process; the development of yeast strains able to overcome inhibitory conditions determined by biomass pre-treatment processes such as the presence of organic compounds and limiting process parameters.

In chapter two, we have described the expression of two bacterial D-xylose oxidative Entner-Doudoroff pathways in *S.cerevisiae*, called Dahms' pathway and Weimberg's pathway. The final products of these two pathways are α-ketoglutarate for Weimberg's pathway, important as precursor in aminoacids production industry, and pyruvate and glycolaldehyde, important as building blocks for wide range of chemical compounds, respectively. The engineered strains

show a slight but reproducible accumulation of biomass having xylose as the sole C source in the medium. Unfortunately, this metabolism is limited by many and different elements. The enzymatic analyses performed on recombinant strains evidenced a poor catalytic ability acquired by the strains. In addition, it is widely known that one of the major problem of *S.cerevisiae* is the absence of specific xylose transporters. Both the attempts of overexpressing the heterologous genes and of expressing possible xylose transporters (homologous as *HXT7* or heterologous as *SUT1-2*) failed in improving the performances of the new strains. It is anyhow important to notice that even the development of *S.cerevisiae* strains bearing the "classical" metabolic pathways for xylose utilization through the PPP needed a long optimization and evolution (Hahn-Hagerdal et al., 2007). So, the results reached in this work can be considered, despite very preliminary, quite promising.

In respect to the heterologous genes expressed, one crucial point to be addressed can be the codon usage, resulting very different between *S.cerevisiae* and *B.xenovorans*. New strains will be developed with ad hoc designed genes. Moreover, the Dahms pathway will be expressed in *E.coli* first of all to finally demonstrate the possibility to transfer it in a cell factory.

Finally, considering the literature data reporting the importance of a direct evolution (as already mentioned), we have also tried to evolve our engineered strains in laboratory shake flasks with repeated re-inoculum. It turned out that, independently from the engineering, there is an intrinsic ability of yest to "learn" how to use D-xylose, despite poorly and accumulating inhibitory compounds. Moreover, the molecular tests confirmed the importance of the gene copy number for a better development of exogenous D-xylose degradation pathways. Despite often and successfully utilized (Kuyper et al., 2004), the shake flask evolution protocol is maybe not the more suitable to obtain the desired result, or it has to be applied after the codon usage optimization mentioned above. In parallel with the development of new strains, we will try to aplly the evolutionary approach in bioreactor, with chemostat cultures.

In chapter three we have described how the *A.thaliana* temperature induced lipocalin (*TIL*) expression in *S.cerevisiae* can improve the strains tolerance against environmental shocks and stresses like oxidative, heat, freezing and organic acids shocks. However when *TIL* is expressed in an engineered *S.cerevisiae* strain, ready for industrial

processes, the positive effect against stresses conditions disappear. We have also seen this behavior when we have tested an industrial strain expressing *TIL* in process conditions like bioreactor fermentation, and we don't see any improvement in ethanol production and productivity at different temperature. *TIL* expression seems, in some way help the cell membrane to restore it when damaged or strengthening it when is under stressful condition, but when *TIL* is expressed in strains with intrinsic high robustness, it doesn't seem give beneficial traits against environmental stresses.

In chapter four we have described the growth performance of different *S.cerevisiae* strains in lignocellulosic pre-treated hydrolysates and we have seen that synergic effect of physical conditions like pH and inhibitor molecules composition can drastically affect the growth, the production and the productivity of yeast strains. Infact at pH 5 all the tested strains are able to growth on formulated medium mimicking spruce pre-treated hydrolysate, but at pH 2.5 acetic acid and formic acid, at concentration tested, determine a strong inhibitory effect on growth and productions of any tested strains. Moreover, if these two strong inhibitors are subtracted to medium at pH 2.5 growth, productions and productivities become possible again, and this condition the *S.cerevisiae* strain engineered for the production and the recycle of L-ascorbic acid performs much better than all the other strains. The ability of *S.cerevisiae* strains to tolerate low pH and the inhibitory effect of organic molecules released after lignocellulosic pre-treatment, are fundamental to develop efficiently industrial strains. Finally, the road to achieve the best industrial *S.cerevisiae* strain able to grow well and able to reach high production and productivity on lignocellulose hydrolysate, in order to meet the industry requests, still appears quite long, but promising and exciting.

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