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Latest Frontiers in the Biotechnologies for Ethanol Production from Lignocellulosic Biomass

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1. Introduction

The development of the industrial and technological society together with the economic and environmental implications, such as global warming and decreasing oil reserves, have been driving worldwide interest in searching for renewable energies to replace fossil fuels. With respect to fossil fuels, biomass-based fuels have the advantage of decreasing greenhouse gas (GHG) emissions. In this context, ethanol produced from biomass, the so called "bioethanol", has become a major energy carrier for a sustainable transportation sector. Bioethanol is an oxygenate fuel with a high octane number (Moon *et al.*, 2009) and it can be used as biofuel either in its pure state (E100) or blended with petrol in various proportions, such as E85, E95, E10 containing 85%, 95% and 10% of ethanol respectively. Among these, E10 not requires any change in engine (Balat, 2009a). In addition, bioethanol has low toxicity and reduces urban air pollution because the carbon dioxide released during its combustion is virtually reused by plants during the chlorophyll photosynthesis. Currently, United States and Brazil are the largest bioethanol producers in the world from corn and sugarcane respectively. However, in some countries with low availability of agricultural lands, the production of biofuels from dedicated crops could lead to direct conflict with food productions. Lignocellulosic materials and, among them, agro-forest residues, could, offer a great potential as biomass source for bioethanol production. In fact, they are virtually abundant and low cost (Perlack *et al.*, 2005). Lignocellulosic materials can be classified in four groups: forest residues (chips and sawdust from lumber mills, dead trees, and tree branches), municipal solid wastes (household garbage and paper products), waste paper and energy crops (Balat, 2010). Lignocellulosic feedstocks are composed primarily of carbohydrate polymers (cellulose and hemicellulose) and phenolic polymers (lignin). Cellulose (C₆H₁₀O₅)_x is a linear polysaccharide polymer of glucose made of cellobiose units that are packed by hydrogen bonds. The structure of this polymer is rigid and compact, so that in order to obtain glucose, the biomass needs pre-treatment that breaks its structure to facilitate the action of the enzymes. The individual cellulose chains are packed and organized into crystalline microfibrils. Within these microfibrils, cellulose is found in two forms, namely amorphous and crystalline. The crystalline form of cellulose is very difficult to degrade. Hemicellulose such as xylan (C₅H₈O₄)_m is a short polymer of pentoses and hexoses

sugars. The dominant sugars in hemicelluloses are mannose (six-carbon sugar) in softwoods and xylose (five carbon sugar) in hardwoods and agriculture residues (Persson *et al.*, 2006). Hemicellulose contains also, galactose, glucose and arabinose. This polymer is amorphous and easier to hydrolyse than cellulose. Lignin $[(C_9H_{10}O_3)(OCH_3)_{0.9-1.7}]_n$ is a phenyl propane polymer that contains many functional groups such as hydroxyl, methoxyl and carbonyl. Unlike cellulose and hemicellulose, lignin cannot be utilized in the fermentation process. In fact, it has high resistance to chemical and enzymatic degradation. Low concentration of various other compounds, such as extractive and ash are also present. Ash consists of minerals such as silicon, aluminum, calcium, magnesium, potassium, and sodium. Extractives include resins, fats and fatty acids, phenolics, phytosterols, salts, minerals and other compounds. The proportions of these constituents vary between different species. Hardwood has a content of cellulose and hemicelluloses around 80% of total feedstock dry matter while softwood contains around 70% of total dry matter (Balat, 2010). On the other side, lignin is more in softwood than hardwood (Balat, 2009b). Table 1 shows the composition of several lignocellulosic materials and their potential ethanol output obtainable from 1 Kg dry biomass of each type. Cellulose generally accounts for 30-60% of the biomass dry weight while the hemicellulose content varies from 10% to 40%, and the lignin content from 10% to 25% except for olive husks in which the lignin content is higher (48.4%, Table 1). Actually, the world's largest ethanol producers are Brazil and USA, which together account for more than 65% of global ethanol production. In Europe (EU), the high oil prices and the ratification of the Kyoto Protocol in 2005 have provided additional incentives to promote the use of alternative fuels. Today, EU is the third producer of bioethanol in the world with a production that in 2009 amounted to 3.7 billion liters (www.platforme-biocarburants.ch).



Fig. 1. European biochemical plants for bioethanol production. Demonstrative plants are marked with a triangle. Pilot plants are indicated with a circle and commercial plants are marked with a square. Information were taken from: <http://biofuels.abc-energy.at/demoplants/projects/mapindex> (TASK IEA 39).

Table 2 shows the detailed bioethanol production in EU for the year 2009 in the major countries.

<i>Biomass</i>	<i>Ash (%)</i>	<i>Hemicellulose (%)</i>	<i>Cellulose (%)</i>	<i>Lignin (%)</i>	<i>Ethanol potential kg/kg*</i>
Poplar		17	49	18	0.37
Eucalyptus		31.8	43.3	24.7	0.42
Maize stalk straw	3	26	38	11	0.36
Wheat straw	1.3	27.6	34	18	0.35
Rice straw	18.9	22.7	37	13.6	0.34
Oat straw	2.6	24.9	37.1	15.4	0.35
Rye straw	1.2	25.7	37.1	17.6	0.35
Barley straw	7.1	44	37	11	0.46
Potato rests	5	11.8	26		0.21
Miscanthus straw	2.7	29.6	44.7	21	0.42
Kenaf			41.9	12.3	0.24
Hemp (wood fiber)		27.5	37.5	22	0.37
Beet tail and beet green	5	10	10	5	0.11
Tobacco stalk	2.4	28.2	42.4	27	0.40
Wood, ailanthus	0.5	26.6	46.7	26.2	0.41
Soybean stalks and leaves		18.5	32.1		0.29
Bagasse		24.6	39.7	25.2	0.36
Tomato plant waste	20.2	6	25.7	19.5	0.18
Garlic waste	17.1	6.9	24.2	8.5	0.17
Vines #		29.42	19.80		0.28
Olive husk	4	23.6	24	48.4	0.27
Agrarian residues		17	32		0.27

Table 1. Composition of some lignocellulosic materials and theoretical ethanol yields. (source: Phyllis database for biomass and waste);* calculated as: 1) cellulose:glucan->glucose->ethanol; 2) hemicelluloses: xylane->xylose->_ ethanol; # data from ENEA

The EU's biggest producer is France with 1250 million liters mainly from beet and molasses. Germany comes second (750 million liters) followed by Spain with 465 million liters. In this country, the goal was reached also thanks the Abengoa's demonstration plant in

Babilafuente (Salamanca). In particular, Abengoa Bioenergy New Technologies has been developing the biorefinery concept to convert a wide range of biomass feedstocks into ethanol, chemicals and energy.

The feedstock includes agricultural residues, wood residues, and energy crops such as switchgrass and poplar (www.abengoabioenergy.com). Table 3 lists some bioethanol plants in the EU using lignocellulosic feedstocks while figure 1 displays the overall distribution of plants, including demonstrative pilot and commercial scale (figure 1), using biochemical conversions to obtain ethanol.

2. Bioethanol production from lignocellulosic raw material

The conversion of lignocellulosic materials to bioethanol via enzymatic hydrolysis can be simplified in four major steps: pretreatment, hydrolysis, fermentation and product separation (figure 2). In the next section the main pretreatment strategies will be overviewed.

COUNTRY	Ethanol production (million liters)
Germany	750
Spain	465
France	1250
Poland	166
Sweden	175
Italy	72
Hungary	150
Lithuania	30
Austria	180
Belgium	143
Czech Republic	113
Slovakia	118

Table 2. Bioethanol production in Europe for the year 2009 (source: www:platformbiocarburants.ch)

2.1 Pretreatments

The conversion of lignocellulosic biomass into ethanol requires a pretreatment step to change the physical and chemical structure of biomass and to enhance the hydrolysis rate. There are several pretreatment strategies, all aimed at opening the structure of the cell biomass and allow the enzymes to access the internal polysaccharides. The available pretreatments can be grouped in chemical, biological, physical and physicochemical processes.

Chemical pretreatments employ different chemical agents like ozone, acids and alkalis. The ozonolysis can degrade lignin and part of hemicellulose but this technology appears quite expensive.

Sulfuric acid is the most applied acid, but other acids such as HCl and HNO₃ were also reported (Taherzadeh *et al.*, 2008). Dilute-acid hydrolysis can be used either as a pretreatment of lignocellulose for enzymatic hydrolysis, or as the actual method of hydrolyzing to fermentable sugars (Taherzadeh *et al.*, 2007, 2008). In general, it has the

disadvantage of the toxicity due to the unspecific and, sometime, harsh degradation of the biomass matrix. Furthermore it is could be corrosive for employed facilities (Abril D. & Abril A., 2009).

Alkali pretreatment is based on the use of alkaline solutions such as NaOH, Ca(OH)₂ or ammonia to remove lignin and part of the hemicellulose, and increase the enzymes accessibility to the biopolymers.

Most promising is also the wet oxidation in which, the material are treated with water and air or oxygen at temperatures above 120°C for a period of e.g. 30 min. The process represents an effective method in separating the cellulosic fraction from lignin and hemicellulose (Taherzadeh *et al.*, 2008).

Biological pretreatment uses microorganisms such as brown, white and soft-rot fungi which degrade lignin and solubilize hemicelluloses (Sun & Cheng, 2002). In recent years, progresses in bioengineering have led to the development of microorganisms which can attack lignin in the biomass. The biological process is interesting for its low energy requirement. However, the rate of hydrolysis in the biological process is very low (Sun & Cheng, 2002).

Among the investigated pretreatment, the *steam explosion (SE)* appears one of the most interesting since it limits the use of chemicals mostly to the use of saturated steam (Ballesteros *et al.*, 1998; De Bari *et al.*, 2002; Ogier *et al.*, 1999).

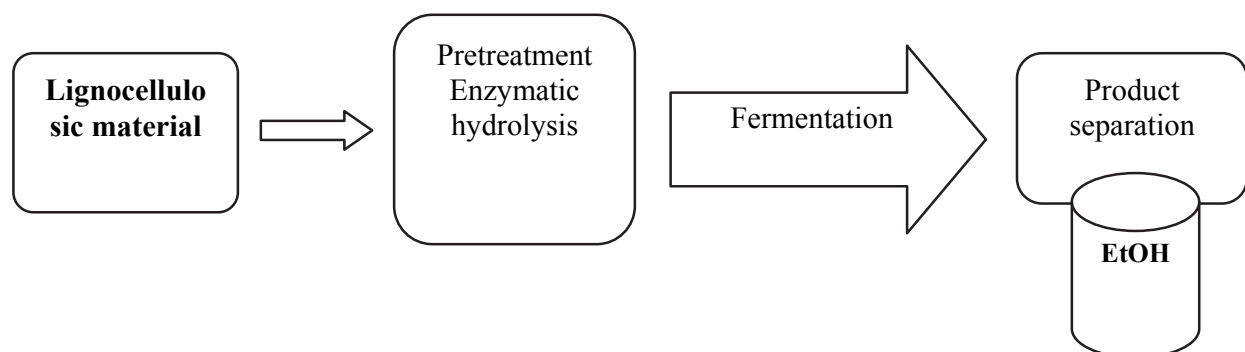


Fig. 2. Main steps of bioethanol production from lignocellulosic materials

Through the saturated water steam at high temperature, SE causes autohydrolysis reactions in which part of hemicellulose and lignin are converted into soluble oligomers. Thus, the lignocellulosic matrix is opened up, and the cellulose surface becomes more accessible to enzymes. The process employs high pressure steam with temperature typically ranging from 160 to 260 °C for few minutes. This is followed by explosive decompression of biomass (Banerjee *et al.*, 2010; Boussaid *et al.*, 1999; Sun *et al.*, 2004; Varga *et al.* 2004).

A number of studies have been already reported in literature describing positive effects in terms of enhancing the enzymatic hydrolyzability of several materials (hardwood and softwood, corn stover, straws etc.) (Cara *et al.* ,2008; Galbe *et al.* 2002; Kobayashi *et al.*,2004; Ohgren *et al.*, 2006; Sun *et al.* 2002; Viola *et al.*,2008) The steam explosion technology, investigated for several years in Italy at the ENEA research Center of Trisaia is now going to be developed at industrial scale thanks to investments from the Italian Mossi & Ghisolfi group. Another physicochemical pretreatment is the *ammonia fiber explosion (AFEX)* in which the biomass is exposed to liquid ammonia at temperature around 90-100 °C followed by instantaneous pressure release. The AFEX process at reduces the lignin fraction but has less effect on the hemicellulose and cellulose fractions. In order to develop improved

lignocelluloses pretreatment strategies the use of CO₂ explosion was also reported (Kumar *et al.*, 2009).

Location	Coordinating organization/Company	Input	Output	Technology	Start - up
Örnsköldsvik (Sweden)	SEKAB		4500 t/a	Enzymes with pretreatment of diluted acid in one step.	2011
Blomsterdalen (Norway)	Weyland AS	0.075 t/h	158 t/a	Strong Acid Process	2010
Ballerup (Denmark)	BioGasol	0.5t/h	10 t/a	Enzymatic hydrolysis and fermentation	2008
Fredericia (Denmark)	Inbicon (DONG Energy)	1t/h		hydrothermal pre-treatment, high gravity hydrolysis, yeast fermentation	2005
Tortona (Italy)	Chemtex-Ghisolfi (Italia)	160.000 t/a	40.000 t/a	Enzymatic conversion. Pretreatment in equipment specifically designed.	2011
Babilafuente, Salamanca (Spain)	Abengoa Bioenergy	35.000 t/a	3950 t/a	Steam-explosion biochemical conversion	2009
POMACLE (France)	PROCETHOL 2G		2700 t/a	Enzymatic hydrolysis followed by yeast fermentation	2011

Table 3. Some bioethanol plants in the EU. (source: <http://biofuels.abc-energy.at>)

On the whole, however, there isn't one general method of pretreatment because different types of raw material require different approaches. For instance, methods such as AFEX and wet oxidation seem to be more successful for agricultural residues whereas steam

pretreatment has resulted in high sugar yields for both forestry and agricultural residues (Hahn-Hagerdal *et al.*, 2006). Table 4 summarizes advantages and disadvantages of some pretreatment processes.

2.2 Hydrolysis step

After the pretreatment, biomass is hydrolyzed to syrups containing monomeric sugars that can be fermented. The most applied methods for hydrolysis can be grouped in two classes: chemical hydrolysis and enzymatic hydrolysis. The latter process is particularly interesting because it is selective in the biomass degradation and can be operated at mild temperature and pH conditions. For several years, the enzymatic hydrolysis of cellulose has been the major target of an international research activity. The main obstacles to the achievement of high process yields have been the existence of crystalline domains within the cellulose and the low efficacy of the enzymes used for the transformation. Considering the specificity of the enzymes action, several components with complementary functions are necessary to attack the different regions in the biopolymers chains. As result, the enzymatic preparations used for the hydrolysis process are complex mixtures of proteins with synergistic actions termed cellulases (Bayer *et al.*, 1998).

Pretreatment	Advantages	Disadvantages
Steam-explosion	Chemical free	Generation of degradation products
AFEX	Low degradation products	Low hydrolysis yields with woody crops
Ozonolysis	Reduction of lignin content, doesn't produce toxic residue	Expensive
Wet-oxidation	Low degradation products	Use of oxygen
Alkalis	Removal lignin, increase accessible surface area	Use of chemicals, long residence time
Acids	Alteration of lignin structure	Equipment corrosion, toxicity
CO ₂ -explosion	Contamination free, increase of accessible surface area	Use of CO ₂
Biological	Low energy requirement	Low hydrolysis rate

Table 4. Advantages and disadvantages of some pretreatment strategies

These are proteins with a molecular weight from 30000 to 60000 AMU with a typical size from min. 30 to max. 200 Å (Fan *et al.* 1987). The surface area of lignocellulosic material is unaccessible to enzymes molecules and this fact implies the need of an initial pretreatment. In fact, the rate of the cellulose enzymatic hydrolysis depends by the structure of cellulose (Balat, 2010) and its crystallinity. In effect, the rate of hydrolysis of amorphous cellulose is 3-30 times faster than that of high crystalline cellulose (Lynd *et al.*, 2002).

Cellulase production is common in a large variety of fungi like *Trichoderma*, *Aspergillus*, *Penicillium* (Galbe *et al.*, 2002). The most frequently reported sources of cellulose is the fungus *Trichoderma reesei* which produces an extracellular and efficient cellulase enzyme system (Jana *et al.* 1994)

In particular, the cellulases mix is constituted of *endo1,4 β -D-glucanase*, *exo1,4 β -glucanase* and *β -glucosidase*. The hydrolysis of hemicellulose is carried out by hemicellulolytic enzymes that include mostly endoxylanase, exoxylanase and β -xylosidase (Saha, 2004).

Most of these cellulolytic cocktails are present in commercial preparations supplied by several biotechnological companies such as Novozymes (Denmark), Genecor (Palo Alto, CA), Iogen (Canada). Recently Genecor launched a new class of enzyme called "Accelerase 1500", which have an enhanced β -glucosidase activity. Similarly, Novozymes has recently produced the Cellin CTEC mixtures having improved activities with respect to the traditional Celluclast.

The commercial preparations are often compared on the base of their activities assayed by standard protocols (e.g. FPU, filter paper units). However the complexity of the lignocellulosic substrates does not make easy the prediction of the enzymes dosage on the base of the standard activities (Kabel *et al.*, 2006). As consequence, the process must be tailored to the specific biomass used.

The enzymes activity mainly depends on the process temperature. An increase of temperature of 20-30°C can introduce a significant improvement of the hydrolysis rate. However, the enzymes are proteins and high temperatures cause their denaturation. In this regard, thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosics. In particular, thermostable enzymes have several advantages like higher stability and higher activity that decrease the optimal dosage needed for the process. Some thermostable enzymes have been isolated from bacteria thermophilic including the *Rhodothermus* strains (Hreggvidsson *et al.*, 1996) and *Thermotoga* (Bok *et al.*, 1998; Bronnenmeier *et al.*, 1995; Evans *et al.*, 2000)

Recently, a new mix of three thermostable enzymes (*cellobiohydrolase*, *endoglucanase* and *β -glucosidase*) were cloned and produced in *Trichoderma reesei* (Viikari *et al.*, 2007). The obtained cellulases mixture was then added with thermostable xylanase and tested at high temperature for the hydrolysis of steam pretreated spruce and corn stover. The results showed that the new enzymatic formulation had an activity at 65°C, 25% higher than the maximum activity of commercial reference enzymes.

3. Fermentation of lignocellulosic hydrolyzates: Conversion of biomass to ethanol by microorganisms

Fermentation of enzymatic hydrolyzates can be carried out by various microorganisms such as several species of bacteria, yeasts and filamentous fungi. Depending on the overall process scheme, mixed or separate C5 and C6 sugars streams can be obtained. While the ethanolic fermentation of glucose, mannose and galactose is well established on large scale (Berg, 2002), the conversion of the pentose sugars, namely xylose and arabinose, is much difficult. However, it was estimated that the complete conversion of pentose sugars to ethanol would reduce the bioethanol production cost by as much as 22% (Sassner *et al.*, 2008). Other essential characteristics required in fermenting microorganisms are high ethanol yields and productivities, minimum formation of secondary metabolites and high tolerance to inhibitors produced during the pretreatment and hydrolysis steps.

The common yeast used for alcoholic fermentation is *Saccharomyces cerevisiae*, which has most of these characteristics. In particular, this specie of yeast catabolizes glucose to ethanol very efficiently by means of the Embden-Meyerhof and Parnas pathway (EMP) followed by alcoholic fermentation under anaerobic conditions (figure 3).

The stoichiometric reaction for glucose conversion into ethanol is described by equation 1:



Considering that the molecular weight of ethanol is 46 g/mole and that of glucose is 180 g/mole and that one mole of glucose produce 2 moles of ethanol, the theoretical yield for ethanol production from glucose is 0.51.

Another microorganism capable to convert glucose into ethanol is *Zymomonas mobilis*, a Gram-negative bacterium which produces ethanol at high yield. Choi *et al.* (2008) reported an ethanol yield of 90.4% from naked barley.

Nevertheless, both *Saccharomyces cerevisiae* and *Zymomonas mobilis* cannot ferment pentoses such as xylose present in the hydrolysates of several abundant lignocellulosic biomass such as residual straws (Keshwani *et al.*, 2009). This inability represents the major obstacle to use these microorganisms for the fermentation of mixed syrups from lignocellulosics.

However, in nature, there are some microorganisms (bacteria and yeasts) which have demonstrated a good capacity of using xylose (table 5). Figure 4 shows the xylose utilization pathways in bacteria and yeasts. Among yeasts, *Pichia stipitis*, *Candida shehatae* and *Pachisolen tannophilus* resulted very interesting for their capacity to ferment xylose. As shown in figure 4, yeasts metabolize xylose by means of the xylose reductase (XR) that converts xylose to xylitol and xylitol dehydrogenase (XDH) that convert xylitol to xylulose. After phosphorylation, xylulose is metabolized through the pentose phosphate pathway (PPP) (Zaldivar *et al.*, 2001).

Generally, XR is an enzyme NADPH cofactor dependent while XDH is NAD⁺ cofactor dependent (Agbogbo & Coward-Kelly, 2008). When the process is carried out under anaerobic conditions, the production of xylitol is favoured and this reduces the final ethanol yield. Among the wild type yeasts fermenting xylose, *Pichia stipitis* was considered the most promising (Agbogbo & Coward-Kelly, 2008) because it has a XR capable to use as cofactor both NADPH and NADH. For this reason, under anaerobic conditions, xylose fermentation in *Pichia stipitis* is carried out by using NADH.

Accordingly, *P. stipitis* produces less xylitol compared to others xylose fermenting yeasts (Agbogbo & Coward-Kelly, 2008). Nevertheless, the use of *P. stipitis* and others wild yeasts for the xylose fermentation is limited by the reduced capacity of using xylose when also glucose is present in the hydrolyzates. In fact, many of these microorganisms have a diauxic growth: when they are in a medium containing mixed sugars, glucose is consumed as first and the others sugars are metabolized after its depletion resulting in a low productivity.

Furthermore, the fermentation capacity of natural *P. stipitis* depends in a critical way on the preservation, through the process of the microaerophilic conditions. Several investigation on the effects of aeration rate on the fermentation of glucose and xylose by *P. stipitis* have established that a low aeration rate is necessary for an optimal conversion of these sugars to ethanol. In detail, an ethanol production rates of 0.35 and 0.13 g g⁻¹ h⁻¹ were reached respectively on glucose and xylose by using oxygen uptake rates below 0.005 mol l⁻¹ h⁻¹; however because the substrate uptake rate is the rate-limiting step, a high cell concentration is needed to obtain high volumetric productivities (Grootjen *et al.*, 1990).

Unlike yeasts, bacteria fermenting xylose directly convert xylose to xylulose (Zaldivar *et al.*, 2001) through the xylose isomerase (XI) (figure 4).

There are some bacteria that have a natural capacity to use pentoses (table 5). *Escherichia coli*, for instance, is a bacterium gram-negative with a facultative anaerobic behavior which

metabolizes pentoses via the PPP. However, the wild strain of this bacterium produces a small amount of ethanol. Thanks to the recombinant DNA technology, it has been possible to transform this microorganism into a bacterium, *E coli B KO11*, capable to produce ethanol with high yields (Ohta *et al.*, 1991).

Some *lactic acid bacteria* (LAB) were also investigated for their ability to produce ethanol. Among them, *L. buchneri* strain NRRL B-30929 can metabolize glucose and xylose simultaneously (Liu *et al.*, 2008) but produce undesirable bio-products such as acetate and lactate.

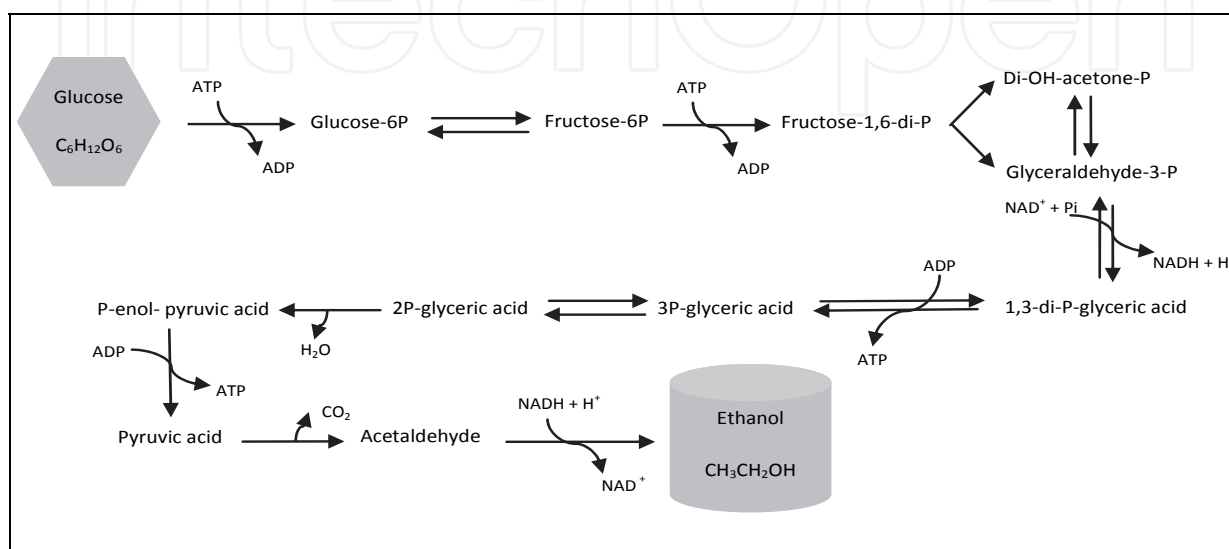


Fig. 3. Metabolic pattern from glucose to ethanol in *S. cerevisiae*. Under anaerobic conditions, pyruvic acid is converted into ethanol by alcohol dehydrogenase.

Thermophilic anaerobic bacteria such as *Clostridium thermohydrosulfuricum* and *Thermoanaerobacter ethanolicus* (table 5) have also been considered for their ethanol production (Balat, 2010). Using thermophilic microbes have several advantages such as the possibility to perform simultaneous hydrolysis and fermentation at high temperature (Knutson *et al.*, 1999). However the low ethanol tolerance of thermophilic anaerobic bacteria represents an obstacle for their industrial application.

Table 5 summarizes the performances of the most common wild type microorganisms. On the whole, the major part of these microorganisms has low productivities. Therefore the scientific community is trying new approaches to achieve the goal of using all the biomass carbohydrates with high efficiency. The following paragraphs describe some breakthroughs obtained in the fermentation of pentoses.

3.1 Cofermentation of mixed hydrolyzates

The simultaneous fermentation of glucose and xylose in the hydrolyzates is one of the most ambitious challenges in the field of bioethanol production because this would simplify some process steps and, as consequence, could reduce capital and management costs.

Certainly, the use of wild yeast co-cultures is a mature approach for the fermentation of mixed syrups. In co-cultures experiments, various combinations of yeasts were tested: the most commonly used co-cultures were constituted by cells of *P.stipitis* and *S.cerevisiae* thanks to the ability of *P.stipitis* to metabolize xylose and the efficient consumption of glucose by *S.cerevisiae*.

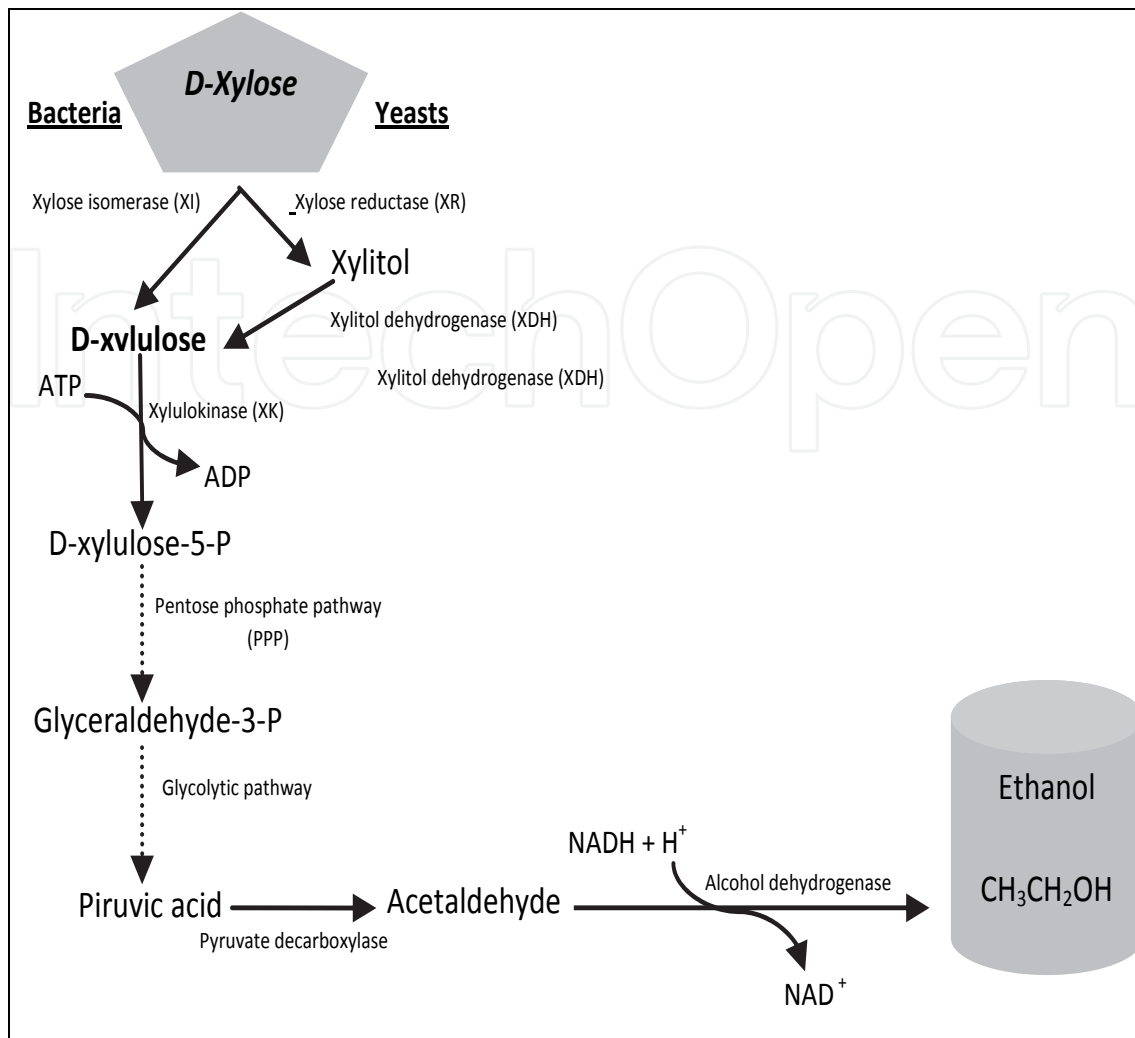


Fig. 4. Metabolism of xylose in yeasts and bacteria.

However, co-cultures of these yeasts do not always ensure the complete conversion of xylose because of the diauxic behavior of *P.stipitis* (Nakamura *et al.*, 2001) and, in batch co-cultures, the production of ethanol from *S. cerevisiae* could worsen the performance of *P. stipitis* whose ethanol toxicity threshold is around 3% (De Bari *et al.*, 2004; Delgenes *et al.*, 1996). Moreover, in the cofermentation process a compromise between the oxygen requirement of the two microorganisms must be used. Often, an efficient ethanol production by co-cultures depends on the competition for oxygen between the two species of yeasts in the medium (Laplace *et al.*, 1991). To favor the xylose consumption by yeast like *P.stipitis* in co-cultures, some researchers proposed the use of a respiratory-deficient strain of *S.cerevisiae* that cocultivated with *P.stipitis* in continuous cultures enabled a substrate conversion rate of 100% (Delgenes *et al.*, 1996). Other researchers proposed the fermentation in immobilized cells bioreactors. (Cuna *et al.* 2008 , De Bari *et al.*, 2004, Lebeau *et al.*, 1998). In particular, enzymatic hydrolyzates from steam treated aspen chips were fermented with *P. stipitis* and *S.cerevisiae* immobilized in Ca-alginate beads. In the best conditions, the process produced 77% of the theoretical yield (De Bari *et al.*, 2004). Moreover, when *P. stipitis* and *S. cerevisiae* are coimmobilized in calcium alginate gel beads, all the cells in the beads external shells metabolize glucose more rapidly than xylose. As consequence, the nutrients flux entering

the internal shells of the beads mainly contain xylose. Furthermore the oxygen level inside the bead is lower than that at the beads surface. These conditions could favor the conversion of xylose to ethanol thus by-passing the *P.stipitis* diauxic behavior.

Recently, others combinations of yeasts were examined to improve the yield of fermentation and the use of xylose (Hamidimotlagh *et al.*, 2007). In detail, co-cultures of two xylose fermenting yeasts, *Kluyveromyces marxianus* and *P.stipitis*, showed process yields of 80% thanks the higher ethanol tolerance of *K.marxianus* than *P.stipitis* (Hamidimotlagh *et al.*, 2007).

3.2 Recombinant yeasts

To overcome the problems related to the inability of wild-type microorganisms to ferment all the sugars in the hydrolysates, several researches were devoted to the development of recombinant organisms which can use both glucose and xylose. In this regard, different metabolic engineering strategies have been explored. The major part of the engineering strategies were based on the construction of recombinants *S.cerevisiae* strains due to its intrinsic robustness and high stress tolerance (Almeida *et al.*, 2007).

The observation that *S.cerevisiae* can ferment xylulose to ethanol (Chiang *et al.*, 1981) led different research groups to develop recombinant strains, cloning the bacterial xylose isomerase (XI) gene in *S.cerevisiae* (table 6).

XI gene from the thermophilic bacterium *Thermus thermophilus* was expressed in *S.cerevisiae*. (Walfridsson *et al.*, 1996) However, the bacterial enzyme XI showed a low activity in the yeast (0.04 U/mg protein⁻¹ Walfridsson *et al.*, 1996) due to an improper folding of the protein in *S. cerevisiae* and to its intracellular precipitation (Gárdonyi & Hahn-Hägerdal, 2003). More recently, the gene XylA encoding the xylose isomerase was isolated from *Pyromyces* sp E2, an anaerobic cellulolytic fungus, and after expressed in *S.cerevisiae*. (Kuyper *et al.*, 2003), The obtained engineered strain, RWB 202, exhibited a xylose isomerase activity of about 1 U/mg protein⁻¹ to say higher than that of the bacterium *Thermus thermophilus*. This finding could be due to the fact that the mechanism of protein folding in *S. cerevisiae* is similar to that of *Pyromyces* (Kuyper *et al.*, 2003). Additional improvements in the RWB 202 were achieved by further genetic modifications. In particular, the strain RWB 218 showed high fermentation rates in mixed syrups, even during anaerobic growth at high sugar concentrations with an ethanol yield of 0.40 g ethanol/g sugars and a low xylitol production (table 6). This engineered strain was obtained from a recombinant strain RWB 217, by prolonging its anaerobic cultivation in automated sequencing-batch reactors on glucose and xylose mixtures. In the recombinant RWB 217 strain the expression of the *Pyromyces* XylA gene was combined with the overexpression of the native *S.cerevisiae* xylulokinase gene and the genes for the conversion of xylulose to glycolytic intermediates. In addition, the endogenous GRE3 gene encoding for a xylose aldolase, was deleted with the effect of reducing the flux of xylose to xylitol, a bio-product that inhibits the activity of XI and decreases the ethanol yields (Kuyper *et al.*, 2005, table 6).

Others strains of *S.cerevisiae* capable to use xylose were generated by expressing the *P.stipitis* genes XIL1 and XIL2 encoding XR and XDH respectively (Jeffries, 2006). The only insertion of these genes enabled *S.cerevisiae* to grow on xylose. However, in most cases, low levels of ethanol were achieved (Kötter & Ciriacy 1993, table 6). In fact, in order to improve the ethanol yields further modifications were necessary.

To obtain this goal, the gene XKS1, encoding xylulokinase XK, from *S.cerevisiae* and the genes XIL1 and XIL2 from *P.stipitis* were inserted into a hybrid host, obtained by breeding of *S.uvarum* and *S.diastaticus*.

The engineered strain obtained in this way, the so called 1400 pLNH32, showed higher yields with respect to recombinant strains containing only XYL1 and XYL2 genes (Ho *et al.*, 1998; Moniruzzaman *et al.* 1997, table 6). Over the years, several recombinant strains of *S.cerevisiae* were obtained by adopting the same approach (table 6, Eliasson *et al.*, 2000; Jeppsson *et al.*, 2002; Karhumaa *et al.*, 2007; Roca *et al.*, 2003; Wahlbom *et al.*, 2003; Zaldivar *et al.*, 2002).

More recently, further improvements of the engineered yeasts performances were obtained by improving the xylose uptake in *S.cerevisiae* through the insertion of genes for xylose transport. In this way, an interesting strain was obtained by the overexpression of the *Opinomyces* xylose isomerase, the *S.cerevisiae* xylulokinase and the *P.stipitis* gene SUT1 encoding for a sugar permease (Madhavan *et al.*, 2009). A more efficient xylose-utilizing strain was isolated from the recombinant strain so obtained, by serial cultivations in minimal media containing only xylose as carbon source.

The xylose adapted strain, ADAP28, showed good performances in the fermentation tests (table 6, Madhavan *et al.*, 2009). Recombinant strains obtained in laboratory were not always applicable at industrial scales because of their instability (Hann-Hägerdal *et al.*, 2007a). In fact only a limited number of engineered strains used at industrial scale have been described in literature. The major part of these strains are genetically modified to express the *P.stipitis* genes XIL1 and XIL2 in the *S.cerevisiae* host and overexpressing the endogenous XK (Hann-Hägerdal *et al.*, 2007b).

Some of the industrial recombinant *S.cerevisiae* strains used in the fermentation of lignocellulosic hydrolysates are summarized in the table 7. With the exception of F12, all of the strains reported in table 7, showed an ethanol yields of more 0.4 g ethanol/g sugars consumed (Hann-Hägerdal *et al.*, 2007b). Finally, given the restriction on GM organisms in many countries, some researchers investigated non-GM strains of *S. cerevisiae* capable to use xylose efficiently (Attfield & Bell, 2006). Attfield and Bell developed a native strain of *S.cerevisiae* capable of using xylose as a sole carbon source by means of natural selection and breeding. The authors claimed that this innovative approach could open new attractive ways to develop yeasts for lignocellulosic substrates.

3.3 Fermentation schemes and technologies

Industrial fermentation processes are traditionally classified in batch, fed-batch and continuous process (figure 5). The choice of the suitable process depends on the type of lignocellulosic hydrolysate and on the properties of the microorganisms employed. Currently, most of the bioethanol process schemes follow the same process employed for centuries in the beverage industry. This strategy is based on the **batch** technology in which substrate and cells are introduced simultaneously into the bioreactor (figure 5). At the end of fermentation, the bioreactor is washed, sterilized and then new medium is introduced. The batch technology is low cost and provides easy operations with reduced risks of contamination given that nothing is added into reactor after the initial inoculation. However, when lignocellulosic biomass is processed, the presence of inhibitor compounds could make the batch process unsuitable (see section 4). The inhibitors effect in the batch reactor can be reduced by increasing the initial cell density in order to exploit the intrinsic capacity of many microorganisms to detoxify the lignocellulosic broths. In **fed-batch** fermentation the substrate is added progressively while fermentation proceeds (figure 5). This process is widely used in industrial applications (Balat, 2010).

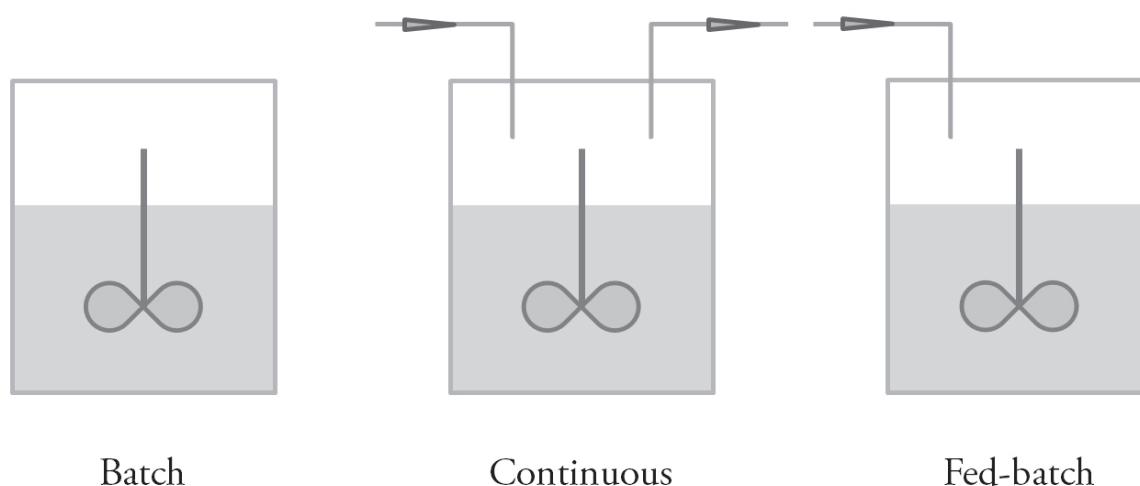


Fig. 5. Schematic representation of fermentation processes.

Fed-batch cultures provide better yields and productivity than batch cultures (Chandel *et al.*, 2007b) thanks to the high cells concentrations during the initial phase of the process. When applied to the fermentation of lignocellulosic hydrolyzates, this approach has the advantage of favoring an “in situ” detoxification through the action of the fermenting microorganisms. In the fed-batch fermentation, the process productivity is influenced by the feed rate of the substrate so that two low feed rates could yield low productivities. (Taherzadeh *et al.*, 1999).

Continuous fermentation is an open system. Sterile medium is continuously added to the bioreactor and an equivalent amount of the converted nutrient solution with microorganisms is simultaneously subtracted from the system (figure 5). Continuous fermentation operations often give higher productivities than batch fermentation (Chandel *et al.*, 2007b), eliminate much of the downtime associated with cleaning and sterilization, and are easier to automate than batch and fed-batch processes. However, the continuous approach is often limited by difficulty of maintaining high cell concentrations in the bioreactor. The use of **immobilized cells** could overcome this problem (Chandel *et al.*, 2007b). Higher ethanol yields compared to free cells were reported in continuous fermentation processes with *S.cerevisiae* immobilized in calcium alginate (Taherzadeh *et al.*, 2001). The next subparagraph contains a survey of the most promising immobilizing matrices and immobilization techniques. In order to make an efficient conversion of biomass to ethanol, several process strategies have been explored, namely Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF) and more recently the Consolidated BioProcessing (CBP). **SHF** consists of two steps: the first involves the enzymatic hydrolysis while the second converts the monomeric sugars into ethanol (Wingren *et al.*, 2003). It offers various advantages such as the possibility to carry out both hydrolysis and fermentation at optimal conditions. In detail, the enzymes can operate at high temperature increasing their performances while microorganisms can work at their optimal temperature and pH. The disadvantages of this method are the risk of contaminations during the process and the inhibition of cellulase and β -glucosidase enzymes by glucose (Xiao *et al.*, 2004). One way to solve the problem of inhibition by glucose is to carry out the hydrolysis and fermentation simultaneously. This process, called **SSF**, combines the hydrolysis step and fermentation in one vessel. As soon as hydrolysis starts, a fermenting microorganism is added into reactor. SSF represents a good strategy with several advantages such as high ethanol yield, lower required amounts of enzymes (Lin & Tanaka,

Microorganisms	Medium	Xilose (g/L)	Glucose (g/L)	Ethanol yield [gp/gs]	Productivity [g/Lh ⁻¹]	References
Yeasts						
<i>Candida shehatae</i> NRRL Y12856	Synthetic	50	n.r	0.45	0.29	Slininger <i>et al.</i> , 1985
<i>Candida shehatae</i> ATCC 22484	Hydrolyzate of hardwood	43.5	9.0	0.14	0.10	Perego <i>et al.</i> , 1990
<i>Pachysolen tannophilus</i> (NRRL Y2460)	Hydrolyzate of hardwood	43.5	9.0	0.21	n.r	Perego <i>et al.</i> , 1990
<i>Pachysolen tannophilus</i> DSM70352	Wheat straw	10.38	16.62	0.44	0.25	Zayed <i>et al.</i> , 1996
<i>Pichia stipitis</i> (NRRL Y7124)	Hydrolyzate of Eucaliptos	30.5	1.5	0.35	0.16	Ogier <i>et al.</i> , 1999
<i>Pichia stipitis</i> NRRL Y-7124	Synthetic	150	n.r	0.39	0.28	Slininger <i>et al.</i> , 1985
<i>Candida shehatae</i> NCIM3501	Hydrolyzate of Sugarcane bagasse treated with ion-exchange resin	21.5	5.84	0.48	0.36	Chandel <i>et al.</i> , 2007a
<i>Pichia stipitis</i> NRRL Y-7124 adapted	Hydrolyzate of Wheat straw overlimed	45	6.40	0.36	0.30	Nigam, 2001a
<i>Pichia stipitis</i> NRRL Y-7124	Hydrolyzate of Eicchornia crassipies treated	54	3.5	0.35	0.18	Nigam, 2002
<i>Pichia stipitis</i> CBS 6054	Synthetic	120.3	n.r	0.381	0.214	Agbogbo <i>et al.</i> , 2007
<i>Candida shehatae</i> FPL-Y-049	Wood hydrolyzate	121.7 (total fermentable sugars)	121.7 (total fermentable sugars)	0.32	0.45*	Sreenath <i>et al.</i> , 2000
Bacteria						
<i>Thermoanaerobacter ethanolicus</i>	Synthetic	10	n.r	0.5	0.12	Carreira <i>et al.</i> , 1983
<i>Clostridium saccharolyticum</i> ATCC 35040	Synthetic	25	n.r	0.21	0.05	Asther & Khan, 1985
<i>Clostridium termohydrosulfuricum</i> 39E	Synthetic	5	n.r	0.39	n.r	Ng <i>et al.</i> , 1981

Table 5. Yeasts and bacteria capable to metabolize xylose; (*calculated from reference, n.r.: not reported)

Recombinant <i>S.cerevisiae</i> Strain	Genotype	Sugar composition	Fermentation conditions	Ethanol yield (g ethanol/g sugars)	Xylitol yield (g xylitol/g xylose)	References
<i>S.cerevisiae</i>	XIL1, XIL2	21.7 g/L xyl		0.07	0.07	Kötter & Ciriacy, 1993
RWB217	XI, XK, del GRE3, overexpressed PPP	20 g/L glu + 20 g/L xyl	Anaerobic batch culture	0.43	0.006	Kuyper <i>et al.</i> , 2004
RWB218	XI, XK, del GRE3, overexpressed PPP, selected for increase glucose uptake	20 g/L glu + 20 g/L xyl	Anaerobic batch culture	0.40	0.003	Kuyper <i>et al.</i> , 2005
RWB202	XI	20 g/L glu + 10 g/L xyl	Anaerobic chemostat cultures	0.39	0.07	Kuyper <i>et al.</i> , 2003
1400 (pLNH32)	XYL1, XYL2, XKS1	50 g/L xylose in YPD	Oxygen-limited batch culture	0.33	0.10	Ho <i>et al.</i> , 1998
TMB3001	XYL1, XYL2, XKS1	50 g/L glu + 50 g/L xyl	Aerobic batch fermentation	0.23	0.08	Zaldivar <i>et al.</i> , 2002
TMB3001	XYL1, XYL2, XKS1	50 g/L xyl	Oxygen-limited batch culture 70 h	0.31	0.29	Eliasson <i>et al.</i> , 2000, Jeppsson <i>et al.</i> , 2002
TMB3001	XYL1, XYL2, XKS1	20 g/L glu + 50 g/L xyl	Anaerobic batch culture	0.33	0.48	Eliasson <i>et al.</i> , 2000, Roca <i>et al.</i> , 2003
TMB3400	XYL1, XYL2, XKS1	20 g/L xyl	Anaerobic batch culture	0.18	0.25	Wahlbom <i>et al.</i> , 2003
TMB3066	XI, XSK1, PPP, del GRE	50 g/L xyl	Anaerobic batch	0.43	0.04	Karhumaa <i>et al.</i> , 2007
ADAP28	XI, XKS1, SUT1, xylose adapted	50 g/L glu + 20 g/L xyl + borate	Fermentation in bottle with a bubbling CO ₂ outlet, 35°C, 40 h	0.48	0.04	Madhavan <i>et al.</i> , 2009

Table 6. Engineered *S.cerevisiae* strain for xylose conversion.

2006; Sun & Cheng, 2002) and contamination reduction during hydrolysis also thanks to the action of ethanol simultaneously produced. However SSF has the disadvantage to operate at temperature and pH conditions that represent a compromise between the optimal conditions for hydrolysis and fermentation. In particular, it is fundamental to consider temperature as the key parameter in the process. In fact, while the cellulase enzymes are more active at 50°C, the yeasts usually work at temperatures lower than 35°C. Several

Strain	Hydrolysate	Fermentation strategy	References
TMB3400	Corn stover steam pretreated	Batch and fed-batch SSF	Ohgreen <i>et al.</i> , 2006
F12	Still bottoms fermentation residue	Batch	Olsson <i>et al.</i> , 2006
TMB3400	Spruce	Fed-batch	Hann- Hägerdal & Pamment 2004
TMB 3006	Spruce	Fed-batch	Hann- Hägerdal & Pamment 2004
424ALNH-ST	Corn stover	Batch	Sedlak & Ho, 2004

Table 7. Industrial *S.cerevisiae* strains fermenting xylose in lignocellulosic hydrolysates.

research efforts were concentrated on the isolation of strains able to work at high temperatures. Good performances have been recently obtained with the thermotolerant strain *Kluyveromyces marxianus* 6556 that showed promising results in the SSF of lignocellulosic agricultural wastes at 37°C (Zhang *et al.*, 2010). In fact various strains of the *K. marxianus* species have the ability to grow at temperature around 40°C and ferment mixed sugars such as glucose, xylose, mannose and galactose (Fonseca *et al.*, 2008). In this regard, Ballesteros *et al.*(2001) carried out several fed-batch SSF tests using *K.marxianus* at 42°C and obtaining ethanol yield of 76% for olive pulp. Rudolf *et al.* (2008), also demonstrated that undetoxified steam-pretreated bagasse could be successfully fermented to ethanol in a SSF process using both natural yeasts (*P.stipitis* CBS6054) that recombinant yeast (*S.cerevisiae* TMB3400). Interesting results were obtained using SSF with other materials such as industrial wastes (Kádár *et al.*, 2004), wheat straw, and sweet sorghum bagasse (Ballesteros *et al.*, 2004). To improve the ethanol yield through the overall consumption of sugars, a variant of SSF has been developed known as Simultaneous Saccharification and Co-Fermentation (SSCF) (Chandel *et al.*, 2007b; Pejo *et al.* 2008) that includes the cofermentation of multiple sugar substrates in the hydrolysates using pentose-fermenting yeast. In conclusion, either SSF or SSCF are preferred to SHF, because both can be performed in the same tank resulting , in lower capital costs, higher ethanol yield and shorter processing time (Chandel *et al.*, 2007b). Recently, a new integrated approach, so called Consolidate BioProcessing (CBP), has been developed. It combines the cellulase production, the cellulose hydrolysis and the sugar fermentation into a single unit operation (Lynd *et al.*, 2005). In other words, CBP combines all the biological steps required for the conversion of lignocellulosic materials to ethanol into one reactor. The process can be drive by a single microorganism or through a microbial consortium capable to ferment pretreated biomass directly (van Zyl *et al.*, 2007). Unfortunately, no natural microorganism exhibits all the features desired for CBP. There are two main strategies to make feasible the CBP process. The first approach consists of cloning and expressing the genes for ethanol production into cellulolytic microorganisms such as *Clostridium cellulolyticum* and *Clostridium thermocellum* (Lynd *et al.*, 2005). Conversely, the other approach constitutes of cloning the genes for cellulolytic activity in the efficient ethanol producing microorganisms such as *S.cerevisiae*. This latter strategy is more viable also thanks to develop of *S.cerevisiae* recombinant strains capable to express cellulases. Most of the cellulolytic enzymes expressed in *S.cerevisiae* are of fungal origin, mainly from *Trichoderma* spp. and *Aspergillus*

spp. (van Zyl *et al.*, 2007). Recently, a promising yeast have been constructed capable to grow on 10 g/L PASC (acid-swollen cellulose) with a subsequent production of 1 g/L ethanol. The recombinant *S.cerevisiae* strain co-expresses a *T.reesei* endoglucanase and a *Saccharomycopsis fibuligera* β -glucosidase (Den Haan *et al.*, 2007). Certainly, further improvements are necessary to optimize the heterologous enzyme expression in order to increase the ethanol yield. For instance, for the conversion of hemicellulose in the CBP process, the microorganism should have also hemicellulase activities. Katahira *et al.* (2004) introduced the genes encoding for xylose utilization from *P.stipitis* into a recombinant *S.cerevisiae* expressing xylanase II from *T.reesei* and β -xilosidase from *A. oryzae*. Despite the noticeable breakthroughs, the CBP approach seems still far from the industrial use and more studies are required to obtain microorganisms capable of producing ethanol from lignocellulosic materials in a single step.

3.4 Innovative bioreactors configurations: Fermentation in immobilized cells bioreactors

Fermentation can be carried in free or immobilized cells bioreactors. The use of the immobilized cells technology (ICT) in the bio-industry has recently received much attention thanks to several advantages: high cell load enhancing the fermentation productivity; feasibility of continuous processing without any interruption. Generally, four categories of immobilization techniques can be distinguished, based on the cell localization and on the interaction mechanisms between cells and supports : “attachment to a surface”, “containment behind a barrier”, “self-aggregation” and “entrapment within a porous matrix” (Karel *et al.* 1985). In the immobilization by **surface attachment**, yeast cells are allowed to attach to a solid support (Verbelen *et al.*, 2006). Cellular attachment to the carrier can be induced through linking agents such as metal oxides, glutaraldehyde or aminosilanes. **Containment of yeast cells behind a barrier** can be obtained through the use of microporous membrane filters or by entrapment into microcapsules. Several polymers can be used as microporous membranes: nylon, polystyrene and polyester. The drawback of this strategy is the membrane fouling caused by the cells growth (Lebeau *et al.*, 1998). The immobilization by **self-aggregation**, known as “flocculation” is based on the natural ability of yeast strains, such as *S.cerevisiae*, to adhere at inert surfaces (Oliveira, 1997). In this process, yeasts form a reversible flocs of thousands of cells (Bony *et al.*, 1997). In particular, adhesion is conferred by a class of special cell wall proteins called “adhesins” or “flocculins” that bind some amino-acid or sugar residues on the surface of the other cells or promote binding to abiotic surfaces (Verstrepen & Klis, 2006). Flocculation is dependent on several parameters, namely the calcium level, the pH and the fermentation temperature (Sampermans *et al.*, 2005). Furthermore, different yeast species present different families of adhesins. The brewer’s yeast *Saccharomyces cerevisiae*, for example has five flocculation genes FLO (Teunissen & Steensma, 1995). For the industrial application flocculation profile was improved through recombinant DNA strategies (Pretorius & Bauer, 2002). Immobilization by **entrapment within porous matrix** is the most widely used method. The matrix is usually composed of agar, agarose, kappa-carrageenan, collagene, alginate, polyurethane, chitosan, polyacrylamide and cellulose. Among these, the most reported in literature is Ca-alginate that is commonly synthesized as spherical polymeric beads with diameter ranging from 0.3 to 3 mm around the cells (Verbelen *et al.*, 2006). Besides the in situ synthesis around the cells, a second entrapment strategy was also reported in which cells are allowed to diffuse into a preformed porous matrix (Verbalen *et al.*, 2006). It was demonstrated that the

immobilization of *S.cerevisiae* in the ICR (Immobilized Cell Reactor) column packed with Ca-alginate beads enables the conversion of concentrated syrups (150 g/L of glucose) with an ethanol yield of 38% in seven hours (Najafpour *et al.*, 2004). More recently, it was found that the use of the *S.cerevisiae* immobilized in the Ca-alginate beads coupled with a permselective separation of ethanol allow to convert diluted hydrolyzates into hydro-alcoholic solutions containing 9 wt% ethanol (De Bari *et al.*, 2009). This process could make to subsequent recovery of ethanol from the fermentation broths more sustainable. In fact, several energetic balances demonstrated that the lowest threshold to make feasible the bioethanol distillation from fermentation broths is 4 wt% (Zacchi & Sassner, 2008). However, the use at industrial scale of Ca-alginate beads is limited by the lack of stability through continuous processes. Other entrapping carriers such as mixed calcium alginate and silica beads, silica film, polyvinyl alcohol (PVA) and tetramethyl orthosilicate (TMOS) were investigated for bioethanol production from mixed sugars syrups by *P.stipitis* (Cuna *et al.*, 2008; De Bari *et al.*, 2007b). In particular, the use of this bioreactor configuration also help to overcome the diauxic behavior of this yeast (Cuna *et al.*, 2008; De Bari *et al.*, 2007b). The obtained results demonstrated that the silica films offer the advantages of immobilizing higher cell concentrations with respect to alginate beads (De Bari *et al.*, 2007b). It was also shown that the ethanol yields obtained by using the TMOS films were higher than those of PVA beads (70% for PVA against 80-82% for TMOS). Another material tested to immobilize cells for ethanol production was the γ -alumina that is a good promoter of ethanol fermentation because of its high porosity and high stability (Kanellaki *et al.*, 1989). The immobilization was carried out by using the spray drier technology. In particular, it was demonstrated that the pre-soaking of γ -alumina particles in a resin solution before the cells immobilization improved the cells uptake and increased the sucrose conversion to ethanol (Isono *et al.*, 1994). Despite the interesting achievements some improvements are still necessary: carriers stability, reduced diffusion coefficients of nutrients and metabolites between the immobilization carrier and the fermentation broth. Furthermore industrial techniques must be optimized for the production of the immobilized biocatalytis at industrial scale.

4. Inhibitory compounds derived from biomass pretreatment: Effect on fermentation step

Fermentation of hydrolyzates represents a critical step in the lignocellulosics-to-bioethanol process not only for the efficient conversion of all the sugars but also for the microbial inhibition due to the pretreatment by-products. In fact, during the pretreatment and the hydrolysis step (chemical hydrolysis) many microbial inhibitors compounds are commonly generated. As reported in literature, these compounds can be classified in three major groups: furan derivates, weak acids and phenolic compounds (Almeida *et al.*, 2007; Palmqvist & Hann-Hägerdal, 2000). **Furan derivates** are mainly constituted by 5-hydroxymethyl-2-furaldehyde (5-HMF) and 2-furaldehyde generated by dehydration of hexoses and pentoses, respectively. These chemical compounds inhibit both the cell growth and ethanol production (Palmqvist & Hann-Hägerdal, 2000).

Several mechanisms were proposed to explain the effect of furfural and 5-HMF on the ethanol fermentation. It was found that in *S.cerevisiae* they inhibit alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH). Furthermore they cause the DNA breakdown resulting in a inhibition of the RNA and

protein synthesis (Modig *et al.*, 2002). Additionally, furan derivatives damage cell walls and membranes (Almeida *et al.*, 2007). However, the inhibition effect of these compounds is dose-dependent (Liu *et al.*, 2004). It was demonstrated that *S.cerevisiae* and *P. stipitis* strains were more sensitive to the inhibition by furfural than 5-HMF at the same concentration (table 8), while combined treatment with furfural and HMF suppressed cell growth (Liu *et al.*, 2004). Nigam (2001a) also found that a furfural concentration of 1.5 g/L interfered in respiration and growth of *P. stipitis*. Delgenes *et al.* (1996) showed that *P.stipitis* growth was reduced by 43%, 70% and 100% when the concentration of HMF was 0.5, 0.75 and 1.5 g/L respectively.

Some microorganisms such as *S.cerevisiae* (Liu, 2006) have the capacity to transform furfural and 5-HMF into less toxic compounds of furfuryl alcohol and 2,5-bishydroxymethylfuran respectively. This process is also known as “in situ-detoxification”.

The **weak acids** such as acetic, formic and levulinic are the most frequent acids present in the hydrolysate from lignocellulosic materials. Acetic acid is produced by de-acetylation of hemicellulose while levulinic and formic acid are formed through the 5-HMF breakdown (Palmqvist & Hann-Hägerdal, 2000). Undissociated acids are liposoluble and therefore can diffuse across the plasma membrane. Once inside the cell, because of neutral pH, dissociation of acids occurs resulting in the cytosolic pH decrease and the cell growth-inhibition. The decrease in the intracellular pH is compensated by the activity of the plasma membrane ATP-ase that pumps proton out of the cell and increases the ATP hydrolysis. This led to a reduction of ATP available for the yeast biomass formation (Russel, 1992, Verduyn *et al.*, 1992). In addition, weak acids reduce the uptake of aromatic aminoacids from the medium (Bauer *et al.*, 2003). The concentration of undissociated acids in the hydrolysate is pH-dependent and, as a result, pH is a crucial parameter during the fermentation step.

Phenolic compounds are produced following the lignin degradation and depend on the biomass source (Almeida *et al.*, 2007). The most common are phenol aldehydes (4-hydroxybenzaldehyde, syringaldehydes and vanillin), phenol ketones and alcohols (hydroquinone, catechol, eugenol, guaiacol). The inhibitor mechanisms of phenolic compounds in the fermenting microorganisms have not yet been completely elucidated (Almeida *et al.*, 2007). Some researches indicated that these compounds partition into cells membranes cause loss of integrity (Palmqvist & Hann-Hägerdal, 2000). It was established that low molecular weight phenolic compounds are more inhibitory than those with high molecular weight (Klinke *et al.*, 2004) and furthermore, the substituent position influenced the compounds toxicity (Larsson *et al.*, 2000). Among the phenolic compounds, vanillin was shown to be a strong inhibitor of growth and ethanol production in *P.stipitis*, *C. shehatae* and *S.cerevisiae* at the concentration of 1 g/L (Delgenes *et al.*, 1996).

On the whole, inhibitor compounds in the hydrolysate from lignocellulosic biomass have a synergistic inhibitory effect (Mussatto & Roberto, 2004). Therefore, removal of inhibitors from hydrolysates is necessary for an efficient fermentation step. In this regard, several methods have been proposed to reduce the inhibitors concentrations (Larsson *et al.* 1999, Mussatto & Roberto, 2004). In general, the inhibitors content in the hydrolyzates can be reduced by using mild pretreatment/hydrolysis conditions; detoxifying the hydrolyzate before fermentation; developing inhibitor tolerant strains, and converting toxic compounds into harmless products (Taherzadeh *et al.*, 2000a). The major part of the detoxification methods are physical, chemical or biological (Mussatto & Roberto, 2004). Among the

physical methods, evaporation removes volatile compounds such as acetic acid, furfural and vanillin (Converti *et al.*, 2000). Chemical methods includes different strategies like overliming treatment and use of ion exchange resins and activated charcoal (De Bari *et al.*, 2004; Lee *et al.*, 1999; Martinez *et al.*, 2001; Nilvebrant *et al.*, 2001.). Biological detoxification is substantially based on the enzymatic treatment using peroxidase and laccase obtained from the lignolytic fungus *Trametes versicolor* (Palmqvist & Hann-Hägerdal, 2000). However, although the detoxification treatments are well established on large-scale processes, they could increase the process cost. According to some economical evaluations, the detoxification costs can constitute 22% of ethanol production cost (Von Sivers *et al.*, 1994). Thus, the use of resistant microorganisms, such as engineered or adapted strains, would be preferable. In particular, laccase gene from *Trametes versicolor* was expressed into *S.cerevisiae* resulting in higher ethanol productivity in spruce hydrolysates and in a media supplemented with coniferyl aldehyde (Larsson *et al.*, 2001). On the other hand, adaptation to toxins could make microorganisms more tolerant (Cuna *et al.*, 2004; Nigam, 2001b). Generally, adaptation is carried out by the sequential transfer of cells in media containing increasing concentrations of inhibitors (Cuna *et al.*, 2004; De Bari., 2005; Liu *et al.*, 2005; Nigam, 2001b). Following the adaptation of *P. stipitis*, De Bari *et al.* (2005) increased the xylose consumption and improved the ethanol yield by 17%. The adaptation strategy also increased the tolerance of *S. cerevisiae* to 5-HMF and furfural (Liu *et al.*, 2005).

The ability to adapt *S. cerevisiae* to lignocellulosic hydrolysates is strain dependent (Olsson & Hann-Hägerdal, 1996). For instance, a strain of *S. cerevisiae* isolated from a sulphite-spent liquor (SSL) was shown to be able to use glucose and galactose simultaneously in the presence of acetic acid in contrast to the behaviour of Bakers' yeast (Linden *et al.*, 1992). On the whole, recombinant technology together with the strains adaptation appears as the most promising approach to develop efficient processes to convert lignocelluloses biomass into ethanol. More insights in the inhibitors mechanisms and in the genomic characteristics of some resistant microorganisms could help the definition of protocols to enhance the yeast robustness.

5. Microbial inhibition by ethanol

The performances of the fermentation microorganism is also affected by the ethanol tolerance. In particular, at low concentrations, ethanol retards the growth rate of yeasts and inhibits cell division, while high ethanol concentrations reduce cells viability and increase their death (Stanley *et al.*, 2010). Furthermore, ethanol stress alters the metabolism (Hu *et al.*, 2007) mostly acting on the plasma membrane and on the cytosolic enzymes (Ansanay-Galeote *et al.*, 2001, Lopes & Sola-Penna, 2001). Exposure to ethanol causes also a disruption of the membrane structure resulting in a loss of electrochemical gradients and transport associated to the membrane (D'Amore *et al.*, 1990).

The xylose fermenting yeasts *P.stipitis* and *C.shehatae* are low tolerant to ethanol and are completely inhibited by ethanol concentration of 30 g/L (Laplace *et al.*, 1991). Conversely, *S.cerevisiae* reasonably tolerates higher levels of up to 70- 110 g/L (Casey *et al.*, 1992). This "ethanol tolerance" property is one of the reasons why *S.cerevisiae* is considered the alcoholic fermentation-organism for excellence. Generally, the yeasts such as *S.cerevisiae* have evolved some protective/adaptive responses to ethanol. One of the ethanol stress response, is the increase of unsaturated fatty acid and sterols in the cell membranes (Beaven *et al.*, 1982). Furthermore when *S.cerevisiae* is exposed to ethanol stress, an increase of the

heat shock proteins (HSPs) expression (i.e. HSP12 and HSP104) was observed (Glover & Lindquist, 1998; Sales *et al.*, 2000). These proteins protect the liposomal membrane integrity and act as remodeling agent in the disaggregation of denaturated proteins. Furthermore, following the exposure to ethanol, yeasts accumulate trehalose. However, this sugar is produced in response to many stresses (Attfield, 1997) and its function in stress tolerance is still not clear. Although this compound has long been considered as a protectant agent in the stress conditions (Ogawa *et al.*, 2000) it was found that a mutant which accumulates high levels of trehalose showed a reduced expression of other adaptive mechanisms (Singer & Lindquist, 1998). According to Lopes & Sola-Penna (2001), the pyrophosphatase inactivation promoted by alcohols is not prevented by the presence of trehalose while 1.5 M urea attenuated this effect.

Inhibitor	Inhibitor concentration (g/L)	Yeasts Strain	Inhibition of ethanol yield (%)	Inhibition of ethanol productivity (%)	References
5-HMF	4	<i>S.cerevisiae</i> Tembec T1	12	45	Keating <i>et al.</i> , 2006
5-HMF	4	<i>S.cerevisiae</i> CBS 8066		40	Taherzadeh <i>et al.</i> , 2000b
5-HMF	4	<i>S.cerevisiae</i> Y-1528	11	40	Keating <i>et al.</i> , 2006
Furfural	4	<i>S.cerevisiae</i> CBS 8066		69	Taherzadeh <i>et al.</i> , 1999
Furfural	1.6	<i>S.cerevisiae</i> Tembec T1		27	Keating <i>et al.</i> , 2006
Furfural	1.6	<i>S.cerevisiae</i> Y-1528		25	Keating <i>et al.</i> , 2006
Acetic acid	4.3	<i>S.cerevisiae</i>	50		Olsson <i>et al.</i> , 1996
Acetic acid	8	<i>P.stipitis</i>	98		Olsson <i>et al.</i> , 1996

Table 8. Effect of inhibitors compounds on fermentation by yeasts

It was established that during acclimatisation to ethanol stress, hundreds of genes are down-regulated and about 100 genes are up-regulated (Alexandre *et al.*, 2001; Chandler *et al.*, 2004).

Ethanol tolerance is not only genetically determined and can be influenced by many factors, such as plasma membrane composition (Mishra & Prasad, 1989), sugars concentration (Meyrial *et al.*, 1995), temperature, osmotic pressure, intracellular ethanol accumulation, byproduct formations. As consequence, it is very difficult to develop more tolerant yeast strains and still few studies are available on the construction of recombinant strains more tolerant to ethanol. In this regard Alper *et al.* (2006) combined mutagenesis and selection to isolate ethanol tolerant strains that showed an increased ethanol yield under a number of conditions and glucose concentrations. Kajiwara *et al.* (2000), also created a recombinant strain of *S.cerevisiae* with a higher unsaturated fatty acid content. This strain showed a higher survival rate than the wild-type strain in broths containing 15% (v/v) ethanol.

Interesting is also the *Saccharomyces diastaticus* (LORRE 316), an ethanol tolerant yeast capable of producing ethanol from corn starch, yielding a final concentration as high as 17.5% (v/v) (Wang & Sheu, 2000).

6. Conclusions

Lignocellulosic materials can be considered an important feedstock for the production of second generation bioethanol. Several breakthroughs have been achieved in the last years in all the process steps thus making this opportunity close to the industrial development. This is confirmed by several demo plants built around the world aiming at exploring the integrated process at significant scale. Depending on the specific biomass composition, some feedstocks, such as softwoods, can be more easily processed at demo scale. In fact, due to the hemicellulose composition of this biomass, the fermentable sugar streams do not contain pentoses and this reduces the process difficulties. The research in this field has given several microorganisms capable of fermenting diverse carbon sources and several process schemes. On the whole, the future development of bioethanol from lignocellulosics can be favored not only by the further optimization of some crucial process steps but also by the full implementation of the biorefinery concept. In this regard, further conversion options might be available for the various biomass streams, including the C5 fraction, and this could make more convenient the entire conversion.

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8. References

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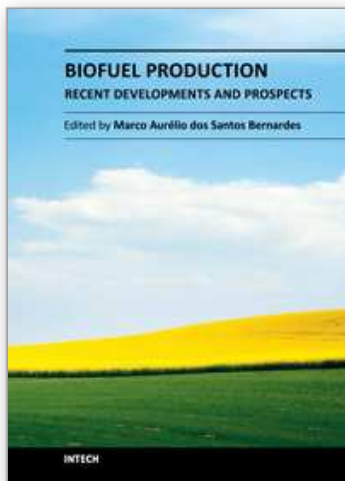
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This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

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