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Research review paper

Technological trends, global market, and challenges of bio-ethanol production

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ABSTRACT

Ethanol use as a fuel additive or directly as a fuel source has grown in popularity due to governmental regulations and in some cases economic incentives based on environmental concerns as well as a desire to reduce oil dependency. As a consequence, several countries are interested in developing their internal market for use of this biofuel. Currently, almost all bio-ethanol is produced from grain or sugarcane. However, as this kind of feedstock is essentially food, other efficient and economically viable technologies for ethanol production have been evaluated. This article reviews some current and promising technologies for ethanol production considering aspects related to the raw materials, processes, and engineered strains development. The main producer and consumer nations and future perspectives for the ethanol market are also presented. Finally, technological trends to expand this market are discussed focusing on promising strategies like the use of microalgae and continuous systems with immobilized cells.

Contents

1. Introduction

The beginning of this century is marked by the large incentive given to biofuel use in replacement of gasoline. Several countries worldwide, including Brazil, United States, Canada, Japan, India, China and Europe, are interested in developing their internal biofuel markets and established plans for use of these biofuels. Such interests are mainly motivated by 1) the rising oil prices and recognizing that the global oil reserves are exhausting fast, 2) concern about fuel emissions, 3) the requirements of the Kyoto Protocol and the Bali Action Plan on carbon emissions, and 4) the provision of alternative outlets for agricultural producers.

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Ethanol, as a clean and renewable combustible, is considered as a good alternative to replace oil (Bai et al., 2008; Almeida and Silva, 2006). Although the energy equivalent of ethanol is 68% lower than that of petroleum fuel, the combustion of ethanol is cleaner (because it contains oxygen). Consequently, the emission of toxic substances is lower (Krylova et al., 2008). Bio-ethanol use as a transportation fuel can also help in reducing CO_2 buildup in two important ways: by displacing the use of fossil fuels, and by recycling the CO_2 that is released when it is combusted as fuel. By using bio-ethanol instead of fossil fuels, the emissions resulting from fossil fuel use are avoided, and the CO_2 content of fossil fuels is allowed to remain in storage. Burning ethanol instead of gasoline reduces carbon emissions by more than 80% while eliminating entirely the release of acid-rain-causing sulfur dioxide (Lashinky and Schwartz, 2006).

This review presents a discussion of current and promising technologies for ethanol production from agricultural products and crops. The market and evolution of worldwide nations on the production of this biofuel are also considered in a subsequent section. Future trends and perspectives to expand this market are discussed mainly emphasizing promissory strategies like the use of microalgae and continuous systems with immobilized cells.

2. Historical background

The use of ethanol as an automotive fuel has a long history. The first prototypes of internal combustion engines built in the nineteenth century by Samuel Morey in 1826 and Nicholas Otto in 1876 were able to use ethanol as fuel (Demirbas et al., 2009). The first car produced by Henry Ford in 1896 could use pure ethanol as fuel and in 1908 the Ford Model-T, the first car manufactured in series, was a flexible vehicle able to use ethanol as a fuel, in the same way as gasoline or any mixture of both (Solomon et al., 2007). The use of bio-ethanol for fuel was widespread used in Europe and the United States until the early 1900s. After the First World War there was a decrease in demand for ethanol, because it became more expensive to produce than petroleum-based fuel, however there was an interest (e.g., from General Motors Corporation and DuPont) in ethanol as both an antiknock agent (i.e., octane enhancer) and as possible replacement for petroleum fuels (Demirbas et al., 2009; Balat and Balat, 2009; Solomon et al., 2007).

Brazil had a pioneering program to produce alcohol for automobile since 1927, when it has installed the first pump alcohol that continued until the early years of the next decade (Bray et al., 2000; Balat and Balat, 2009). However, the fuel ethanol market was revived in the 1970s when, for economic reasons as the global oil crisis and problems in the international sugar market due to overproduction, the National Alcohol Program (ProAlcool) was created in Brazil in 1975. This program was based on the sugarcane use as raw material, and was intended to target the large-scale use of ethanol as a substitute for gasoline (Goldemberg et al., 2008). With substantial government intervention to increase the supply and demand for ethanol, Brazil has developed institutional capacities and technologies for the use of renewable energy in large scale. In 1984, most new cars sold in Brazil required hydrated bio-ethanol (96% bio-ethanol + 4% water) as fuel. As the sugar-ethanol industry matured, policies evolved, and the ProÁlcool program was phased out in 1999, permitting more incentives for private investment and reducing government intervention in allocations and pricing. Although Brazilians have driven some cars that run exclusively on ethanol since 1979, the introduction of new engines that let drivers switch between ethanol and gasoline has transformed what was once an economic niche into the planet's leading example of renewable fuels. Widespread availability of flex-fuel vehicles (promoted through tax incentives) combined with rising oil prices have led to rapid growth in bio-ethanol and sugarcane production since 2000. Today, more than 80% of Brazil's current automobile production has flex-fuel capability (Kline et al., 2008).

In the United States, the combination of raising taxes, a concerted campaign by major oil producers and availability of cheap petrol effectively extinguished ethanol as a transport fuel in the early part of the 20th century (Rossillo-Calle and Walter, 2006). The desire to promote the production and use of bio-ethanol restarted in the early of 1980, largely to revitalize the farming sector at a time of oversupply of agricultural produce (Johnson and Rosillo-Calle, 2007). The United States rebuilt its fuel ethanol industry more gradually than Brazil, and is nowadays the world leader in its production and usage (RFA, 2010). A blended fuel E85 (85% bio-ethanol and 15% gasoline) is used in vehicles specially designed for it. Government has been promoting the development of this blend and several motor vehicle manufacturers including Ford, Chrysler, and GM, have increased the production of flexible-fuel vehicles that can use gasoline and ethanol blends ranging from pure gasoline all the way up to E85.

Currently ethanol is the main bio-fuel used in the world and its use is increasingly widespread, the worldwide prospects are the expansion of the production and consumption of ethanol (Bastos, 2007).

3. Current technologies for ethanol production

3.1. Raw-materials and processes

The biotechnological processes are responsible for the vast majority of ethanol currently produced. About 95% of ethanol produced in the world is from agricultural products (Rossillo-Calle and Walter, 2006). Ethanol production from sugar crops such as sugarcane and sugar beet account for about 40% of the total bio-ethanol produced and nearly 60% corresponding to starch crops (Biofuels Platform, 2010).

Fuel ethanol can be produced from direct fermentation of simple sugars or polysaccharides like starch or cellulose that can be converted into sugars. Thus, carbohydrate sources can be classified into three main groups: (1) simple sugars: sugarcane (Leite et al., 2009; Macedo et al., 2008); sugar beet (Içoz et al., 2009; Ogbonna et al., 2001); sorghum (Yu et al., 2008; Prasad et al., 2007a; Mamma et al., 1995); whey (Dragone et al., 2009; Silveira et al., 2005; Gnansounou et al., 2005; Domingues et al., 2001) and molasses (Roukas, 1996); (2) starches: grains such as maize (Persson et al., 2009; Gaspar et al., 2007); wheat (Nigam, 2001); root crops such as cassava (Kosugi et al., 2009; Rattanachomsri et al., 2009; Amutha and Gunasekaran, 2001); (3) lignocellulosic biomass: woody material (Ballesteros et al., 2004), straws (Silva et al., 2010; Huang et al., 2009), agricultural waste (Lin and Tanaka, 2006), and crop residues (Hahn-Hägerdal et al., 2006).

Ethanol production is usually performed in three steps: (1) obtainment of a solution of fermentable sugars, (2) fermentation of sugars into ethanol and (3) ethanol separation and purification, usually by distillation–rectification–dehydration (Demirbas, 2005). The step before fermentation, to obtain fermentable sugars, is the main difference between the ethanol production processes from simple sugar, starch or lignocellulosic material (Fig. 1). Sugar crops need only a milling process for the extraction of sugars to fermentation (not requiring any step of hydrolysis), becoming a relatively simple process of sugar transformation into ethanol. In this process, ethanol can be fermented directly from cane juice or beet juice or from molasses generally obtained as a by-product after the extraction of sugar (Içoz et al., 2009). In brief, the process of ethanol production from sugarcane consists of preparing, milling of cane, fermentation process and distilling–rectifying–dehydrating. Currently ethanol fermentation is carried out mainly by fed-batch processes with cell recycle, and a small part is produced through multi-stage continuous fermentation with cell recycle (Bastos, 2007).

In processes that use starch from grains like corn, saccharification is necessary before fermentation (Fig. 1). In this step, starch is

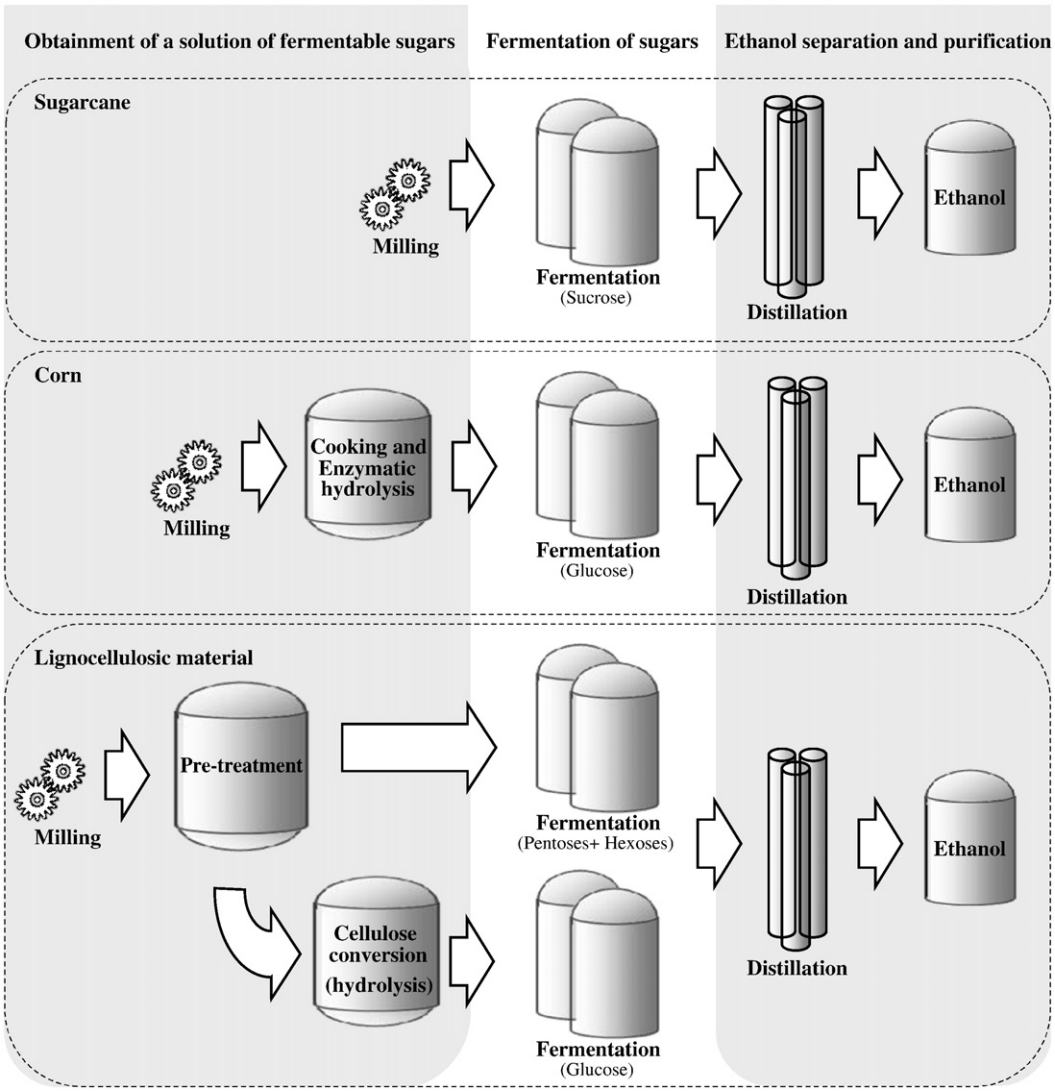

Fig. 1. Flowchart with the main raw materials and processes used for ethanol production.

gelatinized by cooking and submitted to enzymatic hydrolysis to form glucose monomers, which can be fermented by microorganisms. Starch is the most utilized feedstock for ethanol production in North America and Europe. Yeast cannot use starch directly for ethanol production. Therefore, ethanol production from grains involves milling and hydrolysis of starch that has to be wholly broken down to glucose by combination of two enzymes, α-amylase and amyloglucosidase, before it is fermented by yeast to produce ethanol. Corn and wheat are mainly employed with these purposes. In tropical countries, other starchy crops as tubers (e.g. cassava) can be used for commercial production of fuel ethanol (Prasad et al., 2007a; Cardona and Sánchez, 2007). The processes of ethanol production using starchy crops are considered well established. Today, most fuel ethanol is produced from corn by either dry-grind (67%) or wet-mill (33%) process. Recent growth in the industry has been predominantly with dry-grind plants because of less capital costs per gallon and incentives for farmer-owned cooperatives (Bothast, 2005).

Another source of simple sugars that can be used to produce ethanol is whey. Large quantities of whey are produced as by-product during manufacture of cheese. After whey protein has been harvested from whey by ultrafiltration, the remaining permeate is concentrated by reverse osmosis to attain higher lactose content for efficient fermentation. Lactose in whey permeate is fermented with some special strains of the yeast *Kluyveromyces marxianus* that are efficient in fermenting lactose (Dragone et al., 2009; Ling, 2008). In alternative, genetically engineered *S. cerevisiae* strains may be used (Domingues et al., 2001; Guimarães et al., 2008a).

Polysaccharides present in lignocellulosic materials (such as switch grass, wood chips, corn husks and other agricultural wastes), including cellulose and hemicellulose, are of great interest as feedstocks for second generation ethanol production. In this case, the involved technologies are more complex and the ethanol production costs are higher when compared to cane, beet or corn. However, most of lignocellulosic materials are byproducts of agricultural activities and industrial residues and show great potential for the production of fuel ethanol at large scale and a worldwide consumption as a renewable fuel. It is considered that lignocellulosic biomass will become the main feedstock for ethanol production in the near future.

The basic process steps in producing ethanol from lignocellulosic biomass are: (1) pre-treatment to render cellulose and hemicellulose more accessible to the subsequent steps. Pre-treatment generally involves a mechanical step to reduce the particle size and a chemical pre-treatment (diluted acid, alkaline, solvent extraction, steam explosion among others) to make the biomass more digestible; (2) acid or enzymatic hydrolysis to break down polysaccharides to simple sugars; (3) fermentation of the sugars (hexoses and pentoses) to ethanol using microorganisms; (4) separating and concentrating the ethanol produced by

distillation–rectification–dehydration (Fig. 1) (Sánchez and Cardona, 2008).

Some pre-treatments (step 1), such as diluted acid hydrolysis, result in solubilization of sugars from hemicellulose, generally separating the biomass into a liquid fraction containing pentoses and a solid fraction composed of cellulose and lignin The sugar yield is dependent on the kind of pretreatment and of the conditions used. The main technologies proposed for hydrolysis of cellulose (step 2) include concentrated acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis is the most advanced technology, while enzymatic hydrolysis is viewed as the technology with the best chance of reducing the costs of producing ethanol from biomass. A better utilization of carbohydrates in lignocellulosic materials is obtained when the hydrolysis is carried out in two stages. The first stage is performed in conditions that prioritize the hemicellulose hydrolysis, and the cellulose conversion to glucose occurs in a second stage. Both sulfuric acid and nitric acid have been used for acid hydrolysis, although sulfuric acid is the most used (Mussatto and Roberto, 2004).

Enzymatic hydrolysis reproduces a natural process that degrades cellulose to sugars, it is performed by cellulolytic enzymes (produced and secreted by fungi), which allows the process to be carried out in milder conditions than acid hydrolysis. In addition, enzymes offer the advantage of producing higher yields of sugars with little degradation. After the hydrolysis step the obtained sugars can be converted into ethanol by microorganisms (step 3). Hexoses are readily converted by *Saccharomyces cerevisiae*, the conventional yeast used for ethanol production. For a better use of materials containing substantial quantities of pentoses it is interesting to use microorganisms able to convert also these sugars to ethanol, such as yeasts of the genus *Candida* and *Pichia*, or genetically modified *S. cerevisiae* (Agbogbo and Coward-Kelly, 2008; Watanabe et al., 2007). The final step of separation and concentration of the ethanol (step 4) is a well established technology and there are many equipment manufacturers that can provide the distillation systems.

The choice of raw material for bio-ethanol production is strongly related with cultivation conditions of different crops, resulting in the use of a variety of processes, with different production costs, as shown in Table 1.

Table 1
Raw materials, processing and costs of ethanol production in different producers' countries.

Groups	Raw material	Country	Costs (US$/L)	Processing
Simple sugar	Sugarcane	Brazil	0.16–0.22[a,b,c,d]	Milling to extract sugar
		India	–	
	Sugar beet	France	0.60–0.68[e]	
		Europe	0.45[d]	
	Sorghum	India	–	
	Whey	New Zealand	0.42–0.49[f]	
Starches	Corn	United States	0.25–0.40[b,d,e,g]	Milling, liquefaction, and saccharification
		China	–	
		Canada	–	
	Wheat	China	–	
		Canada	–	
		Europe	0.42[d]	
	Cassava	Thailand	0.18[d]	
Lignocellulosic biomass	Mix of lignocellulosic materials	United States	0.43[h]	Milling and hydrolysis of the linkages

[a] Budny (2007).
[b] Goldemberg and Guardabassi (2009).
[c] Balat and Balat (2009).
[d] Moreira et al. (2008).
[e] Içoz et al. (2009).
[f] Ling (2008).
[g] Mcaloon et al. (2000).
[h] Williams et al. (2007).

3.2. Yeast strains development

Yeasts, particularly *Saccharomyces* spp., are usually the first choice for industrial ethanol production, because of their good fermentative capacity, high tolerance to ethanol and other inhibitors (either formed during raw-materials pre-treatments or produced during fermentation) and the capacity to grow rapidly under the anaerobic conditions that are characteristically established in large-scale fermentation vessels. Nevertheless, it is noteworthy that other microorganisms have been considered, namely the bacteria *Zymomonas mobilis* (Rogers et al., 2007) and *Escherichia coli* (Jarboe et al., 2007), which have been the subject of engineering programs with the aim of improving their ethanol fermentation performance.

The microflora of traditional and industrial fermentation processes may constitute a good source of microbial isolates with industrially relevant characteristics. Specifically, stress-tolerant yeast variants may be found in alcoholic fermentation processes, in which the yeast is subjected to several stresses, including osmotic and ethanol stresses (Basso et al., 2008). Such stress-tolerant isolates may be good candidates for further engineering efforts. Several tools are available for the improvement of yeast strains (Fig. 2), namely the genetic and metabolic engineering techniques (Nevoigt, 2008; Nielsen, 2001). In addition, the so-called conventional methodologies of mutagenesis and screening are still very useful in microbial improvement programs (Parekh et al., 2000). The combination of such tools with evolutionary engineering strategies (Çakar, 2009; Sauer, 2001) constitutes a powerful approach for the improvement of strains in the specific harsh environments typical of certain industrial fermentation processes. More recently, novel approaches have been devised and applied to improve ethanol production by *S. cerevisiae*, particularly genome shuffling (Shi et al., 2009; Hou, 2009) and global transcription machinery engineering (gTME) (Alper et al., 2006). Finally, in parallel to the improvement of microbial strains, developments in bioprocess design are essential to establish highly efficient bio-ethanol production systems (Fig. 2). Among the most significant developments in the fermentation field are the implementation of very high-gravity (VHG) technology, the use of lignocellulosic hydrolysates as feedstock and the application of high-cell-density continuous processes. Such technologies benefit from the selection and engineering of more robust yeast strains, with tailored properties for each of the processes.

In VHG fermentations, highly concentrated substrates are used, which allows significant gains in the overall process productivity, minimizing the production costs due to a decrease in the volumes of liquid to handle and the dimension of the equipment. Besides, the ethanol titers obtained are high (generally above 15% v/v), therefore significantly decreasing the distillation costs, which are considered one of the main constraints in industrial processes. Nevertheless, there are several problems associated with the performance of yeasts in VHG fermentations, which are often slow and incomplete. Therefore, the successful implementation of VHG technology in bio-ethanol production requires the development of yeast strains that efficiently ferment high sugar concentrations (>250 g/L) (Bai et al., 2008). Such yeast strains must be resistant to the multiple stresses found in the process, including the osmotic stress that results from the high sugar concentrations, the ethanol stress at the end of fermentation, the anaerobic conditions established in the large-scale bioreactors and the cell recycling procedures for utilization of the yeast for several consecutive fermentation cycles.

Several approaches have been used to select yeast mutants with improved performance under conditions resembling VHG fermentations. Using a strategy that combined UV-mutagenesis with consecutive rounds of batch fermentation under VHG conditions, Blieck et al.

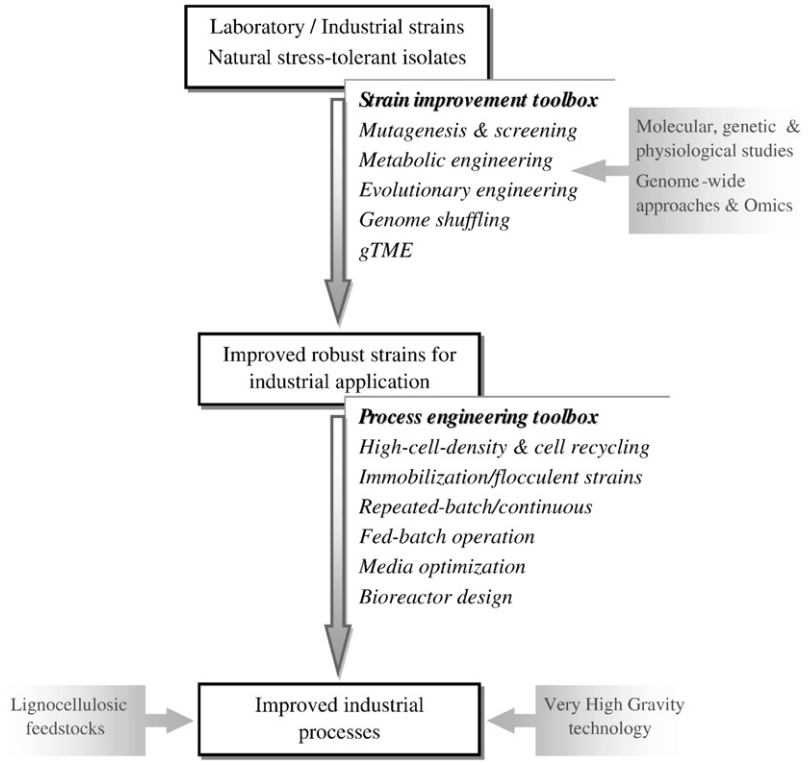

Fig. 2. Integration of yeast strain improvement and bioprocess design for highly efficient industrial bio-ethanol production.

(2007) selected variants of an industrial brewer's yeast strain with significantly improved fermentation capacity. A few recent reports describe the use of genome shuffling for obtaining yeast strains with improved ethanol tolerance and enhanced ethanol productivity in fermentations with high-glucose contents (200–300 g/L) (Hou, 2010; Hou, 2009; Shi et al., 2009). Evolutionary engineering approaches have been used to obtain mutants with higher tolerance to multiple stresses (Çakar et al., 2005), some of which have showed enhanced fermentation kinetics in media with glucose content above 200 g/L and at higher fermentation temperature (Wei et al., 2007). Ooi and Lankford (2009) have also obtained respiratory-deficient mutants of wine yeasts with improved capacity to ferment media with over 200 g/L glucose. Direct rational engineering approaches have also been followed. For instance, Teixeira et al. (2009) recently identified the gene *FPS1* (which encodes a plasma membrane aquaglyceroporin that mediates controlled efflux of glycerol) as a determinant of ethanol resistance in *S. cerevisiae* and found that its overexpression leads to higher ethanol production in fermentations with 300 g/L of glucose. Besides, many other studies have been addressing the identification of genes that confer resistance to ethanol in yeast (Dinh et al., 2009; Yoshikawa et al., 2009; Hirasawa et al., 2007; Hu et al., 2007; Yazawa et al., 2007; Fujita et al., 2006; van Voorst et al., 2006) as well as the molecular and physiological responses of yeast during alcoholic fermentations, particularly under industrial conditions (Argueso et al., 2009; Li et al., 2009a; Sanchez-Gonzalez et al., 2009; Guimarães and Londesborough, 2008; Cheng et al., 2008; Rautio et al., 2007; Hansen et al., 2006). These data will be useful to devise systematic metabolic engineering strategies in order to construct improved robust strains for the industry.

Lignocellulosic raw-materials contain cellulose and hemicellulose polymers built up by long chains of sugar monomers bound together by lignin. After pre-treatment and hydrolysis of these substrates, the resultant sugars can be converted into ethanol by microbial fermentation (Fig. 1). *S. cerevisiae* is one of the most tolerant microorganisms towards the inhibitors formed during biomass pre-treatment and fermentation. However, it can only consume the cellulosic hexose sugars (such as glucose and mannose), and not the hemicellulosic pentoses (particularly xylose and arabinose). Pentose-fermenting strains of *S. cerevisiae* have been constructed using several metabolic engineering strategies involving the introduction of genes encoding for xylose and arabinose pathways from bacteria and fungi (for recent reviews see Van Vleet and Jeffries, 2009; Matsushika et al., 2009; Hahn-Hägerdal et al., 2007). Evolutionary engineering experiments were found most useful to improve the fermenting capacity of the previously constructed strains (Wisselink et al., 2007; Kuyper et al., 2005; Sonderegger and Sauer, 2003). Most recently, an evolutionary engineering strategy was reported for the acceleration of fermentation and co-utilization of glucose–xylose–arabinose mixtures by a recombinant *S. cerevisiae* strain (Wisselink et al., 2009).

In addition to being able to ferment hexose and pentose sugars, the fermenting microorganisms must do this in the presence of the inhibitory compounds that derive from the biomass pre-treatment and hydrolysis steps, including weak acids (particularly acetic acid), furfurals, furan derivatives and phenolic compounds (Stephanopoulos, 2007; Mussatto and Roberto, 2004). Inhibition by these compounds decrease yield and productivity as well as disturbing cell growth (Hahn-Hägerdal et al., 2006). The inherent strategy of yeast to counteract the negative impact of inhibitors, particularly in the case of furfural and other aldehydes is to reduce them to less toxic compounds, such as alcohols (Almeida et al., 2009; Heer and Sauer, 2008). However, limited knowledge is available about the molecular and physiological basis for yeast tolerance towards lignocellulosic hydrolysates. In fact, this tolerance seems to be highly variable among different strains (Albers and Larsson, 2009; Modig et al., 2008). Nevertheless, the mechanisms of tolerance and adaptation of *S. cerevisiae* to the most common inhibitors has been the subject of many studies. In particular, genome-wide approaches (including disruptome screenings, DNA microarrays and proteomic analyses) have been recently exploited to get insights into the molecular and genetic traits involved in the tolerance and adaptation to furfural (Lin et al., 2009a; Lin et al., 2009b; Gorsich et al., 2006), 5-hydroxymethylfurfural (HMF) (Song et al., 2009; Petersson et al., 2006) and vanillin (Endo et al., 2009; Endo et al., 2008).

Evolutionary engineering experiments have been successful in obtaining inhibitor-tolerant strains. Martín et al. (2007) reported the adaptation of an engineered xylose-utilizing *S. cerevisiae* strain to sugarcane bagasse hydrolysates with high concentrations of inhibitors. The adapted strain converted furfural and HMF at a faster rate, presenting higher ethanol yield and higher specific ethanol productivity, as compared to the parental strain. Liu et al. (2008) reported a *S. cerevisiae* strain (NRRL Y-50049) with enhanced biotransformation ability to convert furfural to furan methanol and HMF to furan dimethanol, producing a normal yield of ethanol. Comparative studies between the tolerant mutant and its parental strain allowed these authors to discuss some of the potential mechanisms of detoxification in yeast (Liu et al., 2009a; Liu et al., 2008). Heer and Sauer (2008) isolated representative clones from evolved *S. cerevisiae* populations that exhibited significantly reduced lag phases in medium containing furfural as the single inhibitor as well as in medium supplemented with hydrolysates. Moreover, such clones were able to grow at concentrations of hydrolysates that killed the parental strain. The evolved clones showed increased viability under furfural stress but not increased furfural reduction rates, and consequently it was concluded that their primary advantage is the capacity to withstand and remain active at higher concentrations of furfural or hydrolysates.

The implementation of high-cell-density fermentation systems usually results in improved process productivity, especially when operating in continuous mode. Biomass retention in the bioreactor is accomplished by cell immobilization techniques, including attachment to the surface of a solid support, entrapment within porous matrices or the use of microporous membranes or microcapsules (for a recent review see Verbelen et al., 2006). Besides, the application of flocculent yeast strains constitutes an attractive alternative, particularly for ethanol production (Zhao and Bai, 2009; Domingues et al., 2000). The self-assembling ability results in yeast flocs that may rapidly sediment from the fermenting medium, thus providing a natural immobilization of the cells (Verbelen et al., 2006). When operating in continuous mode, the use of properly designed bioreactors allows for floc retention with consequent biomass accumulation (Zhao and Bai, 2009; Domingues et al., 2000). In addition, flocculation provides an effective and simple way to separate most of the yeast cells from the fermentation broth (Verbelen et al., 2006).

The earlier efforts to transfer the flocculation capacity to non-flocculent *S. cerevisiae* strains by genetic engineering have been reviewed by Domingues et al. (2000). More recently, Cunha et al. (2006) developed conditional flocculent *S. cerevisiae* strains by cloning the *FLO5* gene under the control of promoters that repress gene transcription in the presence of sugar but support strong expression after sugar exhaustion (i.e. at the end of fermentation). Wang et al. (2008a) reported the construction of a stable flocculating strain for fuel ethanol production by expression of the *FLO1* gene. Other authors have cloned a newly found flocculation gene (*FLONS*; Liu et al., 2007) in an industrial strain, thus increasing its flocculation ability (Wang et al., 2008b). The flocculent strain SPSC01, which was obtained by fusion of a flocculent *Schizosaccharomyces pombe* with *S. cerevisiae* K2 (widely used by industry in China), has been intensively studied for continuous ethanol production (Zhao et al., 2009; Ge and Bai, 2006; Xu et al., 2005). In particular, operation with this strain has been validated in a pilot plant and further in an industrial fuel ethanol plant in China (Zhao and Bai, 2009).

A different approach consists in transferring relevant industrial properties to naturally flocculent strains (Domingues et al., 2000). Many *S. cerevisiae* wild strains have the ability to flocculate, and flocculent strains with interesting characteristics may be further isolated from industrial environments. For instance, Andrietta et al. (2008) have tested the performance of 12 *Saccharomyces* sp. flocculent strains, isolated from fermentation tanks environments in Brazilian ethanol production units, in a continuous fermentation system. Moreover, Purwadi et al. (2007) used a flocculating *S. cerevisiae* strain isolated from an ethanol plant in Sweden for the continuous fermentation of lignocellulosic hydrolysates. That strain was able to cope with the toxic hydrolysate, totally converting furfural and >92% of HMF, and therefore requiring no hydrolysate detoxification prior to fermentation. Domingues et al. have cloned the genes for lactose metabolisation from *Kluyveromyces lactis* (Domingues et al., 1999a) and *Aspergillus niger* (Domingues et al., 2002) in a highly flocculent *S. cerevisiae* host (strain NCYC869), thus obtaining flocculent lactose-consuming strains (*S. cerevisiae* wild strains cannot metabolise lactose). These strains proved interesting for the production of ethanol (Domingues et al., 1999b) and ß-galactosidase (Domingues et al., 2005) from lactose-based medium in continuous fermentation systems. The strain expressing the *K. lactis* genes was also able to efficiently ferment cheese whey in continuous operation (Domingues et al., 2001). That recombinant strain was subjected to a long-term evolutionary engineering process that improved its lactose fermentation capacity and also its flocculation ability (Guimarães et al., 2008a; Guimarães et al., 2008b).

In addition to the continuous systems, flocculation has been exploited for repeated batch fermentation processes for the production of ethanol, in which yeast flocs are separated at the end of each batch by sedimentation in the bottom of the bioreactor, thus permitting biomass accumulation and recycling for many batches (Ma et al., 2009; Choi et al., 2009; Li et al., 2009b).

4. Bio-ethanol global market

4.1. Bio-ethanol production worldwide

Ethanol production worldwide has strongly increased since the oil crises in 1970. Its market grew from less than a billion liters in 1975 to more than 39 billion liters in 2006, and is expected to reach 100 billion liters in 2015 (Licht, 2006). Actually, the American continent is the biggest worldwide producer of ethanol, with United States and Brazil representing an important role in this sector. Table 2 shows the larger ethanol producer nations and the production volumes obtained in the year of 2008.

In 1970, Brazil created a National Alcohol Program (ProÁlcool) and today is one of the nations more developed in ethanol, being also one of the largest worldwide producers of this bio-fuel (RFA, 2010). Even so, many efforts have been directed to find alternatives to improve this process. Searches for new sugarcane varieties with higher sugar contents, larger yield per hectare,

Table 2
Worldwide ethanol production in 2008.[a]

Country	Production		
	(MI)[b]	Millions of gallon	% of the total
United States	34,070	9000.0	52
Brazil	24,500	6472.2	37
China	1900	501.9	3
France	1000	264.2	2
Canada	900	237.7	1
Germany	568	150.0	1
Thailand	340	89.8	1
Spain	317	83.7	–
Colombia	256	67.6	–
India	250	66.0	–
Poland	200	52.8	–
Hungary	150	39.6	–
Australia	100	26.4	–
Slovakia	94	24.8	–
Paraguay	90	23.7	–
Others	627	165.6	1
Total	65,362	17,266.8	100

[a] From Biofuels Platform (2010).
[b] MI = megaliter.

higher resistance to diseases and longevity, are continuously being performed. With 90% of the cane genome sequenced in 2003, the research groups were able to produce varieties of transgenic cane with larger productivity, higher resistance to dryness and adaptation to different climates, and higher resistance to low rich soils. A progressive change has also occurred regarding the fermentation mode, from batch to continuous, which promoted an increase in the process efficiency from 88% to 90%. As a consequence of the numerous researches, since ProÁlcool introduction in Brazil a substantial increase in the ethanol production occurred, from 555 million liters in 1975/76 to more than 16 billion liters in 2005/06 (Souza, 2006; Orellana and Bonalume Neto, 2006).

The United States interest in fuel ethanol has also increased since the oil crises in 1970s (Solomon et al., 2007). Similar to Brazil, at that time the United States started to invest in the production of ethanol fuel as an alternative to reduce their dependence to oil. Nowadays, this country is responsible for the highest investments in research related to ethanol. Such investments have been done by governments and companies, aiming the development of a strong market in this sector. Therefore, the ethanol industry in United States has shown a fast growth and development. As result of these efforts, their capacity of ethanol production increased from 1.63 billion gallons in 2000, to 9 billion gallons in 2008, representing a 5.5-fold increase. In 2006, the ethanol production in the United States overcame the Brazilian production, which was the world's largest producer for decades. This rapid growth in ethanol production is predicted to continue at least until 2012 (Solomon et al., 2007), when the United States intend to attain an ethanol production of 28 billion liters/year (Carvalho et al., 2007). Currently, over 95% of ethanol production in the United States comes from corn, with the rest made from wheat, barley, cheese whey, and beverage residues (Solomon et al., 2007).

Ethanol is also produced by other countries besides the United States and Brazil. The ethanol industries in these other countries have lower production scale. However, the increased interest in the bio-ethanol worldwide, will probably favor the expansion of these industries. China, for example, is a country that has invested much in the production of ethanol, and is nowadays one of the largest ethanol producers (Table 2). The experience of the ethanol sector in China started in 2001, using corn as raw material. In 2007, grain-based feedstock was used in four ethanol plants, and their production was about 1.4 million metric tons (MMT). However, due to the grains competition for ethanol and food applications, projects on fuel ethanol based on grain were restricted and development of "non-food ethanol" (ethanol made from non-food crops) was supported by the Chinese government (Fang et al., 2010). Many technologies of ethanol production based on non-food crop, such as cassava, sweet sorghum, sweet potato, Jerusalem artichoke, Kudzuvine root, and others, are being developed (Li and Chan-Halbrendt, 2009). At present, a 5000 ton/year sweet sorghum ethanol demonstration project has been established with the support of the National High-Tech Program, and a 400,000 ton/year cassava ethanol project is under development since 2005. Anyway, the development of fuel ethanol from sweet sorghum, cassava, or sweet potato still remains at the demonstration stage. The common problems encountered by these technologies are storage and pre-treatment of feedstock. In the meantime, to reduce production cost, low energy consumption technology (i.e. the increase of fermentation concentration and the decrease of fermentation time) will be developed in the future (Wu et al., 2009).

In Thailand, which is an agriculture-based country, the government has encouraged production and use of bio-ethanol that is currently obtained from cane molasses and cassava. As well as China, Thailand has also strongly invested in the production of ethanol to reduce the country dependency of fossil energy. In 2007, there were 7 ethanol plants with a total installed capacity of 955,000 L/day, comprising 130,000 L/day cassava ethanol and 825,000 L/day molasses ethanol. In addition, 12 new plants with a total installed capacity of 1,970,000 L/day were being constructed (Silalertruksa and Gheewala, 2009).

In Europe, the major amount of ethanol is produced from wheat and sugar-beet. Some production has also been obtained from wine surplus in France. France, Germany and Spain are the European countries more strongly committed to ethanol production (Prieur-Vernat and His, 2006). The European Union strategy for biofuels is also to decrease their dependence on oil and the negative impact caused to the environment.

India is also one of the largest producers of ethanol and currently all commercial ethanol production in the country uses molasses as feedstock. In 2003, the Planning Commission of the Government in India brought out an extensive report on the development of biofuels and bio-ethanol was identified as one of the main biofuels to be developed for the nation. However, with a huge population to feed and limited land availability, the nation needs to develop bio-ethanol technologies which use biomass feedstock that does not have food or feed value. The most appropriate bio-ethanol technology for the nation would be to produce it from lignocellulosic biomass, such as rice straw, rice husk, wheat straw, sugarcane tops and bagasse, which are the major agro-residues in terms of volumes generated (Sukumaran et al., 2009).

4.2. Bio-ethanol consumption worldwide

The international ethanol market has been stimulated by governmental politics of incentive to the use of renewable fuels. Although in expansion, the international market is very regional, with the largest producers being also the largest consumers (Almeida and Silva, 2006). In ethanol trading, Brazil is the largest exporter, with the United States and Europe being, correspondingly, the largest importers. In 2006, the total trade of ethanol was estimated to be 4.3 gallons (gal). Brazil was the main exporter (3.5 gal), and the United States, Japan and the Europe were the main importers. The ethanol amount imported by the United States (2.5 gal) came in the majority (1.7 gal) from Brazil. In the European Union, the net import of ethanol in 2006 was estimated at 0.5 gal, the Netherlands and Sweden being the largest importers (Heinimo and Junginger, 2009).

In Brazil, the ethanol use as bio-fuel is very common. In the 80s, more than half of the Brazilian cars used 95% anhydrous ethanol, however, the lack of sugar and its high prices decreased this value in the subsequent years (Solomon et al., 2007). Nowadays, almost all the Brazilian vehicles use ethanol, in the pure form or in mixture with the gasoline, where ethanol corresponds up to 25% of the mixture. The high percentage in which ethanol is added to gasoline in Brazil is also an effort made by the government to reduce the imports of oil (Prasad et al., 2007b). In addition, the innovations introduced by the automobile industry with the flex-fuel cars increased the consumers' interest by ethanol. Such cars may be fueled with ethanol and/or gasoline in any proportion, and are sales leaders, representing 90% of the light vehicle sales (Souza, 2006; Anfavea, 2005). The flexibility of using both fuels in a vehicle favored the ethanol consumption in Brazil, which corresponds to 40% of the total fuels used in vehicles (Orellana and Bonalume Neto, 2006; Knight, 2006).

In the United States, ethanol is actually used in two forms: mixed with gasoline in the maximum proportion of 10%, or in mixtures containing 85% ethanol and 15% gasoline, as an alternative fuel (EIA, 2006). In India, the addition of 5% ethanol to gasoline is mandatory in 10 states and 3 territories (Sukumaran et al., 2010). In a next step, the supply of ethanol mixtures with gasoline will be expanded to the whole country and some efforts will be also directed to increase the ethanol percentage in the mixture to 10% (Prasad et al., 2007b). Sweden also uses mixtures containing 5% ethanol in gasoline, while in Canada and some regions of China mixtures containing up to 10% ethanol in gasoline may be found (Souza, 2006). In Japan, the

replacement of 3% of gasoline by ethanol is authorized (Orellana and Bonalume Neto, 2006), but efforts will be also done to increase this value to 10% (Souza, 2006). In Thailand, renewable energy policy promotes the use of a 10% blend of bio-ethanol with 90% gasoline (Silalertruksa and Gheewala, 2009).

4.3. Economic aspects

In average, an ethanol plant of 0.19 hm^3 (50 Mgal) per year requires about US$ 65–100 million in capital cost, give 30–50 jobs, and has an annual operational cost of US$ 45–60 million (Solomon et al., 2007). Among the raw materials already used for ethanol production, sugarcane provides the lowest production costs (Biofuels Platform, 2010), since is the highest productive (6190–7500 L/ha against 3460–4020 L/ha of corn) (Tabak, 2009; Duailibi, 2008; Brown, 2006) and needs an easier processing. In addition, the sugarcane bagasse generated after broth extraction may be burned for energy generation in the plant, contributing for the energy costs reduction (Souza, 2006; Sinício, 1997). For all these reasons, ethanol production in Brazil is cheaper than in the United States or in Europe. In Europe, the production cost of bio-ethanol is on average three times higher than in Brazil and twice as high as in the United States (Biofuels Platform, 2010). In January of 2007, 1 L of ethanol in Brazil was sold at US$ 0.20, while in the United States the value was more than two times higher (US$ 0.47/L) (Notícias Globo, 2007).

The sugarcane use for ethanol production is also more favorable in terms of energetic balance. The energetic balance to convert corn in ethanol is of approximately 1:1.63, i.e., for each 1 kcal of energy consumed for ethanol production, a gain of 1.63 kcal is obtained by the ethanol produced (Kim and Dale, 2005). On the other hand, the energetic balance of the ethanol production from sugarcane bagasse is 1:3.24. Another important aspect is the total expense of fossil energy in the industry to convert the sugars in the same ethanol amount. Sugarcane bagasse requires 4-fold less energy than corn: 1.6 billion kcal against 6.6 billion for corn (Andreoli and Souza, 2007).

Estimating the ethanol production costs from lignocellulosic materials is difficult since different production methods have been evaluated, and there is also some difficulty in accounting the indirect costs involved. According to estimates of ethanol production from cellulose, the largest capital cost components are for feedstock pre-treatment (17%) simultaneous saccharification and fermentation (15%), which can also be performed separately, and energy utilities for boilers and turbogenerators (36%) (Solomon et al., 2007). Recently, developments from Genencor International and Novozymes Biotech have resulted in up to 30-fold drop in the cost of enzymes for hydrolysis process of these materials for ethanol production. Therefore, it is expected that cellulosic ethanol will have potential to compete on a large scale with gasoline without subsidies in the next decade. Several other factors could further lower the production cost of cellulosic ethanol, which include the use of cheap residues for biomass feedstocks lacking other markets, low cost debt financing, or integration into a biorefinery platform to increase the product range including higher-value chemical co-products (Ragauskas et al., 2006; Wyman, 1999). The latter option could potentially increase ethanol yields and further enhance economic competitiveness (Solomon et al., 2007).

Regarding ethanol production from pentoses fermentation, some authors estimated a final production cost of US$ 0.48/L, being the fermentation the most expensive step (31%), followed by the hydrolysate detoxification (22%) and the pre-treatment for the raw material hydrolysis (12.5%). The costs involving distillation, labor, cell mass production (inoculum) would correspond to 35% of the total costs involved (Von Sivers et al., 1994).

4.4. Future perspectives for ethanol market

The biggest expectation for the increase of ethanol market comes from the United States since this nation has invested a lot in this sector in the last years. Europe and Japan are also ethanol markets with great possibility of expansion in a short period. In Japan, for example, the ethanol market has great potential for expansion since fuels consumption for transportation is very substantial. Despite of its prominent role in the global ethanol market, the Brazilian market is still in expansion, with increases of the production capacity of existent units, new units starting operation, and new units to start operating in the future (Souza, 2006). Brazilian market may also require more ethanol due to the constant increase in the sales of flex-fuel cars. Probably, other countries will also invest in this kind of vehicles.

It is also expected that the worldwide production of cellulosic ethanol will amount to at least 16.5 billion gallons in 2020, if the targets set in the United States, China, Europe, Japan and Brazil are achieved. Based on currently proposed and signed legislation, the United States would account for over 63.9% of that market, while Europe and China would account for 10.4 and 11.5% respectively. Although Brazil does not have official legislation on cellulosic ethanol, based on UNICA (União das Indústrias de Cana-de-Açúcar) estimations for market penetration, it should amount to around 2.1 billion gallons (12.9%) in 2020. Japan would account for only 1.3% based on currently proposed legislation (Openpr, 2010).

5. Technological trends and challenges of bio-ethanol production

Currently, almost all bio-ethanol is produced from grain or sugarcane. It is known that the renewable energy production from agricultural feedstocks by cultivation in set aside areas or in even larger available marginal areas worldwide has a large positive impact on rural development, like the creation of new jobs and supplementary incomes. However, as this kind of feedstock is essentially food, bio-fuel production from these crops, especially corn, has attracted criticism due to rising food prices and the global food shortage. In addition, the ethanol production from grains like corn has several important environmental impacts, including the soil erosion, loss of biodiversity, and high volatile organic compound and NO_x pollution. This ethanol production technology has also an energetic balance disadvantageous, and requires a significant area for plantation and water (Solomon et al., 2007; Giampietro et al., 1997). For all these reasons, the worldwide nations are continuously searching for new technologies and processes for the production of this biofuel, without causing these adverse effects to the environment.

In this sense, bio-ethanol from lignocellulosic biomass is one of the important alternatives being considered for large scale production and are expected to be major feedstocks for the production of this biofuel in the near future. However, while technologies to produce ethanol from sugar or starch are well established, the technologies to produce bio-ethanol from lignocellulosic biomass are still under development all over the world. Despite substantial progress in cellulosic ethanol research and development, many challenges remain to be overcome, such as: 1) Development of a suitable and economically viable hydrolysis process step. This stage requires the use of enzymes to convert the cellulose in sugars, and such enzymes present an elevated cost for commercial utilization; 2) High energy consumption for biomass pretreatment remains a challenge, though the cost of energy consumption for woody biomass pretreatment can be reduced to the level used for agricultural biomass; 3) Improvement in the conversion rate and yield of hemicellulose sugars is still needed; 4) Process scale-up is one of the key challenges for commercial production; 5) Capital equipment required for commercial demonstrations of some technologies, such as steam explosion, does not exist; 6) The recovery of pretreatment chemicals and wastewater treatment are also important

issues in selecting pretreatment technologies in commercial production (Wu et al., 2009; Zhu and Pan, 2010; Souza, 2006).

For cellulose use in ethanol production to be performed in large scale and in a competitive level, major research efforts are still needed in order to: 1) develop more efficient technologies for the lignocellulose biomass pre-treatment, such as the application of new, engineered enzyme systems for cellulose hydrolysis; 2) To develop microorganisms able to metabolize all pentose and hexose sugars and simultaneously withstand the stress imposed by the process inhibitors; 3) determine how to maintain stable the performance of genetically modified microorganisms in fermentation operations in commercial scale and; 4) integrate the optimal conditions in a economic system for ethanol production (Margeot et al., 2009; Prasad et al., 2007b). The economic viability of ethanol production from cellulose is very important because it will allow an increase in the production of ethanol fuel, which will strengthen the international bio-ethanol market.

Besides the development of an efficient and economically viable technology for ethanol production from lignocellulosic materials, other processes based on the use of microalgae and continuous reactors with immobilized cells have also been strongly evaluated being considered as promissory strategies for a future development of this sector.

5.1. Microalgae as a feedstock for bio-ethanol production

Microalgae are a large group of fast-growing prokaryotic or eukaryotic photosynthetic microorganisms that can live in harsh conditions due to their unicellular or simple multicellular structure. Examples of prokaryotic microorganisms are Cyanobacteria (*Cyanophyceae*), and eukaryotic microalgae include for example green algae (*Chlorophyceae*) and diatoms (*Bacillariophyceae*) (Li et al., 2008).

These microorganisms convert sunlight, water and CO_2 to algal biomass (composed mainly by carbohydrates, proteins and oils) and can double their biomass in periods as short as 3.5 h presenting high growth rates in cheap culture media (Chisti, 2007). While the mechanism of photosynthesis in microalgae is similar to that of higher plants, they are generally more efficient converters of solar energy due to their less complex structure. Furthermore, since microalgae are microscopic in size and grow in liquid culture, nutrients can be maintained at or near optimal conditions potentially providing the benefits of high levels of controlled continuous productivity, similar to those achieved in microbial fermentations (Walker et al., 2005).

Microalgae can provide feedstock for several different types of renewable fuels such as biodiesel, methane, hydrogen, ethanol, among others (Fig. 3). Using microalgae as a source of biofuels is not a new idea (Chisti, 1980), but it is now being taken seriously because of the increasing cost of petroleum and, more significantly, the emerging concern about global warming arising from burning fossil fuels (Sawayama et al., 1995). The ability of microalgae to fix CO_2 has been proposed as a method of removing CO_2 from flue gases from power plants, thereby reducing the greenhouse gas (GHG) emissions (Abbasi and Abbasi, 2010). Approximately half of the dry weight of microalgae biomass is carbon derived from CO_2 (Chisti, 2008).

Other advantages of microalgal systems are: (1) microalgae can be harvested batch-wise nearly all-year-round; (2) they grow in aqueous media, but need less water than terrestrial crops, therefore reducing the load on freshwater sources; (3) microalgae can be cultivated in brackish water on non-arable land, and therefore may not incur land-use change, minimizing associated environmental impacts, while not compromising the production of food, fodder and other products derived from crops; (4) microalgae biomass production can effect biofixation of waste CO_2 (1 kg of dry algal biomass utilize about 1.83 kg of CO_2); (5) nutrients for microalgae cultivation (especially nitrogen and phosphorus) can be obtained from wastewater, therefore, apart from providing growth medium, there is dual potential for treatment of industrial and domestic sewage; (6) microalgae cultivation does not require herbicides or pesticides application; (7) they can also produce valuable co-products such as proteins and residual biomass, which may be used as feed or fertilizer and (8) the biochemical composition of the microalgal biomass can be modulated by varying growth conditions, therefore, the oil or starch yields may be significantly enhanced (Brennan and Owende, 2010).

Certain species of microalgae have the ability of producing high levels of carbohydrates instead of lipids as reserve polymers. These species are ideal candidates for the production of bio-ethanol as carbohydrates from microalgae can be extracted to produce fermentable sugars. It has been estimated that approximately 5000–15,000 gal of ethanol/acre/year (46,760–140,290 L/ha) can be produced from microalgae (Cheryl, 2008). This yield is several orders of magnitude larger than yields obtained for other feedstocks (Table 3). Even switchgrass, considered as the cellulosic "second generation" of biofuels, achieves ethanol yields that are a fraction of microalgal yield.

The microalgae *Chlorella vulgaris*, particularly, has been considered as a promising feedstock for bio-ethanol production because it can accumulate up to 37% (dry weight) of starch. However, higher starch contents can also be obtained for optimized culture conditions (Hirano et al., 1997). Under favorable climate conditions, yields of 80–100 ton dried *Chlorella* biomass per ha area for a 300-day culture season can be reached (Doucha and Lívanský, 2009). According to these authors the *Chlorella* sp. strain is able to accumulate starch up to

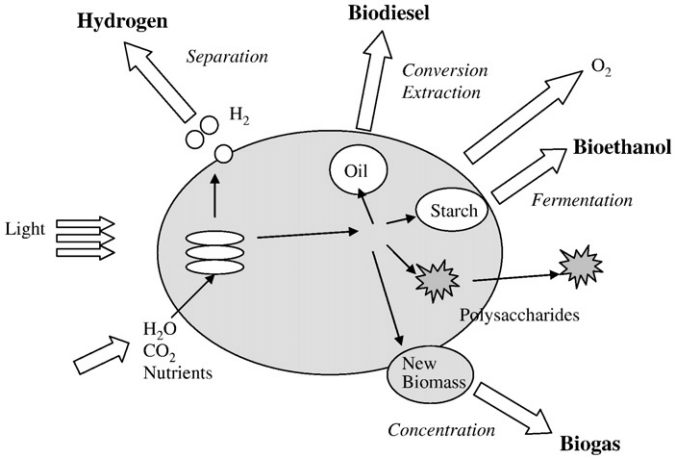

Fig. 3. Potential pathways from microalgae to fuels (adapted from Posten and Schaub, 2009).

Table 3
Ethanol yield from different sources.

Source	Ethanol yield (gal/acre)	Ethanol yield (L/ha)	References
Corn stover	112–150	1050–1400	Tabak (2009)
Wheat	277	2590	Cheryl (2008)
Cassava	354	3310	Cheryl (2008)
Sweet sorghum	326–435	3050–4070	(Lueschen et al., 1991; Hills et al., 1983)
Corn	370–430	3460–4020	Tabak (2009)
Sugar beet	536–714	5010–6680	(Hills et al., 1983; Brown, 2006)
Sugarcane	662–802	6190–7500	(Brown, 2006; Duailibi, 2008)
Switch grass	1150	10,760	Brown (2006)
Microalgae	5000–15,000	46,760–140,290	Cheryl (2008)

70% of algal dry weight under conditions of protein synthesis suppression. *Chlorococum* sp. was also used as a substrate for bio-ethanol production under different fermentation conditions. Results showed a maximum bio-ethanol concentration of 3.83 g/L obtained from 10 g/L of lipid-extracted microalgae debris (Harun et al., 2009).

Production of ethanol by using microalgae as raw material can be performed according to the following procedure (Fig. 4). In the first step, microalgae cultivation using sunlight energy is carried out in open or covered ponds or closed photobioreactors, based on tubular, flat plate or other designs. In the second step, the biomass needs to be concentrated by an initial factor of at least about thirty-fold, requiring very low-cost harvesting processes. After harvesting, microalgal starch is extracted from the cells with the aid of mechanical equipment or enzymes. Following starch extraction, amylolytic enzymes are used to promote formation of fermentable sugars. *S. cerevisiae* is then added to begin alcoholic fermentation. At the end of fermentation, fermented broth containing ethanol is drained from the tank and pumped to a holding tank to be fed to a distillation unit (Amin, 2009).

Apart from that, instead of extraction, there are also algal species able to conduct self-fermentation. Ueno et al. (1998) reported that dark fermentation in the marine green algae *Chlorococcum littorale* was able to produce 450 μmol/g dry-wt ethanol, at 30 °C. Seambiotic, in collaboration with Inventure Chemicals, successfully demonstrated the production of ethanol by fermentation of microalgal polysaccharides. Seambiotic is the first company in the world that is utilizing flue gases from coal burning power stations as a source of CO_2 for microalgae cultivation (Shen Goh and Lee, 2010).

Algenol Biofuels Inc. claimed that its plant is able to produce ethanol at a rate of over 6000 gal/acre/year and is expected to improve to 10,000 gal/acre/year. With further refinement, the microalgae cells would have the potential to increase production rates to 20,000 gal/acre/year in the future. The company also stressed on the tolerance of the engineered algae to high temperature, high salinity, and alcohol levels present during ethanol production (Algenol Biofuels, 2010).

5.2. Bio-ethanol production in continuous fermentation systems using immobilized cells

Fermentation systems operated in continuous mode offer a number of advantages compared to batch processes, generally resulting in enhanced volumetric productivity and, consequently, smaller bioreactor volumes and lower investment and operational costs (Brethauer and Wyman, 2010). These continuous processes can benefit from whole cell immobilization techniques in order to retain high cell densities inside the bioreactors. Such immobilization techniques can be divided into four categories: attachment or adsorption to solid surfaces (e.g. wood chips, brewer's spent grains, DEAE cellulose, and porous glass), entrapment within a porous matrix (e.g. calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatine, chitosan, and polyacrilamide), mechanical containment behind a barrier (e.g. microporous membrane filters, and microcapsules) and self-aggregation of the cells by flocculation. Many aspects related to the application of these distinct immobilization methodologies and carriers have been recently discussed (Kourkoutas et al., 2004; Brányik et al., 2005; Verbelen et al., 2006), including their impact in microbial growth and physiology, internal and external mass transfer limitations, product quality and consistency, bioreactor design, bioprocess engineering and economics.

Industrial application of continuous fermentation systems with immobilized cells has up till now been scarce, mainly due to process engineering and microbial activity problems as well as unrealized cost advantages (Verbelen et al., 2006; Brányik et al., 2005). Nevertheless, some successful applications have been reported. In Brazil, although the majority of the distilleries producing ethanol from sugarcane still employ the fed-batch Melle–Boinot process, there have been efforts to implement efficient continuous processes (Brethauer and Wyman, 2010). Modern third-generation continuous processes use optimization methods and kinetic models with the objective of designing new plants with maximized productivity and higher process flexibility and stability (Zanin et al., 2000). Similar to the Melle–Boinet process, most often such continuous systems use centrifuges to recover the yeast cells for biomass recycling. However, some Brazilian industrial plants have implemented a continuous process using flocculent *S. cerevisiae* that permit biomass recovery by sedimentation in settlers, thus avoiding costly centrifugations (Zanin et al., 2000). The use of flocculent strains (see Section 3.2) has been frequently mentioned as advantageous over carrier-based immobilization systems, due to the simplicity and low costs associated with the concept (Domingues et al., 2000; Verbelen et al., 2006; Bai et al., 2008; Brethauer and Wyman, 2010).

In China, pilot and commercial scale industrial plants have been constructed for continuous ethanol production using the flocculent yeast strain SPSC01 (Bai et al., 2008; Zhao and Bai, 2009). In the United States, continuous fermentation systems are common along large industrial units producing ethanol from corn. A study done in 2002 identified 4 plants using continuous fermentation out of the 21 dry-mill ethanol plants surveyed (Shapouri and Gallagher,

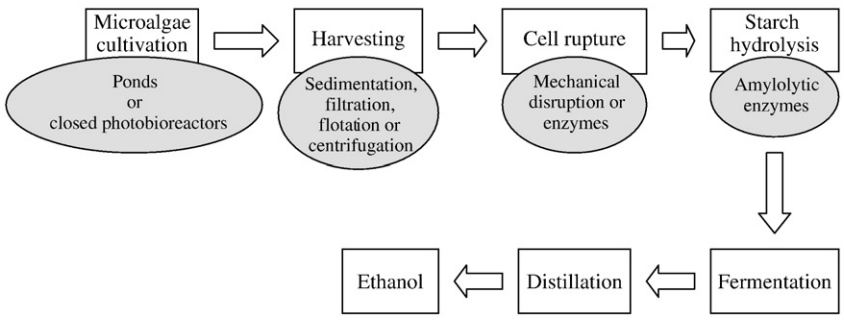

Fig. 4. Procedure for bio-ethanol production from microalgae.

2005). In brewing, industrial continuous systems using immobilized cells have been successfully implemented for the production of alcohol-free beer and for beer maturation (Mensour et al., 1997; Linko et al., 1998; Virkajärvi and Linko, 1999). Although often pointed as very promising, the industrial experience with continuous systems for the main brewing fermentation has been mostly limited to pilot scale demonstration plants (Mensour et al., 1997; Virkajärvi and Linko, 1999).

Despite of the slow incorporation by the industry, research in the field of continuous bio-ethanol fermentation with immobilized cells has been quite active. For instance, in the last few years, an increasingly number of reports were released on continuous systems for the production of bio-ethanol from lignocellulosic raw-materials (reviewed by Brethauer and Wyman, 2010). Continuous fermentations should reduce costs with detoxification of the hydrolysates due to *in situ* detoxification abilities of yeast, especially at high cell densities that can be established with immobilized or flocculent yeasts. Moreover, these processes should allow better handling of solids, which may constitute a considerable fraction in concentrated hydrolysates (Brethauer and Wyman, 2010). Purwadi et al. (2007) reported the successful continuous fermentation of a dilute-acid biomass hydrolysate without prior detoxification using a flocculating *S. cerevisiae* strain that accumulated to high cell concentration in the bioreactors. The formation of yeast colonies during flocculation or encapsulation was described to give a possibility for the cells to work in concert and protect each other against the hydrolysate inhibitors. Possibly, cells at the outer layer of the population are sacrificed and protect the other cells by converting toxic compounds (Talebnia and Taherzadeh, 2006; Purwadi et al., 2007).

The development and testing of new carrier materials for microbial immobilization and application in continuous fermentations is also an active research topic. Novel supports recently described include natural materials such as brewer's spent grains (Brányik et al., 2001) or sorghum bagasse (Yu et al., 2007), as well as synthetic materials like microporous divinyl benzene copolymer (Karagöz et al., 2009) or magnetic particles for use in a magnetically stabilized fluidized bed reactor (Liu et al., 2009b). The exploitation of microorganisms other than *S. cerevisiae* for continuous alcoholic fermentations has also been reported, namely *Z. mobilis* entrapped into polyvinyl alcohol hydrogel (Rebros et al., 2009), engineered *E. coli* immobilized on porous glass beads (Martin et al., 2006; Zhou et al., 2008) and the thermophilic anaerobic bacterium *Thermoanaerobacter* strain BG1L1 immobilized on granular carrier material for the fermentation of glucose and xylose in undetoxified lignocellulosic hydrolysates (Georgieva and Ahring, 2007; Georgieva et al., 2008).

In summary, continuous fermentation processes based on immobilized microbial cells have the potential to maximize the volumetric productivity while minimizing the production costs in the bio-ethanol industry. Increasingly advanced studies in the physiology and metabolic activity of immobilized cells, bioreactor and bioprocess design, development of carrier materials and flocculent strains, shall progressively mitigate the main problems and the industry resistance to the widespread adoption of such systems.

6. Conclusions

Ethanol has experienced unseen levels of attention due to its value as fuel alternative to gasoline, the increase of oil prices, and the climatic changes, besides being a renewable and sustainable energy source, efficient and safe to the environment. Currently, worldwide ethanol production is in high levels, and corn is the main raw material used for this purpose, but this scenario may change due to technological improvements that are being developed for production of low cost cellulosic ethanol, as well as for ethanol production from microalgae. Is important to emphasize that, to be a viable alternative, bio-ethanol must present a high net energy gain, have ecological benefits, be economically competitive and able to be produced in large scales without affecting the food provision. The use of various wastes (such as wood and agricultural wastes) and unconventional raw materials (such as microalgae) can solve the problem without sacrificing food demands.

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