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Review

Current Trends in Metabolic Engineering for the Production of Advanced Bioethanol

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Abstract

Bioethanol produced from various biomass materials such as wood, agricultural and forest residues has the potential to be a valuable substitute to gasoline. This review article reviews the research activities on development of the technology for bioethanol production from biomass. Based on its importance in current research and its promising output, lignocellulose as the most abundant biological material feedstock on earth, has been more highlighted. This review article focuses on ethanol production from biomass, analysis of conversion pathways from technical, economic and environmental points of view. This bioprocessing integrates enzyme production, microbial activities and fermentation process. Based on the current and future prospects, this review emphasizes the advances in bioethanol production and possible involvement of genetic technology which can contribute for better production of bioethanol.

Keywords: Biomass, lignocellulose feedstock, metabolic engineering, fermentation, bioethanol.

Introduction

Bioethanol is included in biofuels together with biohydrogen, biomethanol, biochar, biobutanol, biogas and biodiesel (Domínguez-Barroso *et al.,* 2016; Asikin-Mijan, 2017; Choia and Hwang, 2018). Ethanol (ethyl alcohol, C_2H_5OH, melting point–114°C, boiling point-78.4°C) is soluble in water and has a density of 789 g/L at 20°C. Bioethanol is derived from alcoholic fermentation of sucrose or simple sugars, which are produced from biomass. Absolute 95% ethanol are good solvents and are used in many industrial products such as paints, perfumes and tinctures (Madheshiya and Vedrtnam 2018; Stephen and Periyasamy, 2018). Solutions of ethanol (70-85%) are used as disinfectants in medicine. Ethanol can be used as a petrol additive but can also be used as a petrol substitute (Awole, 2009; Kumar *et al.*, 2017; Onukwuli *et al.,* 2017; Iulianelli *et al.,* 2018). Except for the uses in motor fuels, synthetic ethanol got the largest market share in industrial applications, as it is cheaper than ethanol derived from biomass. Bioethanol is a renewable energy source produced through fermentation of sugars unlike fossil fuels (Manochio *et al.*, 2017). Interest in the bioconversion of abundant and renewable cellulosic biomass into fuel ethanol as an alternative to petroleum is rising around the world owing to the realization of diminishing natural oil and gas resource. Conventional techniques to achieve this bioconversion include the acid or enzymatic hydrolysis of cellulose followed by fermentation of the resulting soluble sugars into ethanol (Balusu *et al.,* 2005; Mandade *et al.,* 2015). Many people have difficulties to distinguish biomass from fossils. Biomass, in terms of energy, means plant based material. The main difference between biomass and fossil fuels is one of time scale. Biomass takes carbon out of the atmosphere while it is growing, and returns it as it is burned. This process maintains a closed carbon cycle with keeping stable CO_2 levels in atmosphere (Pimentel *et al.,* 2005).

Lignocellulose biomass, including forestry residue, agricultural residue, yard waste, wood products, animal and human wastes, etc., is a renewable resource that stores energy from sunlight in its chemical bonds (Edwards and Doran-Peterson, 2012; Prasetyo and Park, 2013; Bumbudsanpharoke and Ko, 2018). It has great potential for the production of affordable fuel bioethanol, since it is less expensive than starch (e.g. corn) and sucrose (e.g. sugarcane) producing crops and available in large quantities (Pimentel *et al.,* 2005). Lignocellulose is mainly composed of cellulose (40-50%), followed by hemicelluloses (25-35%) and lignin (15-20%) is extremely resistant to enzymatic digestion (Kaparaju *et al.,* 2009). According to the Kyoto agreements, total CO_2 emissions from industrialized nations are to be reduced by 5% by 2010, relative to the 1990 level. In the longer term, a reduction of more than 50% will be required to stabilize the CO_2 level in the atmosphere.

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One of the major strategies to achieve these objectives is the large-scale substitution of petrochemical fuels and products with CO_2-neutral alternatives derived from biomass (El-Asli and Qatibi, 2009). One such fuel is ethanol produced from biomass such as agricultural waste and forest residues. Although CO_2 is emitted during the combustion of bioethanol, the same amount will be assimilated when new biomass is produced. Thus, total net emissions of CO_2 are essentially zero. Bioethanol has also several other advantages: It is non-toxic; it is a liquid at room temperature; it can be blended (up to 20% without modifications of the engine) with gasoline. Ethanol as a renewable and environment-friendly fuel has attracted significant interests. In recent years, ethanol production in the USA has enhanced from 4.89 billion gallons in 2006 to 14 billion gallons in 2011, indicating an almost tripled increase. Similar increases have occurred in other countries such as China and India (Ru-Bo and Xiao-Jun, 2005).

Ethanol fermentation can be performed using only specific strains of different microorganisms as the fermenting microorganism. These particular strains of microorganisms have higher thresholds for ethanol stress and therefore can tolerate higher ethanol concentrations. It has been shown that these specific fermenting microorganisms can even yield ethanol concentrations at the 15% (v/v) level with initial glucose of more than 250 g/L (Mathew *et al.,* 2015). This review article will summarize the current trend in bioethanol production. This review also highlights some possible room of improvement and possible technologies to be incorporated in order to link the current trend to future prospects in bioethanol production.

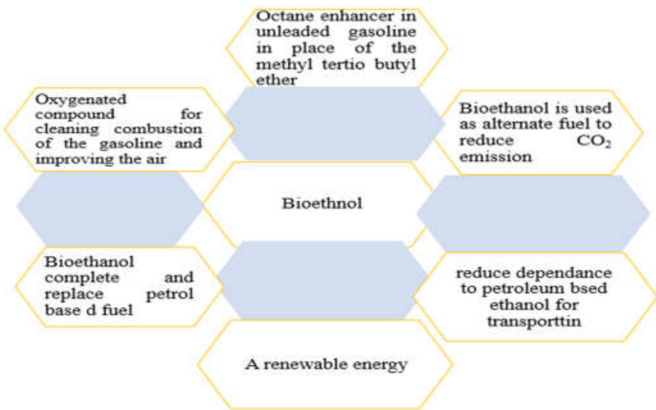
The main reasons of the appreciation of bioethanol

Currently, the limitations, disadvantages and exhaustive petroleum based fuel coupled to many advantages of biofuel are stimulating researchers to carry out different researches to replace and complete fossil based fuel by biofuel. One of the possible candidates is bioethanol.

Biochemical conversion of biomass-to-ethanol

Large-scale biomass-to-ethanol industry mainly exploits sugarcane or sugar beet juice, corn or wheat. Lignocellulose biomass is envisaged to provide a significant portion of the feedstock for bio-ethanol production in the medium and long term due to their low cost and to their high availability (Sosa *et al.,* 2013). Biomass-to-ethanol crops comprise: (1) multipurpose crops that are also reserved to food markets; and (2) dedicated ethanol crops. While the latter are cultivated especially for ethanol production on non-agricultural lands (fallow or undeveloped lands), the former provide almost all the feedstock used to date for ethanol production (sugarcane in Brazil and corn in the United States).

Fig. 1. Summary of different motivations pushing researchers to work on bioethanol production: The shaded surface area connects bioethanol to its different uses but also connect related or interconnected utilities and advantages of bioethanol. For instance being used as oxygenated compound for cleaning combustion of the gasoline reduce CO_2 emission , reduce dependence to petroleum, replace and complete gasoline, limit to exhaust fossil based biofuel. All of these are added to advantages of being source of octane enhancer and renewable energy source.



In most industrialized countries, the development of biomass-to-ethanol conversion emerged as alternative markets for sugar and grain surpluses (Manochio *et al.,* 2017). As the feedstock cost often represents more than 75% of the ethanol production in these cases, the economic viability of multipurpose crops to ethanol depends on the food markets situations (Kostin *et al.,* 2018). This correlation between food and ethanol markets may generate a volatility of the ethanol prices. In developing countries, the possible competition with food is one of the risks when using agricultural crops for ethanol production. Obviously, the developing countries should focus first on cellulosic biomass in order to limit competition between bioethanol production and food need (Yu *et al.,* 2012). Main Steps in biomass-to-bioethanol processes once the biomass is delivered to the ethanol plant, it is stored in the warehouse and conditioned to prevent from early fermentation and bacterial contamination (Fig. 2). Through pre-treatment, carbohydrates are extracted or made more accessible for further extraction. During this step, simple sugars may be made available in proportions depending on the biomass and the pre-treatment process. A large portion of fibers may remain for Saccharification through hydrolysis reactions or other techniques, in order to obtain simple sugars, which are then fermented (Sedlak and Ho, 2004). In the batch fermentation, the hydrolysates, the yeasts, nutrients and other ingredients are added from the beginning of the step. In case of a fed batch process, one or more inputs are added as fermentation progresses.

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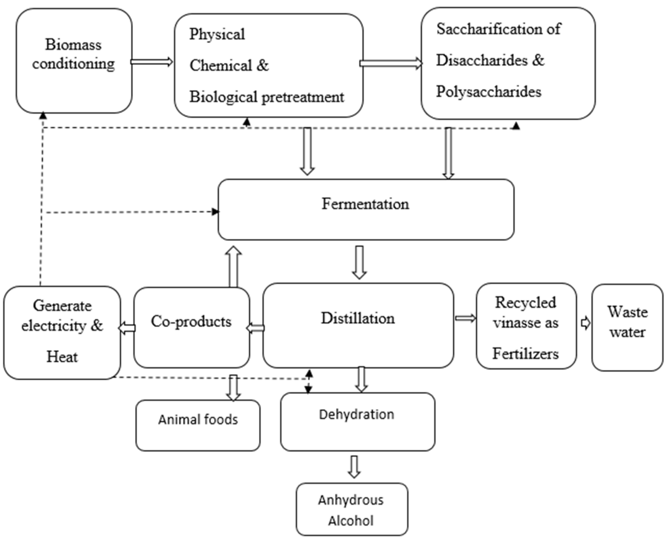
Continuous processes, in which ingredients are constantly input and products removed from the fermentation vessels, are also used (Olofsson *et al.,* 2008).

Fig. 2. Schematic outline of biomass to ethanol process. The main steps in this bioconversion of biomass to ethanol are pretreatment, Saccharification, distillation and dehydration. In the batch fermentation, the hydrolysates, the yeasts, nutrients and other ingredients are added from the beginning of the step. In case of a fed batch process, one or more inputs are added as fermentation progresses. Continuous processes, in which ingredients are constantly input and products removed from the fermentation vessels, are also used (Olofsson *et al.,* 2008).



Processing of disaccharides-to-ethanol

The most common disaccharide used for bioethanol production is sucrose, which is composed of glucose and fructose. Fermentation of sucrose is performed using commercial yeast such as *Saccharomyces ceveresiae*. Chemical reaction is composed of enzymatic hydrolysis of sucrose followed by fermentation of simple sugars. First, invertase (an enzyme present in the yeast) catalyzes the hydrolysis of sucrose to convert it into glucose and fructose.

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}+H_2O} \xrightarrow{\text{invertase}} \underset{\text{Glusose or Fructose}}{C_6H_{12}O_6} \xrightarrow{\text{Zymase}} \underset{\text{Ethanol}}{2CH_3CH_2OH+H_2O}$$

Biochemical processing of starch-to-ethanol

Starch stored in grains is long chains of α-glucose monomers, 1000 or more monomers for one amylose molecule and 1000 to 6000 or more monomers for amylopectin.

To convert starch to ethanol, polymer of α-glucose is broken into glucose through a hydrolysis reaction with glucose-amylase enzyme (Devi , 2012).

$$\underset{\text{Sucrose}}{(C_6H_{10}O_5)n+(H_2O)n} \xrightarrow[\text{amylase}]{\text{Gluco-}} \underset{\text{Glusose or Fructose}}{n(C_6H_{12}O_6)} \xrightarrow{\text{Zymase}} \underset{\text{Ethanol}}{(2CH_3CH_2OH)n+nH_2O}$$

The enzymatic hydrolysis is then followed then followed by fermentation, distillation and dehydration to yield anhydrous ethanol. In the bio-ethanol fuel industry, grains (corn, wheat or barley) mainly provide starch (Tanimura *et al.,* 2015). Starch makes 60-70% starch of corn feedstock in starch-to-ethanol industry worldwide. In dry milling process, grain is grinded to a powder which is hydrolyzed and the sugar contained in the hydrolysates is converted to ethanol while the remained flow containing fibre, oil and protein is dried and converted into a by-product known as Distillers Dried Grains when it is combined to process syrup. Distillers Dried Grains are a very valuable by-product of dry mills sold as animal feed. Another by-product may be the CO_2 that can be sold for different applications for instance CO_2 is used to make carbonated beverages or dry ice. Dry mills are dominant in Grain-to-Ethanol industry. (Li *et al.,* 2018), in a number of large facilities, the mills are kinds of bio-refineries in which the grains are wet milled for separating first the different components before converting these intermediates into final co-products (Ramchandran *et al.,* 2017).

The current situation of bioethanol production

The recent alternative introduced in the production of biofuel is the use microalgae for bioethanol production (Oh *et al.,* 2018). The advantages include being low cost effective and have the capacity of playing dual role when used to produce electricity they are able to act both as electron donor and electron acceptor). Other advantages include high lipid content per cell, areal lipid productivity, cultivation availability on non-arable land and ocean. More importantly the biomass production of algae is 5-10 times greater than soil based plants (Bibi *et al.,* 2017). These advantages are additional to its high growth rate and its ability to fix high amount CO_2 (Sikarwar *et al.,* 2017). Moreover, the use of algae can be another way of cleaning water bodies like lakes and pond as they are known to be potential pollution vectors and can cause eutrophication. The big challenge is that algae need to be in symbiotic relationship with bacteria for their growth. This disadvantage is added to its high sensitivity to environmental conditions change (Rashid *et al.,* 2013). So these are the issues to be addressed before thinking about the optimization of the culture of these algae. Due to its ease handling and processing, the present mechanisms of producing bioethanol depend largely on starch grains, sugarcanes and tubers and crops bagasse (Dar *et al.,* 2018).

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The use of these food staffs causes competition significantly in developing countries where shortage of food is a big problem. The main raised issue is the interference between food security and bioethanol production (Herrmann *et al.,* 2017; Moula *et al.,* 2017). In some countries the agriculture can be self-sufficient to supply footstock need for biofuel but still the choice of good culture is still a priority (Moula *et al.,* 2017). The establishment of the use of lignocellulose biomass presents advantages as there is no competition with the food market (Wasiak, 2017). The only problem to deal with when used lignocellulose biomass is the estimated price which is higher than corn-base and other food related feedstock (Shields-Menard *et al.,* 2018). For instance, cassava exploited to produce bioethanol in four provinces of china is compensated by the imports from Taiwan. This is a highlighted evidence to show that the same cassava cannot satisfy the feedstock for biofuel production and the food needs in china (Ye *et al.,* 2018). To overcome these problems, the recent practices exploit the lignocellosic materials for bioethanol production. (García *et al.,* 2011). These second and the third generation process still need some improvement in laboratory practices in order to allow it to replace the first generation (crop based biomass) (Xavier *et al.,* 2010; Kang *et al.,* 2014; Church *et al.,* 2017; Servaes *et al.,* 2017).

Biochemical processing of lignocellulose-to-ethanol structure of lignocellulose materials

Lignocellulose, which is the principal component of the plant cell walls, is mainly composed of cellulose (40-60% of the total dry weight), hemicellulose (20-40%) and lignin (10-25%). Cellulose molecules consist in long chains of β-glucose monomers assembled into micro-fibril bundles to polysaccharides (Restolho *et al.,* 2009). Depending on the types of plants, the hemicelluloses can be xyloglucans or xylans. The hemicellulose backbone consists of chains of β-glucose monomers from which chains of xylose branch. The latter are predominantly made of xylose bonded to arabinose or/and other compounds that vary from one biomass source to the other. The hemicellulose molecules are attached to the micro-fibrils by hydrogen bonds. Lignin are compounds formed by polymerization reaction of three different types of monomers (p-coumaryl, coniferyl and synapyl alcohols), the proportion of which differs significantly depending whether the plant is from gymnosperms, woody angiosperms or grasses. Lignin provides to the cell wall with mechanical strength and stiffness (Lange, 2007). Lignocellulose is abundant in nature and does not compete with food. Typical sources of lignocelluloses biomass are agricultural and forestry residues (bagasse of sugarcane or sweet sorghum, corn stover biomass) industrial wastes and dedicated woody crops.

Once the lignocellulose biomass is pre-treated, and hydrolyzed, the released sugars are fermented and the down- stream process is similar to that of sweet juice and starch (Falter *et al.,* 2015).

Pre-treatment

The aim of the pretreatment is to alter the lignocellulose structure and increase the rate of enzymatic hydrolysis primarily the cellulose. This should be done with a minimum formation of compounds, which inhibit the fermenting microorganisms (Olofsson *et al.,* 2008). Pre-treatment consists of delignification of the feedstock in order to make cellulose more accessible in the hydrolysis step, using physical, physic chemical, chemical and biological treatment (Rijal *et al.,* 2014). Performance of a technique is assessed with the aim to the yield of fermentable sugars, inhibitors, the recycling of chemicals, the production of wastes and the investments. The commonly used methods are steam explosion and dilute acid pre-hydrolysis before enzymatic hydrolysis. In steam explosion method, the lignocellulose materials are treated with high- pressure saturated steam for several seconds to a few minutes. Then, the pressure is suddenly dropped to atmospheric pressure causing the materials explosion. Most of the hemicellulose is solubilized during the process whose efficiency depends on the temperature and residence time (Rijal *et al.,* 2014). Lower temperature and longer residence time give a higher efficiency. Sulphuric acid or carbon dioxide is often added in order to reduce the production of inhibitors and improve the solubilisation of hemicellulose (Parekh, 1988; Xavier *et al.*, 2010).

Enzymatic hydrolysis of cellulose and fermentation of simple saccharides

Enzymatic treatment of cellulose is achieved using celluloses, which are usually a mixture of groups of enzymes such as endoglucanases, exoglucanases and β-glycosidase acting in synergy for attacking the crystalline structure of the cellulose, removing cellobiose from the free chain-ends and hydrolyzing cellobiose to produce glucose (Chen and Wan, 2017). Cellulases are produced by fungi, mainly *Trichoderma reesei*, besides *Aspergillus*, *Schisophyllum* and *Penicillium*. High concentration of cellobiose and glucose inhibits the activity of cellulase enzymes and reduces the efficiency of the Saccharification (Schuster and Chinn, 2012; Hou *et al.,* 2018;). One of the methods used to decrease this inhibition is to ferment the reduced sugars along their release. This is achieved by simultaneous Saccharification and fermentation (SSF), in which fermentation using yeasts (*Saccharomyces cerevisiae*) and enzymatic hydrolysis are achieved simultaneously in the same reactor (Mattila *et al.,* 2017; Rastogi and Shrivastava, 2017).

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Table 1. Summary of the steps of bioethanol production from lignocellosic feedstock.

Steps	Description	References
Pretreatment of lignocellulose biomass	Breaking down biomass materials. It can be: Physical (milling) Biological (using microbes such as *Basidiomycete* to degrade lignin to organic hemicellulose Chemic (use of acids and organic solvents	García *et al.*, 2011 Shirkavand *et al.*, 2016
Saccharification	Involves hydrolysis of cellulose and hemicellulose to generate simple fermentable sugars	Dauriat, 2005
Fermentation	Hexoses and pentose sugars are fermented under aerobic and anaerobic conditions to generate ethanol. Here specific microbes are used for instance *Saccharomyces cerevisiae* is used for hexose and *Candida shehatae* used for both hexose and pentose	Parekh and Wayman, 1986
Distillation	Separation of bioethanol and its co-products (lignin containing materials)	Algayyim *et al.*, 2018

The fermentation of the xylose released from the pre-hydrolysis process can be processed in a separate vessel or in SSF reactor using a genetically modified strain from the bacterium *Zymomonas mobilis* that can convert both glucose and xylose. The latter method is named simultaneous Saccharification and co-fermentation. Compared to the sequential Saccharification and fermentation process, SSCF exhibits several advantages like lower requirement of enzyme, shorter process time and cost reduction due to economy in fermentation reactors because only one reactor compared to the three sets. (Edwards and Doran-Peterson, 2012).

Use of bioethanol in spark ignition internal combustion (IC) engines and the challenge

In compression of engine, one of the possible substitutes of diesel replacement as it reduces environmental pollution is less toxic and less corrosive compared to other used fuel such methanol (Algayyim *et al.*, 2018). When using bioethanol in engine one of the following technics are adopted: adaptation of bioethanol to engine or adaptation of engine to bioethanol use (Kumar *et al.*, 2013). Ethanol has good properties for spark ignition IC engines. Its motor octane number and research octane number are respectively 90 and 109 leading to an average octane number of 99 compared to 88 for regular gasoline. Lower heating value of ethanol (21.2 MJ/L) is two-thirds that of gasoline (30.1 MJ/1L). Bio-ethanol fuel is used in IC engines as 5-26% anhydrous ethanol blends to gasoline (5% maximum in Europe and in India, 10% in USA, 22-26% mandatory blends in Brazil) or as pure fuel (100%) of hydrated ethanol in dedicated vehicles (Onukwuli *et al.*, 2017). When anhydrous bio-ethanol is blended to gasoline in small proportion (upto 15%), influence of lower heating value has no significant effect. For higher blend levels, fuel economy is reduced compared to that with conventional gasoline.

Ethanol dedicated vehicles are optimized so that the engine efficiency is improved by running at higher compression ratios to take advantage of the better octane number of ethanol compared to gasoline. Therefore, for pure hydrated ethanol used in optimized vehicles, ethanol can achieve about 75% or more of the range of gasoline on a volume basis (Mahesh *et al.*, 2017). Furthermore bioethanol use in engine proved to result in less Co_2 emission (Mohsin *et al.*, 2014).

Use of ethanol in compression ignition (CI) engines and challenges ahead

Due to its low cetane number, ethanol does not burn efficiently by compression ignition. Moreover, ethanol is not easily miscible with diesel fuel. Three directions are followed to improve the use of ethanol in CI vehicles (Demirbas, 2009). The first that consists in direct blend of ethanol with diesel needs addition of an emulsifier in order to improve ethanol-diesel miscibility. Other additives are used such as ethylhexylnitrate or diterbutyl peroxide in order to enhance the cetane number. Most of blends of ethanol to diesel (E-diesel) limit by up to 15% ethanol and up to 5% emulsifiers. The second way is a dual fuel operation in which ethanol and diesel are introduced separately into the cylinder. Finally, modification of diesel engines has been experienced in order to adapt their characteristics of auto-ignition and make them capable to use high blends such as 95% ethanol (Mahesh *et al.*, 2017).

Competence and drawbacks

In 2016, 80% of Brazilian energy comes from renewable resources where the bioethanol from biomass is the most important (Oliveira, 2016). Brazil has achieved the economic competitiveness of ethanol by configuration of multi-product industries. In this technology, the production of ethanol is coupled to the production of sugar. This has an economic advantage as the installed plant is not limited to one product (Kostin *et al.*, 2018).

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Except from Brazil, bioethanol is not presently competitive. This situation is critical on an energy basis; as low heating value of ethanol is one-third lower than that of gasoline even though at lower incorporation rates, this factor has little or almost no effect. Therefore, in several countries, government ensures subsidies and tax reductions in order to promote introduction of bio-ethanol. Some countries, such as Thailand, have succeeded to train people on use of agriculture by products for bioethanol production instead of discarding them as waste (Papong *et al.,* 2017). An alternative way to promote bio-ethanol introduction in the market is to cross-subsidies ethanol by fossil fuel. This approach increases the price of fuel for consumers and is neutral from a taxation point of view. When the difference between the production cost of ethanol and fossil fuels is low and the blend level is about 5 percent, the increase in price is not significant as the oil price is very volatile. In case of high ethanol production cost as in Europe, direct stimulation is required in order to make ethanol introduction appreciable for most of consumers (Dauriat, 2005).

Greenhouse gas and other environmental influence

As ethanol contains more oxygen than gasoline, its use favors more complete combustion and reduces the emissions of particulate matter and hydrocarbons, which result from incomplete combustion of gasoline. Tailpipe emissions of carbon monoxide and sculpture dioxide are also improved. However, low levels blends of ethanol with gasoline can increase the emissions of volatile compounds and nitrogen oxides. These emissions favor ozone formation. Still the wide spread use of bioethanol instead of biomass based biofuel has been proven to reduce by 21% greenhouse gas emission (Bayrakci Ozdingis and Kocar, 2018). Emissions of aldehydes and peroxyacetyl nitrate also increase to an extent that depends on weather conditions. The use of catalytic converters reduces the emissions of aldehydes. Reducing in refinery the vapour pressure of gasoline, which is blended with ethanol, can prevent emissions. Experiments about different percentages of ethanol-diesel blends show significant advantages concerning the above mentioned chemicals. Ethanol is more corrosive than gasoline and diesel, and at high concentration, can damage fuel system components. For high concentrations of ethanol, compatible materials are used in dedicated designed vehicles (Parekh, 1988; Honig, 2014). The average net CO_2 balance of bio-ethanol production is not presented here, because of the additional issues of system boundaries and variations of the incorporation rate of ethanol to gasoline (Nakashima and Ishikawa, 2016). Indeed, if the common practice for comparing the net energy balance of ethanol to that of conventional fossil fuels does not generally include the utilization phase of the fuels, it is more often the case as far as the CO_2 balance is concerned (Mandade *et al.,* 2016). Tailpipe emissions of carbon monoxide and sulphur dioxide are also improved. However, low levels blends of ethanol with gasoline can increase the emissions of volatile compounds and nitrogen oxides. These emissions favor ozone formation. Emissions of aldehydes and peroxyacetyl nitrate also increase to an extent that depends on weather conditions. The use of catalytic converters reduces the emissions of aldehydes (Xavier *et al.,* 2010).

Molecular advances in bioethanol production

The significance of biofuel in substituting and completing petroleum based biofuel have attracted the implication of advanced technologies for achieving the aimed target- increasing the production of biofuel and improving its desired properties. The necessity of engineered microorganisms is required because natural microorganisms are known to suffer from low growth rate and intolerance to the high concentration of produced biofuel added to toxicity of such biofuel and incomplete carbon source usage for instance a big number of microorganisms is not able to metabolize xylose containing feed stock at a given extent (Unrean and Srienc, 2010; Zhang *et al.,* 2011). Other parameters such as feedstock prices, processing technologies, production quality, and differences in varieties, production processes, and market acceptance have to be taken into consideration when thinking about engineering microorganisms for biofuel production (Yamada *et al.,* 2017). The existing metabolic engineering technics include shifting metabolic flux towards synthesis of new desired products, speeding up the rate of governing each step, extending the existing pathway to produce novel product, engineering the arrays of enzymatic activities that synthesizes new structure (Zeng and Sabra, 2011; D'espaux *et al.,* 2015; Cheon *et al.,* 2016). This genome engineering involves gene deletion, gene disruption or addition in targeted manner in order to change the physiology of a given microorganism. For instance, under strong promoter, the genes encoding for alcohol dehydrogenase II and pyruvate decarboxylase from *Z. mobilis* have been inserted into *E. coli* and resulted into increased growth and high production of bioethanol (Hou *et al.,* 2017). Another example is the genetic manipulation of *E. coli*, *Saccharomyces cerevisiae*, *Pichia pastoris* and *P. stipitis* which has made them robust and well-performing in the way that they can metabolize pentose sugars as they metabolize hexose sugars during the production of biofuel (Hou *et al.,* 2017). The above mentioned microorganisms many others like *Clostridium thermocellum*, *C. phytofermentas*, *Thermoanaerobacterium saccharolyticum* among others have been modified by means of adaptive evolution and recombinant tools such as direct mutagenesis , genetic and metabolic engineering to enhance bioethanol production as well as tolerance (Lee *et al.,* 2015).

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Future prospective

The molecular technology and evolution in selecting and processing of efficient microorganisms involved in biofuel production, along with possible combination of genetic manipulation will allow enhancing bioethanol production and its improvement for energy use and transportation application. This will be further made possible by the application of established genetic engineering techniques of the appropriate microorganisms such as *Saccharomyces cerevisiae* and bioprocessing techniques. Going ahead with the development of new microbial library creation, screening more powerful strains by direct evolution which will lead to explore the safe production and application of bioethanol in different sectors.

Conclusion

Due to its environmental merits, the bio- ethanol market will grow fast in the next future. Brazil benefits from bioethanol production and particularly favorable conditions with related to agricultural feedstock, sugarcane. Especially, Brazil exhibits the lowest production costs of bio-ethanol fuel worldwide and is in position to capture a large share of the international market in the future. However, the market price of bio-ethanol fuel will fluctuate as a result of the balance between demand and supply of bioethanol, oil and sugar. It is likely that the trend will be for an increase as a consequence of the fast growth of the world demand in the future. Apart from challenge related to production limitations, another big challenge is that bioethanol is used as additive to gasoline fuel and a blend over 20% can impact the function of engine. So an Adaptation of engine to ethanol concentration could be another room of improvement. In a longer term, lignocellulose-to-ethanol is very promising due to its independence from food markets. It is expected that the production cost will decrease as a result of the research works are carried out in several countries. Finally, due to the limited production resulting from inhibitors and limited resistance of use microbes in ethanol production, genetic engineering of these microbes could be a better solution for improved production.

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