REVIEW ARTICLE

TITLE

BIOETHANOL PRODUCTION FROM SUGARCANE MOLASSES AN INDUSTRIAL WASTE PRODUCT

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REVIEW: ETHANOL FUEL BASED ON A SUGAR INDUSTRY WASTE-MOLASSES

ABSTRACT

Due to recent increase in price hike of petroleum products, different efforts are being done throughout the world in search of an alternative source of fuel. For this reason, various agricultural countries are trying to produce their own fuel from plant based materials. Different types of biofuels like bioethanol, biodiesel, biohydrogen, biogas etc. are being investigated throughout the world. The type of the biofuel being produced depends upon the availability of the substrate. This review mainly focused on sugar industry waste called molasses, which is rich in fermentable sugars. The easiest form of the fuel that can be produced from sugar rich substrate is bioethanol, thus the review mainly focused on different methods of bioethanol production from molasses and the challenges related to this process. There are several factors that can affect the production of bioethanol that includes the type of substrate, the fermenting microorganisms, optimization of physicochemical parameters like pH, temperature, and the nutrient requirements of fermenting microbes for the conversion of sugar rich substrate into bioethanol. Moreover, the current scenario of the utilization of bioethanol as a fuel in various countries throughout the world was also discussed in this review.

Keywords: Bioethanol, Biofuel, Molasses, Sugar Industry, Fermentation

1. INTRODUCTION

Ethanol is the most advanced liquid considered as renewable fuel, which is able to substitute gasoline. Recently, major portion of this fuel is being produced by biological fermentation of sugars present in sugarcane molasses, fruits and cereals. Many investigations are also being done on cellulosic waste utilization for ethanol production **(Badger 2007; Karimi 2007)**. Ethanol is environment friendly fuel that can help in reduction of greenhouse effect produced by fossil fuels. Ethanol is most widely used in industrial applications due to its relative high affinity to water and organic compounds **(Anxo*et al*., 2008)**. Most often it is used as a recreational drug. Historically, ethanol was widely used as a medicine for depression and anesthesia. Despite of the fact that it seems quite simple chemical, it has vast variety of uses. Alcoholic beverages are common, but not the only use of ethanol. It is also used in thermometers and as a solvent to dissolve different chemicals. One of the most important and common use of ethanol is its use as spirit for disinfecting purposes. For this purpose a little amount of methanol (5%-10%) and some colors are added to ethanol to sell it as methylated spirit. Ethanol is most widely used as an antiseptic agent. About 62% (v/v) is used in labs to kill microbes. It destroys bacteria, fungi and viruses by denaturing their proteins and lipids but it is ineffective against resistant bacterial spores **(McDonnell, 1999).**The most recent advances in alcohol industry are its increasing demand to use it as fuel. Recently, the ethanol has been used as a substituent for fuel or fuel additive as it emit less pollutants **(Carvalho*et al*., 1993)**.

Microorganisms that carry out fermentation can't tolerate the higher ethanol and sugar concentrations as well. The high osmotic pressure created due to presence of higher sugar concentration may affect the health of cells and they can't carry out normal fermentation process. Thus, it is necessary to determine the amount of sugar which gives maximum ethanol yield. The amount of ethanol production increases as the concentration of sugar is increased up to certain limit that can be tolerated by microorganism. There is specific amount of sugar concentration for each strain when ethanol is produced to the maximum possible amount. At higher sugar concentration the production of ethanol starts decreasing due to unfavorable osmotic pressure for microorganism **(Peña-Serna *et al*., 2011)**.

In global market, Brazil is the country which produces 15 billion liter ethanol from 300 plants per year and supplies about 3 million of the car with pure ethanol. In US 80 plants are operating producing 10 billion liter ethanol per year. Similarly, China, India, Eastern Europe, Western Europe and Canada produce 3, 2.7, 2.5, 2 and 1.4 billion liters of ethanol per year respectively **(Klein, 2005)**.

2. CHEMICAL NATURE OF ETHANOL

Ethanol production and utilization have been known since ancient times. In alcoholic beverages it has been used widely as an intoxicating agent. Now it is positioned second to the water due to its best solvent properties thus increasing its demands in industrial sector. Ethanol acts as a solvent for wide range of products like lacquers, dyes, paints and oils. Further, it is widely used in chemical industries for production of different chemicals and a little for fuel purposes **(Rose, 1961)**.

Ethanol is actually 2-Carbon alcohol with the molecular formula CH_3CH_2OH. It is a colorless liquid, having agreeable smell. It is volatile in nature and quite inflammable. Ethanol is one of the volatile compounds with the boiling point of 78.5°C and melting point of -117.3°C **(Kaur and Kocher, 2002)**. It is actually a monohydric alcohol having single –OH group at the end of small 2 carbon chain. The hydroxyl group has the abilities to make hydrogen bonding which lowers its viscosity and polarity and makes it more viscous as compared to other polar organic compound of similar molecular weight.

Furthermore, the hydroxyl group makes ethanol more hygroscopic due to which it readily takes up water from the atmosphere. Ethanol is quite a versatile solvent, dissolves water as well as many organic solvents like acetic acid, benzene, toluene etc. The hydroxyl end of ethanol has polar nature which makes it capable of dissolving many polar and ionic compounds like KCl, $MgCl_2$, and $AlCl_3$ etc. However, the other non-polar end of ethanol makes it quite miscible with non-polar compounds like oils, medicinal agents, flavoring etc.

3. HISTORY

Ethanol production and utilization have been known since ancient times. In alcoholic beverages it has been used widely as an intoxicating agent. Dried residues found on nine thousand years old china pottery also showed that alcoholic beverages were also utilized by Neolithic people **(Roach, 2005)**. In Greek and Arabs, distillation was quite common. It was School of Salernoalchemists in the 12th century that was first reported for the alcoholic production from distilled wine **(Forbes, 1948)**.

Ethanol was first reported to be purified by passing distilled alcohol through activated charcoal, the method developed by **Johann Tobias Lowitz (1976)**. However, first synthetic ethanol was prepared in 1826 by Henry Hennel (Britain) and S.G Sérullas (France), both did independent efforts in this regard. Two years later, in 1828, Michael Faraday got succeeded in preparing ethanol by acid catalyzed hydration of ethylene, the method still used in industries for ethanol production. In 1826 ethanol was used as an engine fuel for the first time. However, since 1850s ethanol was mainly used as fuel for lamps. Years later, in 1876, Nicolaus Otto, the inventor of modern quadricycle combustion engine used ethanol for the first time for its engine. Later in 1896, first automobile was built by Henry Ford, in which pure ethanol was used as a fuel. Ford also designed an automobile model, which ran on mixture of gasoline and ethanol, where ethanol acts as octane booster. The main advantage of using this blend was its biodegradability. Ethanol is more easily degradable in nature as compared to gasoline. Furthermore, less than 10% ethanol addition enables the automobile engine to run smoothly even in absence of harmful chemical additives like lead. Ethanol addition also helps in reduction of pollution like release of carbon monoxide and other toxic pollutants. As ethanol is produced by crops, it utilizes carbon dioxide and released oxygen thus in turn ethanol can be considered as involved in reduction of greenhouse effect.

Now the interest of modern world in petrol is decreasing due to its price hike and the wide differences among supplies and demands. In addition, it is one of the major contributors of air pollution thus causing serious health hazards. Thus the limited global supply of oil is stimulating the search for alternative fuel to substitute the fossil fuel. In present state, due to energy crises, efforts are being done to find the renewable energy sources. Bioethanol can be used as one of the renewable energy source, produced by agricultural or agro industrial wastes and its byproduct **(Aristidou and Penttila 2000; Jeffries and Jin 2000; John 2004; Kerr 1998; Wheals *et al*., 1999; Zaldivar*et al*., 2001).**

Today the ecofriendly bioethanol utilization as a substituent for the petroleum based products has attracted worldwide interest for its production at large scale because this is the only fuel which can be used in current unmodified petrol engines by blending with gasoline in different proportions **(Hansen *et al*., 2005; Shanmugam, 2008; Klein, 2005).** In gasoline-ethanol blend the ethanol component of the blend act as oxygenator for gasoline **(FNB, 2007; Soliclima, 2007; Peña-Serna *et al*., 2011).**

4. METHODS OF ETHANOL PRODUCTION

There are several methods used for the production of ethanol, synthetic method or biological method i.e. fermentation. Now a day's ethanol is produced by both methods in industries. However the method of production is depends on the way of utilization.

4.1. Chemical Method

The ethanol that is used for the non-beverage purposes or solvent is produced from acid-catalyzed hydration of ethylene, a petrochemical feedstock. The ethanol produced by this method is referred as 'synthetic' and represented by the following chemical equation:

$$\mathbf{C_2H_4 + H_2O \rightarrow CH_3CH_2OH}$$

4.2. Biological Method

Most of the ethanol utilized in alcoholic beverages and fuel is produced by fermentation. In process of fermentation different *Saccharomyces* strains are used for the conversion of sugar in to ethanol and carbon dioxide in the absence of oxygen. One of the most common yeast used for this process from ancient times is *Saccharomyces cerevisiae*, whose name also shows its importance in history for ethanol production as ‘Saccharo’ refers to ‘sugar’ and ‘*myce*s’ means ‘fungus’ in Greek language whereas the word ‘*cerevisiae*’ is used for ‘beer’ in Latin. The overall reaction was expressed by a scientist Gay-Lussac that forms the basis of calculating fermentation efficiency.

$\mathbf{C_6H_{12}O_6}$		$\mathbf{2C_2H_5OH}$	+	$\mathbf{2CO_2}$
Glucose	$\Longrightarrow$	Ethanol		Carbon dioxide
(1 Kg)		(0.511 kg)		(0.489 kg)

Ethanol is more environmentally friendly fuel as compared to gasoline. If ethanol is combusted it causes less release of volatile organic compound, carbon monoxide (CO) and nitrogen oxides **(Wyman and Hinman, 1990)**. As ethanol is produced from the natural resources thus it is also believed that it is involved in reduction of greenhouse effect because the overall process utilizes CO_2 and release O_2. If ethanol is added gasoline different carcinogenic additives like lead are not necessary to use **(Lynd 1996).**

5. PRODUCTION OF BIOETHANOL

Bioethanol production mainly contains four important components that have the major role in production efficiency i.e. (i) fermentable sugars (ii) an efficient microbial strain (iii) nutrients and (iv) Optimized cultural conditions for best fermentation. Almost 80% of the world’s ethanol supply meets by the fermentation of either sugar/starch containing crops or byproducts of agricultural based industries.

5.1. Role of Different Substrates in Bioethanol Production

The substrate for the ethanol production is of immense importance for the fermentation process. The nature of the substrate used has a great influence on the process **(Prescott and Dunn, 1987; Baptista*et al*., 2006)**. Ethanol can be made from variety of agricultural feedstock like fruit juices, grains, cereals, whey, molasses etc by certain yeast’s species which converts sugar into ethanol and carbon dioxide under anaerobic conditions **(Nigam *et al*., 1998).**

Molasses can be obtained from different sources like sugarcane, sugar beet and fruits etc. The molasses is actually a thick material that is obtained after the sugar is crystallized and removed from mother liquor. The molasses contains different sugar like sucrose, glucose and fructose thus making total concentration of sugar 45 to 60% (w/v). Three types of molasses are mostly available i.e. black strap, refinery and invert or high test molasses. Sugarcane molasses contains less sucrose but more invert sugar i.e. fructose and glucose is present in it. Moreover it contains very little nitrogen which is necessary for different metabolic activities and amino acid generation in fermenting organism and raffinose content is also less. Molasses has very intense brown or black color and more buffer capacity **(Wang *etal.*, 1985; Borzani*et al*., 1993; Borzani 2001).** Now different investigations are being done on ethanol production by using free and immobilized cells of different microbes **(Gikas and Livingston, 1997; Yamada *et al.* 2002).** Pretreated sugar cane molasses is used for ethanol production; however, molasses requires very little pretreatment as compared to other substrates like cereals, grains or cellulosic materials etc.

There are several kinds of natural feedstock which can be used for ethanol production, however, sugar cane molasses are the most widely used in Brazil **(Bose and Ghose, 1973)** and India **(Sharma and Tauro, 1986; Bulawayo *et al.*, 1996)**. This byproduct of sugarcane industry mainly contains fermentable sugars therefore it is relatively much easier to produce ethanol and requires limited pretreatment as compared to other starchy or cellulose containing materials. In addition molasses are very cheap and available in plenty in India; therefore it is preferred as substrate for ethanol production **(Sharma and Tauro, 1986).** Other important substrates used for this purpose are sweet sorghum **(Bulawayo *et al.*, 1996)** sugar beet and beet molasses (**EI-Diwany*et al.*, 1992; Agrawal*et al.*, 1998).**

Ethanol is mainly produced by fermentation of sugar crops, in which sugar is converted in to ethanol. In US corn is the most predominant crop that is utilized for ethanol production whereas the world's largest ethanol producer i.e. Brazil produces its ethanol from sugarcane **(Morimura*etal.*, 1997; Agrawal*et al.*, 1998).** Recently efforts are being done on producing ethanol from cellulosic waste. In this way grasses, woods and other cellulosic waste can be used to produce ethanol to meet energy demand. Some easily fermentable materials contain whey, milk and cheese. Ethanol production from these materials can be possible by using those yeast strains having capability to hydrolyze lactose sugar **(Silva *et al.*, 1995; Ghalay and Ben-Hassan 1995).** Some starch containing materials are also used as raw material for ethanol production. Among them the most commonly used substrate is sweet potato **(Maisch*et al*., 1979; Sree*et al.*, 1999).** Other starchy material like Corn cobs and hulls **(Wilke *et al*., 1981; Beall*et al.*, 1992; Arni*et al* 1999; Kadam and McMillan, 2003)** are also used in some countries. Ethanol production from wheat has also been reported by different strains of saccharomyces **(Dabas*et al*., 1997; Lindeman and Rocchiccioli, 1979; Sasson, 1990).**

If ethanol has to be produced from cereal grains, first of all, it is necessary to convert starch into fermentable sugar by the action of amylase enzymes (Hsu, 1996). For this purpose, during beer production, grains are first allowed to be germinated, so that when the grains are mashed, the amylase produced during germination may convert starch into fermentable sugars like glucose and fructose. For fuel ethanol, this conversion of starch into glucose can be accelerated by treating the starchy material with either dilute sulfuric acid or fungal amylase. Sometimes combination of both is also used for this purpose **(Badger, 2007).**

Another method for the production of ethanol is by utilizing cellulosic waste. This can be used to obtain sugar from cellulosic material for ethanol fermentation **(Taherzadeh, 2007).** The first cellulose-based ethanol plant was established by Canadian firm logen in 2004. United state department of energy along with Canadian government are highly interested in commercializing the cellulose bases ethanol. Different enzymes companies are also finding different ways for designing genetically modified organisms to enhance enzymes production i.e. cellulase, xylanase and hemicellulase thus in turn reduces the enzyme's costs necessary for biological pretreatment of cellulosic material before fermentation **(Shoemaker, 1984; Ogier*et al*., 1999; Sonderegger and Sauer, 2003; Yu and Zhang, 2004; Kaar and Holtzapple, 2000; Maiorella, 1985; Sun and Cheng 2002).** Cocoa, Pineapples and sugarcane baggase are also now used as raw material for bioethanol production **(Othman *et al* 1992)**. However this is very expansive method for ethanol production and not a well-established one for commercialization. The substrates that are points of main focus are those that provide easily fermentable sugars **(Clines, 2006; Polman, 1994; Brown *et al*., 2001; Kaylen*et al*., 2000; Zhuang*et al*., 2001).**

However, the ethanol production from starch or cellulosic material is difficult because the substrate in this case is supposed to be hydrolyzed to fermentable sugar by physiochemical and enzymatic pretreatment before fermentation **(Zertuche and Zall, 1982).** The substrates that are points of main focus are those that provide easily fermentable sugars like molasses.

5.1.1. Pretreatment of Molasses

The effect of Pretreatment on sugarcane molasses before fermentation was studied by **Yadav*et al* (1997).** Molasses was treated with sulfuric acid H_2SO_4 and $K_4Fe(CN)_6$ before yeast inoculation and it was noticed that this is an effective method for the reduction of various inhibitory compounds. This chemical pretreatment reduces the inhibitory substance like iron (Fe), calcium (Ca) and copper (Cu) hence improved ethanol production.

In molasses the sugars that can easily be fermented include glucose, fructose and sucrose. Most of the yeast contain special enzyme i.e. invertase which convert the sucrose molecule into simple sugar. The non-sugar substance present in molasses may include polysaccharide, gums sterols, pigments, wax, different salts of magnesium (Mg), Potassium (K) and calcium (Ca) **(Rao, 1983).**

5.2. Role of Microbes

There are number of microorganisms including fungi, bacteria, and specifically yeasts which carry out the fermentation process for ethanol production. An extensive investigation has been done on the microorganisms especially yeast cells which have the ability to produce bioethanol by fermenting sugar present in different substrates **(Bajaj *et al*., 2001)**. Among all the microorganisms *S. cerevisiae* has remained the most favorite one for the researchers because this is the same species that is used for baking and brewing as well (**Walker *et al*., 1990; Converti*et* al., 2003; Moreira *et al*., 2005)**. Other organisms of main interests are *S. uvarum, S. pombe, S. vini, S. acldodevoratus*and *Kluyveromyces* sp. **(Tao *et al*., 2005; Haq and Ali, 2007).**

The main hurdle comes while production of higher concentration of ethanol is the intolerance of microbes against product. The higher ethanol concentration is quite harmful for microorganism. Ethanol denatures the enzymes necessary for fermentation which inhibits the further production. Baker's yeast can only withstand the ethanol not more than 5 or 6% (w/v). Many of the yeasts in industries can tolerate the ethanol up to 18% **(Balat et al., 2008).**

Another crucial factor that plays a major role in fermentation is the selection of potent microorganism. Different varieties of microorganisms including bacteria fungi and yeasts have been used for the ethanol production **(Vallet et al., 1996).** Some important yeast strains used in distilleries for alcohol production are S*.cerevisiae, Zygo.saccharomycessp., S.ellipsoids, Schizo.pombe, Schizo.mallaeri*producing percentage alcohol of 5.8-11.16, 4.2, 9.7, 8.7 and 7.8 (w/v), respectively **(Kalra, 2004).**

Among bacteria the most promising one for the ethanol production is *Zymomonasmobilis* commonly used in making palm wines. **Skotnicki *et al* (1981)** worked on growth and ethanol production by 11 different strains of *Zymomonas* and observed that some of the strains are more tolerant to high sugar and ethanol concentration and resist temperature higher than others.

Uma and Polasa (1990) also isolated *S. cerevisiae* from Palm wine. **Bertolini*et al* (1991)** isolated another strain of *S. cerevisiae* from alcohol industries and cultivated them on a basal medium having 48% sucrose. The isolated strain was found to have the ability to convert concentrated sugar solution with conversion efficiency of 89 to 92%. **Bansal and Sing (2003)** compared *Saccharomyces cerevisiae* with *Zymomonasmobilis*formaximum ethanol production from molasses and they found that yeast is more tolerant to ethanol concentration.

All the previous studies show that *S. cerevisiae* was the most potent microorganism for ethanol production as compared to others **(Ergun & Ferda, 2000).** The main reason for this difference different level of production by different strains is that some species adopt different metabolic pathway for ethanol production in special genes encoding special enzymes like invertase are involved. This enzyme has an important role in conversion of sugar to ethanol or other metabolites **(Fregonesi*et al*., 2007).**

Microbes have specialized enzymes like invertase and zymase that convert sugars to reducing sugars then to high concentrations of ethanol respectively. There are certain specific conditions for each strain at which its enzymes perform at its best. In extreme conditions which are unfavorable for microbes, enzymes become inactive and ethanol production is reduced.

5.2.2. Genetically Modified Organisms

The Plant Biotechnology Unit of the Corporación para Investigaciones Biológicas(CIB) genetically modified the *S. Cerevisiae* strain by inserting optimized *pdc*and *adhII*genes from *Zymomonasmobilis*which have shown greater ethanol production than CBS8066 (parental strain) when glucose was used as a carbon source **(Vásquez et al., 2007).**

The enzymes i.e. pyruvate decarboxylase (PDC) and alcoholic dehydrogenase (ADH) are important for ethanol production **(Gunasekaranand Chandra, 1999; Gottschalk, 1986; Matthew *et al*., 2005).** These enzymes are present in both organisms i.e. *S. cerevisiae* and *Z. mobilis* however they have some differences according to the origin of microorganisms. It was found that the enzyme possess by *Z. mobilis* showed high affinity to their respective substrates **(Sprenger, 1996; Brenda database, 2007).** It was observed that despite of lower enzyme affinity yeast has more tolerance against ethanol. Therefore ethanol yield can be enhanced by inserting genes of *Z. mobilis* to *S. cerevisiae* **(Zhang *et al*. 1995; Ingram and Doran 1995; McMillan *et al*. 1999; Lynd *et al*. 2002; Sun and Cheng 2002).**

The culturing of yeast for the production of ethanol is termed as brewing. Brewing process is only able to produce very dilute amount of ethanol because yeast can tolerate ethanol up to certain limit. The higher ethanol concentration affects the catalytic activity of yeast enzymes. Baker's yeast can tolerate ethanol not more than 5-6% (w/v). However, the yeast used in several wine industries stops reproducing when ethanol concentration reaches up to 12-15%. The most tolerant yeast is reported to withstand the alcohol content of 18 percent **(Balat*et al.*, 2008).**

6. OPTIMIZATION OF PHYSICOCHEMICAL PARAMETERS

The optimization of different physicochemical parameters for maximum ethanol yield is of great interest to ensure the efficient utilization of carbon source **(Wyman and Hinman, 1990).** The importance of optimized temperature and pH is quite clear from the previous studies. It shows that a specific condition of temperature and pH plays a vital role in preventing contamination in fermenting material **(Wang *et al*., 1998; Sugawara *et al*., 1994; Murtagh*et al.* 1999).** In previous studies the lower yield of ethanol was attributed to shortage of trace elements in molasses **(Wang *et a*l., 1998; Sugawara *et al*., 1994; Murtagh*et al.*, 1999)**. Different studies were also done to solve this problem.

6.1. Effects of Sugar Concentration

The main hurdle to fulfill the desire of obtaining higher ethanol production is the intolerance of yeast towards high sugar concentration and final product i.e. ethanol as well. The metabolic cycles of the yeasts are inhibited at too much higher sugar concentration. Therefore it is quite important to determine the amount of sugar that could give the maximum ethanol production with minimum substrate utilization **(Peña-Serna *et al.*, 2011).**

The amount of ethanol production is increased with the use of higher sugar concentration. The comparison of different substrates i.e. sugarcane molasses and banana juice showed that higher final ethanol yield obtained while using sugarcane molasses as it contains more sugar as compared to banana juice. Furthermore, greater biomass was also produced when sugarcane molasses was used as a substrate **(Peña-Serna *et al.*, 2011).**

Borzani*et al* (1993) studied the effect of various sugar concentrations for final ethanol yield. They also expressed the logarithmic relationship between the initial sugar concentrations and fermentation time. In industries, 16-18% (w/v) sugar concentration is usually used and further increase in concentration adversely affects the fermentation efficiency due to high osmotic pressure development.

Bertolini*et al* (1991) studied different strains of yeast capable of utilizing 30% of sucrose and producing ethanol in different concentrations. These strains were isolated from Brazilian alcohol factories hence are more tolerant to high sugar and ethanol concentration. The fermentation efficiency of these strains varied from 89% to 92%. The final yield of ethanol was obtained as 19.7, 18.0 and 15.6% (w/v). Thus it is quite important to isolate and identify those strains which are more tolerant to substrate and product concentration to obtain maximum ethanol yield.

When high sugar concentration is present in fermentation broth, it increases the ethanol yield **(Thatipamala*et al.*, 1992)** because in the presence of higher concentration the yeast (even under high dissolved oxygen concentration) changes its oxidative metabolism to oxidoreductive or fermentative metabolism (Lei *et al.*, 2001). This phenomenon is referred as Crabtree effect **(Converti*et al*. 1985, Lei *et al.* 2001, Thatipamala*et al*. 1992).**

Xin *et al* (2003) studied that maximum 16.5% (w/v) of ethanol production was obtained by using 35% (w/v) of glucose concentration. When this concentration exceeded 45% the bacterial growth was totally inhibited due to high osmotic pressure.

Sree et al (2000) carried out batch fermentation to determine the ethanol production by using different sugar (glucose) concentrations i.e. 150, 200 and 250 (g/L) at 30°C. Immobilized osmotolerant*S.cerevisiae*(US3) was used for this process. The ethanol yield obtained for the three concentrations were 72.5, 93 and 83 (g/L) respectively at 30ºC after 48h. Hence the maximum yield was obtained when initial sugar concentration was 20% (200g/L) giving fermentation efficiency of 90%.

An inhibition effect was also observed due to some minor sugar (arabinose, rhamnose and galactose) that are slowly metabolized or not at all in fermenting material. **Converti et al (1998)** observed the inhibition in fermentation of oak hemicellulose acid hydrolysates by minor sugars. Sánchez and Cardona (2008) suggested that the lesser ethanol production from banana juice as compared to sugarcane molasses could be due to oligosaccharide that can’t be taken up by the yeast cells thus their growth and final ethanol yield decreased.

6.2. Effect of pH

Another important parameter that has a huge impact over the fermentation and final yield is the determination of the most favorable pH for fermenting material. The pH control is very important for fermentation process for two reasons. First, yeast prefer to grow at slightly acidic pH range. The most favorable pH range for the yeast growth is 4.0 to 5.0. Secondly, bacterial contaminant of fermenting material can't survive the acidic pH i.e. below 5. The grain mashes mostly have the slightly acidic pH i.e. 5.4 to 5.6. However the saccharine substances that are directly fermented like sugarcane molasses or fruit juices have naturally alkaline pH and should be acidified prior to fermentation to favor the yeast growth and preventing contamination. The main contaminant bacteria of fermenting material are *Lactobacillus spp*. which grows best at pH range 5.5 to 6.0. These bacteria produce lactic acids thus alter the taste. Although during fuel production taste of the product is not concerned therefore lactic acid can be simply subtracted from final ethanol yield. However the contamination should be avoided as much as possible. As the growth of these contaminants are greatly repressed at pH below 5 therefore the pH of fermenting material should be adjusted between 4 to 5 to prevent contamination and efficient yeast growth as well **(Mathewson, 1980).**

It is evident that the best pH for fermentation is slightly acidic in which yeast grows at best and contamination can be prevented. The studies show that the contaminants produce waste materials which make the environment unfriendly for other organisms even for the yeasts. At first yeast outgrows the other organisms but as the yeast growth slows down the contaminant growth increases due to competition of nutrient. In some cases such substances are produced during fermentation which alters the pH of fermenting material. Once the pH crossed the optimized range all the efforts to readjust the pH are in vain. Therefore pH should be periodically adjusted to obtain maximum ethanol yield only possible due to efficient yeast growth **(Yadav *et al*1997; Periyasamy*et al*. 2009).**

6.3. Effect of Temperature

During the fermentation process heat is evolved which raises the temperature of fermenter. Temperature exerts noticeable effects on growth of the organism, its metabolic activities and in turn fermentation process. Thus it is quite important to cool down the large fermenters to maintain the optimized conditions. However, this maintenance of temperature is quite expensive and a laborious process. In industries, fermentation is usually carried out at moderate temperature i.e. between 25°C to 35°C. The temperature above 40°C in some moderately hot regions may affect the viability of yeast cells and the fermentation process as the viability of cells is important for the fermentation process. The thermo-tolerant cells that can tolerate the temperature above 35 °C or 40°C can reduce the operational costs of temperature control for maximum ethanol production.

Therefore lots of investigations are being done to search those organisms that could survive higher temperature up to 40-45°C **(Szczodrak and Targonski 1988).**

Laluce*et al.* (1991) determined the effect of temperature on three yeast strains by using sugarcane juice having 15% sugar. The fermentation was carried out at 40°C. He observed the complete conversion of sugar within 12 hours. It was also noticed in all the three strains that the fermentation was inhibited above 40°C. Further findings showed that the growth of organism and ethanol formation depends on the composition of medium and strain used. At higher sugar concentration of 20%, the best temperature was found as 35°C for maximum ethanol production.

Mauricio *et al.* (1989) reported that the fermentation at lower temperature produces volatile acids. The fermentation stops before whole sugar utilization at 30°C when 342 (g/L) sugars were used. The temperature below 30°C causes the adverse effects on survival of *Saccharomyces cerevisiae*.

Saeki *et al.* (1997) reported some thermotolerant bacterial strains that give 2 to 3 times more ethanol yield at higher temperature as compared to mesophillics strains at 30°C.

Torija et al. (2001) reported different strains of *Saccharomyces* and observed the difference in optimum temperature for each strain. He carried out the fermentation at temperature range 25°C to 30°C and found that some gives better yield at lower temperature while others perform best at higher temperature. Those strains that perform better at lower temperature can't withstand higher temperature thus by increasing temperature the amount of secondary metabolites increased.

Singh *et al.* (1998) reported several strains of *Kluyveromyces marxiaus*var. *Marxianses* which are capable to grow at high temperature and produce more ethanol from molasses and glucose. Fermentation at the temperature above 30°C stuck the fermentation; however in some findings the ethanol yield increases from 6% to 7% at the temperature 35°C **(Yalçin and Özbas, 2004; Dhaliwal*et al*., 2011).**

Phisalaphong *et al.* (2005) showed the effect of temperature on *Saccharomyces cerevisiae* M30 by using sugarcane molasses as a raw substrate. He designed a mathematical model to explain the kinetic parameters of ethanol affected by higher temperature. The model explains that how the higher temperature adversely affect the fermentation efficiency and final yield.

6.4. Nutrient's Requirement

Once there was a wrong perception regarding ethanol tolerance that it does not depend on nutritional requirement but now the idea has been changed. It is now evident that by altering the nutrients supply to the microorganism its ethanol tolerance can also be altered and more ethanol yield can be obtained this way **(Casey *et al*., 1983).**

In industrial scale, the fermentation efficiency calculated is based on amount of sugar introduced in fermenter. The ethanol yield from *S. cerevisiae* can be 90% to 93%. The studies show that the ethanol tolerance and sugar utilization can be improved by changing the amount of nitrogen source i.e. urea in fermenting material **(Thomas and Ingledew, 1990; Thomas et al., 1993; 1996, Yalçin and Özbas, 2004).**

According to **Bafrncová et al. (1999),** nitrogen plays a vital role in growth of the organism, its tolerance towards ethanol and the final ethanol yield. Thus the nitrogen source i.e. urea was examined and it showed the profound effect on fermentation rate. **Gough *et al*., 1996** provided different concentration of urea to the fermenting media and found the concentration of 3 (g/L) as the best. At this concentration 7.7% (w/v) final ethanol yield was obtained. However, alteration in urea concentration showed no effect on the growth of yeast cells.

The studies revealed that the nitrogen supply can be more effective in increasing the ethanol yield even at the higher temperature. Currently, 0.5 (g/L) of urea is used in the industries. However according to studies 3(g/L) urea gives more ethanol yield. Normally at higher temperature i.e. 40°C the ethanol yield is limited as compared to lower temperature. However, if urea is supplemented at the amount of 3(g/L) then the ethanol yield becomes 65% which is 30% higher than the yield while using 0.5 (g/L) at the same temperature. Thus the importance of nitrogen for *S. Cerevisiae* has become evident. It was also observed that the higher urea concentration may cause the adverse effect on fermentation efficiency because of the toxicity created in the medium due to more waste accumulation.

7. LIMITATION TO ETHANOL PRODUCTION

Ethanol produced by the fermentation arise from the biotechnological industries is the most valuable product in terms of both the amount and value **(Nissen *et al.*, 1999). Oderinde *et al.* (1986)** reported that if the metal ions are removed from the molasses it enhances the final ethanol yield. In 1996, total production of ethanol reached to 31.3 billion liters **(Berg *et al.*, 1998)** and more than 80% of it was produced by anaerobic fermentation of different sugars sources by *Saccharomyces cerevisiae.*

Among the last two decades lots of investigations have been done in order to improve the fermentation technology. Contamination, availability of substrates and the process design in fermentation are the limitations that have a great impact over yield and quality of final product **(Rückle, 2005).** The price of raw material is also a determining factor for the estimation of profit in this process **(Bai*et al.,* 2008; Carlos *et al.,* 2011).**

The awareness regarding the importance of ethanol in its use as a substituent for fuel created a huge interest in increasing the fermentation efficiency from last two decades **(Vega *at al*., 1987).** All the factors should be checked which are responsible for poor fermentation **(Walker *et al.,* 1996).** The qualities of the molasses should be monitored from the time of cultivation to the harvesting of sugarcane so the possibilities of all the impurities could be judged **(Eggleston *et al.,* 2008).**

The presence of heavy metal ions like potassium causes the adverse effect on yeast growth and metabolism while the availability of micronutrients like zinc and magnesium are vital for the enzyme activity of yeast cell **(Walker *et al.,* 1996; Ryan and Johnson, 2000).** Since yeasts are unable to produce required microelements and nutrients supply make them more tolerable to high alcohol levels **(Walker *et al*., 1998).**

The temperature control should also be monitored in fermenter because the high temperature can affect the cell viability or denature the enzyme so that yeast cell loss its enzyme activity and the final ethanol yield becomes low. Further some measures must be adopted to prevent the fermenting media from contaminating bacteria and wild yeasts that may also adversely affect the ethanol production **(Wang et al., 1999).**

There are number of lactic acid and acitic acid bacteria that are present as contaminant in molasses but the most problematic one is *Leuconostoc mesenteroides* which is harder to detect. The bacteria polymerize the sucrose into larger molecule i.e. dextrin which is unable to ferment by *Saccharomyces Cerevisiae* but count as a reducing sugar in total sugar as invert (TSAI). **(Eggleston *et al*., 2008)**. Now the economic incentives have been taken in industrial process for ethanol production thus causing remarkable increase in final yield **(Carlos *et al.,* 2011).** The most recent investigations are done for the use of genetically modified substrate (containing high sugar content) and organisms as well that improve the ethanol yield and efficient fermentation process **(Riley *et al*., 1996; Wood *et al.* 1997; Wooley*et al*., 1999; Cao *et al*., 1996).** Other research are also focused on enzymetic hydrolysis of several substrate, use of magnetized fluid bed reactor, and immobilized cells of *Zymomonasmobilis* and fermentation of molasses by *Zymomonasmobilis* **(Ghasem*et al*. 2004; Takamitsu*et al.* 1993, Herna´ndez-*Salas et al*., 2009; Chun-Zhao *et al.,***

2009). Most of the industries rely on the ability of yeast to ferment the sugar even under aerobic conditions thus developed different signaling and sensing mechanism to stop the use of alternative carbon source other that may favor the ethanol yield **(Badotti et al., 2008).**

8. WORLD'S ETHANOL PRODUCTION

Since 1978, Brazil is too much concerned about its environment because disposal of ethanol in water was creating serious health problems. Some of the diseases are curable while others are incurable. However, after years of researches Brazil succeeded in handling the problems caused by biofuel. Now it is quite safe and sound and they have set the tradition to use it as biofuel. Secondly, the use of bioethanol can be quite beneficial for the farmers as they can increase their income by providing natural feed-stocks for the production of bioethanol.

Brazil is the country that meets 45% of its fuel demands from ethanol **(Agama Energy, 2003).** More than 20% of the Brazilian vehicles have flex fuel engine or ethanol-only engine having the ability to use 100% pure ethanol. In Brazil the flex fuel engine can use either pure ethanol or ethanol-gasoline blend containing at least 25% ethanol. In Brazil ethanol is produced from sugarcane molasses, having 30% more sugar as compared to corn which is widely used in United States of America (USA) for the production of bioethanol **(Rosillo-Calle and Cortez 1998; MacDonald *et al*., 2001)**. In USA the flex-fuel automobiles can use the ethanol from 0 to 85% in fuel. Almost 65% of the world's total ethanol is supplied by USA and Brazil. It is also used as a fuel in rockets **(Denise, 2010).** The main hurdle in shipping ethanol from modern pipeline (like petroleum products) is its high miscibility with water **(Horn, 2006).**

Pakistan has recently announced the policy for utilization of ethanol as biofuel. They have decided to use the blend of gasoline and ethanol in which 10% ethanol will be mixed with gasoline. Ethanol is considered as much cheaper fuel as compared to gasoline. Test runs are on the way under different companies. PSO (Pakistan state oil) is taking different steps to initiate the use of alternative fuel source in Pakistan under Government of Pakistan policies.

Pakistan produces 2 million tons molasses each year and exported 1.5 million of it at the price of $35 per ton, earning about $45 million annually. About 240 to 270 liters of ethanol can be produced from 1 ton of molasses (depending upon the quality of molasses). If whole amount of the molasses is fermented and processed to produce ethanol, more than 500 million liters ethanol can be produced from 200tons of molasses. If the country exported the ethanol (at the price of $360 per ton) instead of molasses then it can get the profit $144 million **(Rashid and Altaf, 2008).**

In Pakistan the utilization of molasses for commercial purposes is very limited. Major amount of is exported out. Recently, 13 distillatory are working in Pakistan. Out of which 10 distilleries are attached with sugar mills with 8 functional and 2 non-functional. The ethanol production in Pakistan is not comparable to European market. Due to sanctions on Pakistan, two out of seven distilleries have been closed and five more distilleries have also lost the interest in operating due to unsure conditions of market **(Bendz et al., 2005).** The ethanol that was exported to EU can be now converted to industrial fuel. However, the conversions of ethanol into fuel ethanol dependent on the government's indigenous fuel ethanol program **(Rashid and Altaf, 2008).**

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