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11

Biofuel From Cellulosic Mass with Incentive for Feed Industry Employing Thermophilic Microbes

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1. Introduction

Drastically depleting fossil fuels' supplies and the associated environmental concern dictate for immediate, renewable and environmental friendly alternatives. Cellulosic biomass has a great potential for bioethanol production. Many problems of the way have been solved by isolating and employing thermophilic, cellulolytic and ethanologenic microorganisms. Many workers have established simultaneous saccharification and ethanol fermentation from agro-industrial wastes rich in cellulosic material and some soluble sugars. The latter substances provide quick carbon and energy sources to the bacteria and yeasts inoculants. In Pakistan sugarcane bagasse is a very appealing potential agro-industrial waste in this regard. Owing to the great sun shine in majority of the country area throughout the year, solar energy has been dreamt for disinfecting the fermentation facility as well as providing steam for pretreatment of the substrate.

Another source of clean fuel is H_2. We have also been able to isolate and cultivate purple-non sulfur bacteria for the production of H_2 employing certain agro-industrial waste including the one just referred as major media ingredients. Biohydrogen can be obtained economically, by employing bacteria capable of fixing sun energy and utilizing agroindustrial wastes including cellulosic material heterotrophically.

A very appealing notion about the potential of microorganisms isolated from industrially contaminated aquatic and soil habitats form developing countries is their pollutants' resistance. This ability renders such microorganisms capable of biofuel generation from industrial effluents containing biomass as well as different chemical pollutants. It is very right time to conserve such pollutants' resistant microbial diversity before the developing countries progress for in situ treatments plants for their industries and the polluted areas recover back to their uncontaminated nature, alongwith losing the pollutants selective pressures mediated and thus evolved microbial communities. This journey is expected to be completed earlier than the time frame the developed countries had passed through. As the developing countries are benefitting from the experiences of the developed nations and thus are striving to escalate the process of progress.

This chapter outlines the possibilities of ethanol and hydrogen fermentations for the application of agro/food industrial wastes. The related issues have been dealt in depth and the chapter comprises two major sections i.e.bioethanol and biohydrogen.

2. Ethanol a renewable biofule

Energy needs of most nations of the world have increased over the time. Following industrial revolution in the late 18th century, societies that had been based largely on agriculture turned to industry to meet the needs of their growing populations. Energy plays an essential role in modern society. Fuel consumption has not only increased by factories, rather more fuel is required to distribute the market products. Ever increasing human population density and the desire for higher life standards, demanding more and more comforts, had necessitated large scale exploitation of fossil fuel energy resources, in the recent centuries. Between 1900 and 2000, word energy consumption increased by a factor of fourteen while the population increased threefold. Owing to the facts of ever increasing consumption and rapidly depleting resources of fossil fuels, scientists have rightly sensed that to feed and provide other requirements to the human population at a reduced environmental cost is a real target for future biotechnological improvements. Fueling both the humans and the required mechanical engines necessitates various developments in the agricultural and energy sectors, respectively. (Enger & Smith, 2002; Gray *et al.,* 2006; Smith, 1996).

Besides environmental deterioration, one of consequences of fossil fuels usage, their supply is being exhausted rapidly. Priorities are being shifted from building power stations, oil fields and coal mines, to active pursuit of energy and efficiency improvement and identifying renewable energy sources. One such resource is the bioconversion of plant biomass to ethanol. Motor cars in some countries are being driven by gasoline-alcohol mixture (4:1) called gasohol (Bernstein *et al.,* 1996; Preuss *et al.,* 1998; Van Haandel, 2005).

Biofuels represented by biologically produced alcohols, gasses, and oil represent renewable energy resources, unlike petroleum, coal and nuclear fuels. Rising energy and environmental problems have led to increased interest in the production from diverse routes and resources and utilization of alcohols as fuel (Atiyeh & Duvnjak, 2002; Lawford *et al.,* 2001; Von *et al.,* 1994).

The subject mater is reviewed here under the following headlines:

1. Ethanol as fuel
2. Ethanologenic fermentations
3. Ethanol from lignocellulosic biomass
4. Consolidated bioprocess: Simultaneous Saccharification and Fermentation (SSF).
5. Thermophilic ethanologenic microbes.
6. Sugarcane bagasse a resource rather than a waste.
7. Single Cell Protein (SCP) from agro industrial wastes.

Some of the highlights regarding the above referred topics are described in the forthcoming pages.

3. Ethanol as fuel

Ethanol has been used as biofuel in the United States, Europe and Brazil. In Brazil industrial scale ethanol is produced from sugarcane for blending with gasoline.While in the U.S. corn is used for ethanol production and is then blended with gasoline to produce gasohol (Enger & Smith, 2002; Lynd, 1995; Wheals *et al.,* 1999). Apart from being a renewable fuel made from plants, with high octane at low cost, ethanol is a much cleaner fuel than petrol. Ethanol blends dramatically reduce emissions of hydrocarbons, major

sources of ground level ozone formation, cancer-causing benzene and butadiene, sulphur dioxide and particulate matter. Moreover, ethanol blends can be used in all petrol engines without modifications (Miller, 2003).

Lynd (1995) has condensed valuable information in his essay on biological fuel production.Accordingly, ethanol is the most widely used biologically produced transportation fuel. Major ethanol industries arose during the 1980s in Brazil and the United States. Ethanol has a higher economic value in low level (e.g., 10%) gasoline blends than in neat (unblended) form. However, the fuel properties of neat ethanol are in general excellent and decreased emissions of ozone precursors are expected for neat ethanol.

Brazil is the largest producer of bioethanol, and sugarcane is the main raw material. In this country ethanol has been used as an octane enhancer in gasoline in the form of 22% anhydrous ethanol at 99.6 Gay-Lussac (GL) and 0.4% water or in neat ethnaol engines in the form of hydrated ethanol at 95.5 GL. In other countries gasohol blends typically contain only 10% ethanol. Ethanol makes an excellent motor fuel: it has a research octane number of 109 and a motor octane number of 90, both of which exceed those of gasoline. Ethanol has a lower vapour pressure than gasoline, which results in lower evaporative emission. Ethanol's flammability in air is also much lower than that of gasoline, which reduces the number and severity of vehicle fires. These properties of ethanol have led to the development of dedicated (E-100) and modified (E-22) engines for the ethanol-gasoline mixture in Brazil (Goldemberg & Macedo, 1994; Zanin *et al.,* 2000).

Sixty eight percent of the ethanol produced in the world is used as fuel. Production of ethanol is not evenly distributed throughout the world. North America contributes for 66%, Asian and Pacific Ocean countries for 18%, Europe for 14% and Africa for 2%. Brazil and United States contribute a great share of global production with 53% and 19%, respectively. Brazilian sugarcane ethanol is now a global energy commodity that is fully competitive with motor gasoline and appropriate for replication in many countries (Goldemberg, 2007; Zanin *et al.,* 2000).

4. Ethanologenic fermentations

Ethanologenic fermentation is the microbial conversion of sugars into carbon dioxide and ethyl alcohol. Regarding the provision of sugars for ethanol fermentation, it is pertinent to note that development of several novel sweeteners, many times sweeter than sucrose could ultimately lead to a reduction in the traditional sugar market for sugarcane and sugar beet. In this way, these economics predominately in developing countries could experience severe financial and employment discretion with alternatives difficult to find (Smith, 1996). The ethanol fermentations meant to generate biofuel would then be amongst the considered alternatives. Sugars may also be derived from starches and cellulosic materials in addition to black strap molasses, a by-product of cane sugar manufacture. Once simple sugars, the monomeric units are formed, enzymes from yeasts and bacteria can readily ferment them into ethanol.

Moat *et al.* (2004) have summarized the fermentative pathways occurring in some of the major groups of microorganisms (Fig.1). They have described that a thorough evaluation of the pathways of carbohydrate fermentation requires qualitative identification of and quantitative accounting for the amount of products recovered. To assess the accuracy of the analytical determinations, a carbon balance or carbon recovery is calculated. Oxidation-reduction (O-R) reactions play a major role in the fermentative metabolism of carbohydrates.

The O-R balance provides an indication as to whether the formed products balance with regard to their oxidized or reduced states. It may not be possible to balance the hydrogen and oxygen of the substrate directly because hydrations or dehydrations may occur as intermediary steps in the fermentation pathways.

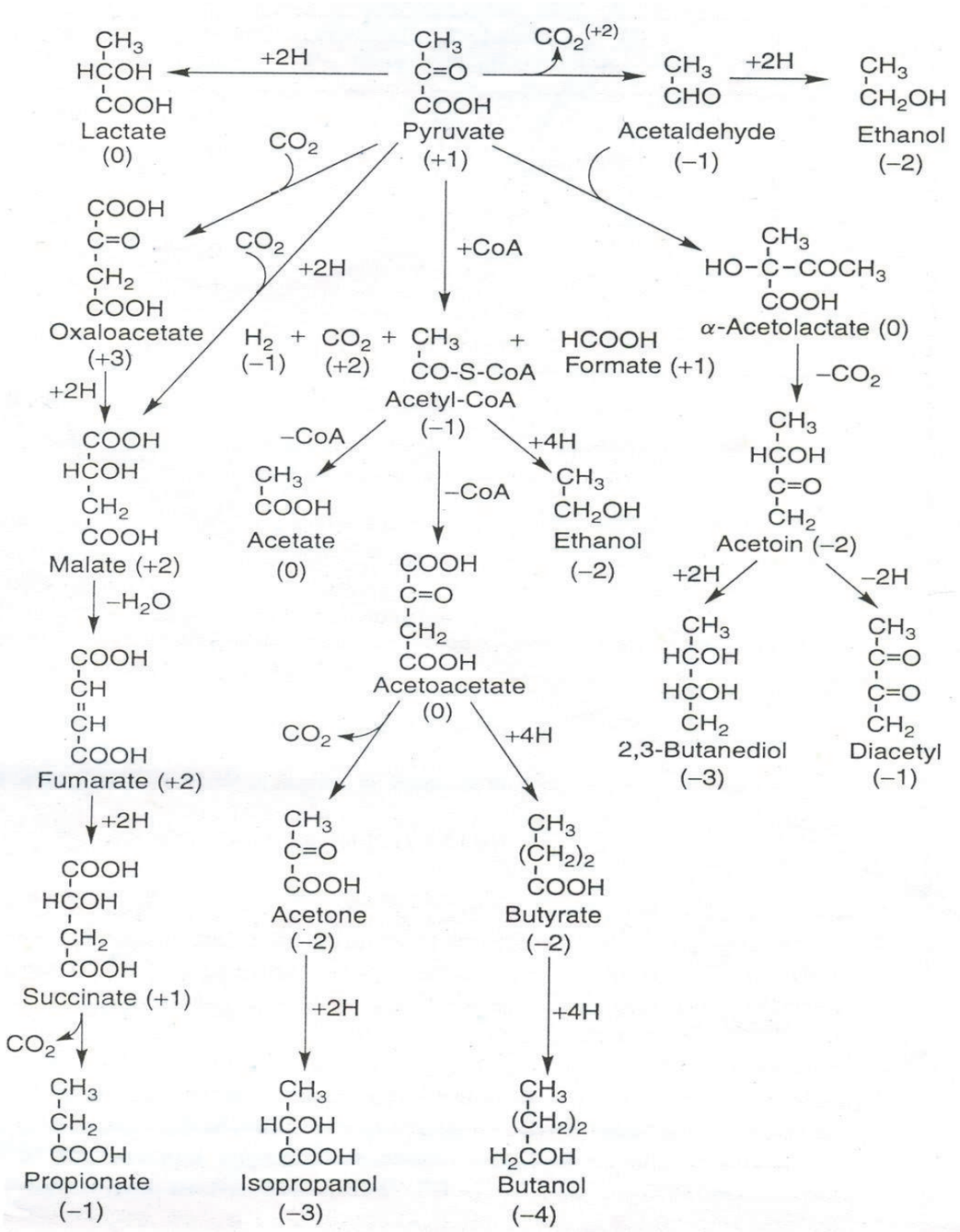

Fig. 1. Major pathways of fermentation products formation from pyruvate. Number in parentheses are the oxidation values calculated on the basis of the number of oxygen atoms less one-half the number of hydrogen atoms (Moat *et al.,* 2004).

If the ratio of oxidized products to reduced products is close to the theoretical value of 1.0, this provides further indication that the products are in balance. The oxidation value of a compound is determined from the number of oxygen atoms less one-half number of hydrogen atoms. For glucose, which has 6 oxygen atoms and 12 hydrogen atoms the oxidation value is 0. Thus, glucose is referred to as a neutral compound, and to be equally balanced the fermentation products should contain equivalent amounts of oxidized and reduced products. Another aspect of fermentation balances is the C_1 balance. The amount of expected C_1 product is calculated from the amounts of those products for which CO_2 or format is expected as an accompanying product. For example, if a C_2 compound such as ethanol or acetate is among the final products, an equal amount of CO_2 will be expected, since ethanol is derived from pyruvate by decarboxylation.

5. Ethanol from lignocellulosic biomass

Having technologically difficult boundaries but abundantly available cellulosic material has been conceived by contemporary biotechnologists for bioethanol production. For instance, Wheals *et al.* (1999) while discussing commercial viability of fuel ethanol had described that there will only be sufficient, low-cost ethanol if lignocellulose feddstock is also used. Similarly, Farrell *et al.* (2006) have explained that large-scale use of ethanol for fuel will almost certainly require cellulosic technology.

Amongst the plant biomass, cellulose is the primary substance that is produced following chemical transformation of the solar energy. It is one of most abundant organic compound in the biosphere. Some 10^{15} Kg of cellulose is synthesized by converting more than 100 billions metric tons of CO_2 and water into this and other plant products. A comparable amount of cellulose is also degraded on earth every year. It is an unbranched polymer of glucose residues joined by β-1,4 linkages consisting of 10,000-15,000 D-glucose units (Stryer, 1995). The β-1, 4-linked glucose polymer occurs in crystalline or amorphous forms and is usually found along with other oligosaccharides in the walls of plants and fungi. Cellulose-digesting microorganisms in the rumen of herbivorous animals are responsible for the ability of ruminants to use cellulose as a source of energy and building blocks for biosynthesis. The ubiquitous distribution of cellulose in municipal, agricultural and forestry wastes emphasizes its potential use for conversion to useful products such as single-cell protein or fermentation products such as methane or alcohol. As a consequence, the degradation of cellulose has been a continuing subject of intense study. Cellulose constitutes much of mass of wood and cotton is almost pure cellulose. Many manufactured products; including paper, cardboard, rayon and insulating tiles are derived from cellulose. (Moat *et al.,* 2004; Nelson & Cox, 2000; Stryer,1995). When first discovered, it was believed that polysaccharide bound and trapped in the cellulose structure of plant extractable with alkali, comprised of smaller molecules, which would be eventually converted to cellulose by plant. For this reason they were termed hemicelluloses. It is now known that this was an erroneous belief. Upon hydrolysis hemicelluloses may yield pentoses and hexoses or both, together with uronic acids. The most abundant polysaccharides in this group are the xylans, which occur particularly in all land and some marine plants. They constitute some 15-30% of corncobs, grains and nuts etc. They are composed of almost D-xylose (Oser, 1965). Five-carbon sugar xylose is stereo-chemically similar to glucose but one carbon shorter, bind to hexokinase, but in a position where it cannot be phosphorylated. Xylose is sufficient to induce a change in a hexokinase (Nelson & Cox, 2000).

Hemicelluloses are closely associated with cellulose and occur in matrix of plant cell wall. They include, for example, glactomonnas, glucomonnas, mixed beta glucans, xylans and xyloglucans etc. In plant cell wall the cellulose fibers are embedded in and cross-linked by a matrix containing other polysaccride and lignin, a plastic like phenolic polymer (Singleton & Sainbury, 2001; Voet *et al.,* 1999). Lignin is perhaps second to cellulose in term of biomass. It protects cellulose and hemicellulose from enzymatic attack. Lignin is also important structural component of plants. It provides structural rigidity and resistance to the compression, bending and resistance to pathogens. They are richest sources of aromatic compounds in nature (Coyne, 1999).

While considering production of ethanol from cellulosic biomass complete conversion of its suitable constituents is critical for the development of an efficient and economically feasible fermentation process. Since pentose sugar can comprise up to 30% of the biomass substrate, therefore there is considerable economic incentive to develop strains of yeast that will efficiently ferment this biomass component too. If xylose were converted, in addition to glucose to ethanol, the final ethanol yield would be expected to increase several folds (Ho *et al.,* 1999; Wilke *et al.,* 1981). Moat *et al.* (2004) have described that cellulose degradation requires the combined activities of three basic types of enzymes (Fig.2). Initially, an *endo-β-*1,4-glucanase cleaves cellulose to smaller oligosaccharides with free-chain ends. Then *exo-β-1,* 4-glucanases remove disaccharide cellobiose units from either the reducing or nonreducing ends of the oligosaccharide chains. Cellobiose is then hydrolyzed to glucose by *β*-glucosidases.

The cellulolytic enzymes may be produced as extracellular proteins by organisms such as *Trichoderma*, *Phanaerochete* (filamentous fungi), *Cellulomonas, Microbispora,* and *Thermomonaspora* (Actinomycetes). Rumen bacteria such as *Ruminococcus flavofaciens* and *Fibrobacter succinogenes,* or gram-positive anaerobes such as *Clostridium thermocellum, C. cellulovorans,* or *C. cellulolyticum,* produce a cell-bound multienzyme complex called the cellulosome. With the aid of the electron microscope, cellulosomes can be seen as protuberances on the cell surface. The cellulosome of *C. cellulovorans* contains three major subunits: a scaffolding protein, CipA; an exoglucanase, ExgS; and an endoglucanase, EngE. Also present are endoglucanases EngB, EngL, and EngY, and a mannanase, ManA. The scaffolding protein serves as a cellulose-binding factor. Another component, present in duplicate and referred to as dockerin, mediates the association of cellulose fibers with the scaffolding protein. Various models have been proposed to conceptualize the complete interaction of the cellulosome with cellulose fibers during the digestion process. A wide diversity of actively cellulolytic organisms is important in industrial applications, in the rumen of animals, and in the digestive systems of arthropods that degrade wood. Termites and other arthropods that degrade wood owe their ability to digest cellulose in the presence of specific microbial symbionts in their digestive tract (Moat *et al.,* 2004).

Future processes will increasingly make use of organic materials that are renewable in nature and/or occur as low value wastes, valueless or adding negative value to the produce, that may presently cause environmental pollution. Currently more than ten times more energy is generated annually by photosynthesis than is consumed by mankind. On a worldwide basis land plants produce 24 tons of cellulose per person per year. Definitely, lignocellulose is the most abundant and renewable natural resource available to humanity throughout the word. It has been documented that massive technological difficulties such as expensive energy-demanding pretreatment processes have to be overcome before economic

use may be made of the plentiful renewable resource. Following its chemical and/or enzymatic hydrolysis soluble sugar products of cellulose can then be converted to form ethanol, butanol, acetone, single cell protein and methane, etc. (Anderson *et al.*, 2005; Nelson & Cox, 2000; Smith, 1996). Hill *et al.* (2006) have described that negative environmental consequences of fossil fuels and concerns about petroleum supplies have spurred the search for renewable transportation biofuels, but to be a viable alternative, a biofuel should provide a net energy gain, have environmental benefits, be economically competitive and be producible in large quantities without reducing food supplies. These authors have reported that dedicatedly even if all the U.S. corn and soybean productions were dedicated to produce the biofuels, it would only cover 12% of gasoline and 6% of diesel demands. Thus, fuels such as celllulosic ethanol produced from low-input biomass grown on agriculturally marginal land or from waste biomass could provide much greater supplies and environmental benefits than food-based biofuels. Likewise, Taherzadeh & Karimi (2007) have recently indicated that lignocelluloses can be expected to be major feedstocks for ethanol production in future. Evans (2005) has earlier explained that being 50% of the total dry matter of plants, the cellulose is potentially a huge renewable energy store, and vast amounts of this material are routinely thrown away. However, until recently, the prospect of realizing this potential fuel source was viewed as difficult and expensive; the combination of cellulase-resistant links and its close association with lignin discouraged its large-scale hydrolysis to sugars. Energy involved in rendering various cellulosic materials into acceptable from had been considered a major limiting factor. Nevertheless contemporary technologies employing whole organisms and isolated enzyme technique appear to be promising to make the commercial processing of cellulose to alcohol a reality.

Xylose is represented by 20 to 40% of the contents of different cellulosic materials (Bicho *et al.*, 1988). Economic ethanol fermentations of cellulose are required to use this pentose sugar along with glucose following the saccharification of the fibrous matter. As majority of the well known microbial diversity in this regard has been reported capable of utilizing only glucose. Previously reported scarcity of xylose fermenting pathways in the microorganisms has been discouraging for cellulosic materials to be employed for economic ethanol generation. However, recently naturally occurring as well as genetically engineered xylose fermenting microorganisms have also been well documented (Chaudhary & Qazi, 2006a; Sonderegger *et al.*, 2004; Toivari *et al.*, 2001). It has been, however, reported variously that glucose is preferred by fermenting microorganisms capable of fermenting the both categories of the monosaccharides i.e., the glucose and xylose. In such cases glucose depletion within a fermenting substrate may allow for xylose utilization (Govindaswamy & Vane, 2006). Many strategies can be attempted to overcome co-substrate inhibition of xylose consumption by glucose considering the nature and diversity of fermenting microorganism(s). For instance, in case of microbial consortia first the glucose be utilized and then the residual material be attempted with xylose utilizers. Regarding other sugars, microbes could be found capable of co-utilization. Karhumaa *et al.* (2006) have reported simultaneous co-utilization of xylose and arabinose in recombinant strains of *S. cerevisiae*. This is well clear that economic ethanol generation from lignocelluloses requires the maximum utilization of all the diverse sugars monomers derived through any feasible saccharification process.

Responding above referred situations requires the isolation and construction of microorganisms capable of fermenting glucose and xylose at appropriate levels. Fermenting

microbes that would prefer xylose and/or be incapable of glucose utilizations may find increasing utilization in one or two-chambered fermentative processes. In the latter case, a fermented matter in which glucose has been used maximally would serve feed stock for xylose-fermenting microorganisms. Mutants or genetically modified organisms with derived characteristics would be required to develop processes for obtaining ethanol from cellulose.

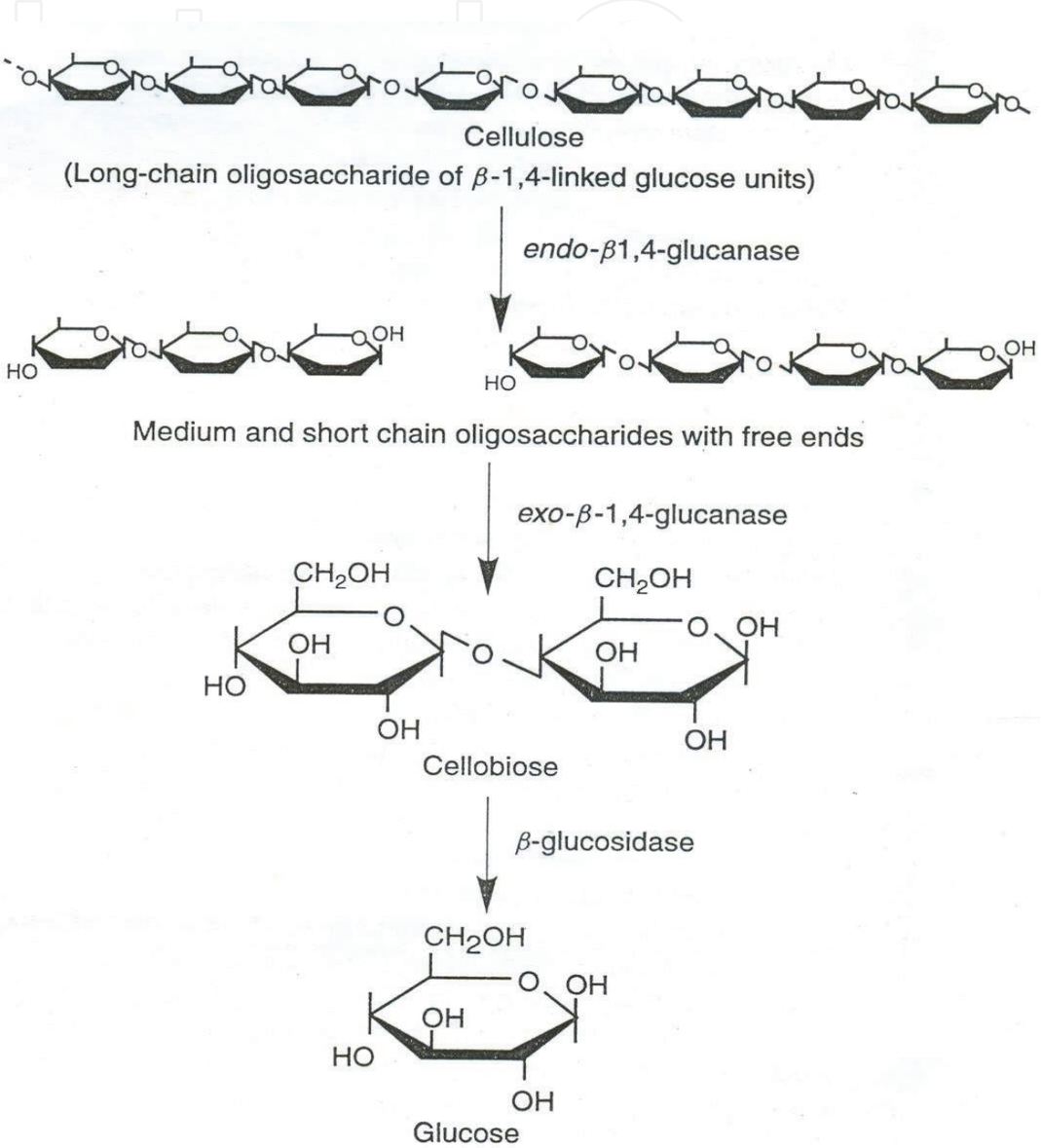

Fig. 2. Enzymatic degradation of cellulose (Moat *et al.*,2004).

Infact, the afore-mentioned approach could play a major part in addressing the largest environmental issue of our time, energy and waste. Energy extraction accompanied by environmentally safe disposable of a cellulosic waste may render the process economically feasible. A large number of point and non-point/plant biomass loads, to the natural water

systems result in higher BOD values. Processing such wastes for ethanol fermentation is highly appealing to reduce the pollutants (Evans, 2005). Converting cellulose to ethanol is accomplished in essentially four stages, discussed below:

Acid/microbial hydrolysis: This process breakdowns the cellulose into a slurry of sugars in acid water and solid lignin particles. While reviewing acid-based hydrolysis processes for ethanol from lignocellulosic material, Taherzadeh & Karimi (2007) have summarized that concentrated-acid processes operated at low temperatures gave high sugar yield. On the other hand, dilute-acid processes operated at high temperature gave low sugar yield. Both categories of the hydrolyses result, however, into equipment corrosion.

Acid recovery: Following acid hydrolysis sugary liquid is separated from the previous stage and the process acid is recovered partly and reused. Van Groenestijin *et al.* (2006) have claimed that recovery of sulphuric acid in the form of H_2S from anaerobic waste water treatment has a low overall cost for ethanol production.

Fermentation: The derived sugars are fermented by yeast and/or bacteria into alcohol. As already indicated efficient fermentation of lignocellulosic hydrolyzates requires employing microorganisms capable of fermenting the maximum variety of monomeric sugars and to resist inhibitory products of the hydrolyses process as well.

Distillation: It is required to collect the market grade ethanol. Economizing these four phases necessitates understanding and optimizing the diversity of the processes involved.

Fortunately, a large number of efforts in this regards have been continuously reported in the literature (Anderson *et al.,* 2005; Ballesteros *et al.,* 2001; Negro *et al.,* 2003). For instance, the latter authors have described that crops such as switch grass, bermudagrass, or napiegrass have the capacity to produce large quantities of lignocellulose for biofuel. To facilitate use of lignocellulosic material for the production of ethanol, it will be necessary to determine cost efficient pretreatments to enhance of the substrate conversion to fermentable sugars.

6. Consolidated bioprocess: Simultaneous Saccharification and Fermentation (S.S.F.)

Apart from the distillation step, which is required to separate the produce from the fermentation chamber, actual process of obtaining ethanol from lignocellulosic material essentially comprises of two phases i.e., saccharification of the substrate and the efficient utilization of all types of monosaccharides by suitable microbe(s) for producing the ethanol. Usually the two processes are accomplished in separate facilities. Science of process economics has dictated for developing some consolidated processes to allow the individual phases of the two processes to be completed simultaneously. This has been elaboratively described as Simultaneous Saccharification and Fermentation (SSF). Lynd (1995) described that biological conversion of cellulosic biomass typically involves four stages: production of cellulose enzymes, enzymatic hydrolysis of cellulase, hexose fermentation and pentose fermentation. And if a single organism or system of organisms were to carry out all four elements of ethanol production with high rates and yields, process economics would benefit profoundly.

The SSF has been conceptualized by many workers as a promising economical strategy for converting plant biomass to ethanol. In case of cellulosic ethanol production, developing genetically engineered microbes with the traits necessary for one-step processing of cellulosic biomass to ethanol appear to achieve the goal. Genetic improvements of microorganisms have been made either to enlarge the range of substrate utilization or to

channel metabolic intermediates specifically toward ethanol for simultaneous saccharification and fermentation of lignocellulosic biomass from various sources (Chandrakant & Bisaria, 1998; Greer, 2005; Teixeira *et al.,* 2000). In their findings, Stenberg and co-workers (2000) pointed out that economic optimization of the production of ethanol by SSF requires knowledge about the influence of substrate and enzyme concentration on yield and productivity. These investigators obtained highest ethanol yield 68% of the theoretical based on the glucose and mannose present in the original wood at 5% substrate concentration. Compared with separate hydrolysis and fermentation, SSF gave a higher yield and doubled the productivity.

Lynd *et al.* (2002) have described that developing microorganisms capable of substrate utilization and product formation required for consolidated bioprocess can be pursued by two strategies. The native cellulolytic strategy involves engineering naturally occurring cellulolytic microorganisms to improve product related properties, such as yield and titer. While, the recombinant cellulolytic strategy involves engineering non-cellulolytic organisms that exhibit high product yields and titers so that they express a heterologous cellulose system that enables cellulose utilization.

Fujita *et al.* (2004) achieved efficient direct fermentation of amorphous cellulose to ethanol by developing a yeast strain and have reported the role of whole-cell biocatalysts for reducing the cost of ethanol production from cellulosic biomass. They described the advantages of the engineered yeast strain displaying three types of cellulolytic enzymes. The advantages included: conversion of cellobiose and glucose, which inhibit cellulase and β-glucosidase activities; lower sterilization requirements, as glucose was immediately taken up by the cells for ethanol production in a single cell reactor.

Considering process kinetics is very important. Microorganisms tend to disturb the optimum conditions provided to them due to their own growth and metabolic activities. Further feed back inhibition is an important limiting factor for both saccharification and ethanologenic levels. Thus microorganisms with a wide range of activities are to be worked out. Different bacteria and yeast have varying levels of ethanol tolerance. Here thermophilic ethanologenic microorganisms become important as the produce, recovery can be achieved under elevated temperatures, while not stopping or influencing negatively the fermentation process. Last but no the least, is the requirement for large scale microbial decontamination of the process material for controlled microbial hydrolysis and subsequent fermentation. This is surely energy demanding activity. A plant that has recently been proposed will work by intensifying solar radiations to provide heat for decontaminating the substrate and enabling cellulose substrate to be hydrolyzed as well as fermented to ethanol by thermophilic microorganisms (Chaudhary & Qazi, 2007). The proposed plant has technically been designed; its outcome is likely to be reported soon. The designed plant derives maximum benefits from sun heat and in this regard pivotal role of thermophilic ethanologenic microorgansisms has been discussed.

7. Potential of thermophilic ethanologenic microbes

Production of ethanol from low cost plant biomass is influenced by a number of phenomena. Majors of which are saccharification and fermentation efficiencies of the microbial culture(s) involved. The fermentation efficiency is in part influenced negatively with the raising levels of product accumulation and its inhibitory effect for further production. This can be overcome by employing thermophilic fermenting microorganisms as con-comittant removal of product, the

bioethanol, at elevated temperature may delay or practically keep away accumulation of inhibitory level of the produce. Moreover, employing thermophilic microorganisms can bring support to the process economics by reducing the efforts required to keep the process facilities decontaminated by mesophilic bacteria and to reduce the cost of cooling that must be provided to maintain the correct temperature range required for optimal functioning of the mesophilic bacteria. These considerations have well earlier been taken into account by various workers (Budden & Spencer, 1993; Lamed & Zeikus, 1980; Thomas *et al.,* 1981).

While, commenting on yeast in their book "Thermophilic Microbes in Ethanol Production" Slapack *et al.* (1987) concluded that it is evident that thermotolerant yeasts would offer many advantages to the fermentation industry. Energy costs (cooling, distilling, and mixing) would be minimized and theoretically, productivity and growth rates should increase. Thermotolerant yeasts would be especially attractive in tropical countries where cooling costs are very expensive, and they are paramount for efficient simultaneous saccharification fermentation processes currently being investigated. In view of their many advantages, it is surprising that so few attempts have been made to select for thermotolerant yeasts and in particular, yeasts that can produce ethanol efficiently at high temperatures.

Sree *et al.* (1999) reported a novel solid substrate fermentation system to produce fuel ethanol from sweet sorghum and sweet potato using a thermotolerant *Saccharomyces cerevisiae* strain (VS#) and an isolate of amylolytic *Bacillus* sp. (VB9). They recorded maximum amount of ethanol production in co-culture with a mixed substrate as to be 5g/100 g of substrate at 37°C and 3.5 g/100g of substrate at 42°C. Likewise, Ueno *et al.* (2002) have evaluated a thermotolerant, fermentative yeast strain (RND 13) from a hot spring drainage for ethanol producing ability at elevated temperatures at 15% concentration of glucose. The RND 13 utilized glucose almost completely at 40°C with increasing inoculum size producing ethanol upto 6.6% (w/v). These workers found maximum rate of ethanol production of 9.00 g/L at 40°C with 5% inoculum size in batch fermentation.

It appears that further research on thermophilic microbes both prokaryotes and the eukaryotes with higher ethanologenic potential would continue. And the present, incipient successes are promising to dig more in this field for isolating, optimizing and developing thermophilic ethanologenic microbes for designing economically and environmentally friendly strategies. For obtaining the bioethanol form agro-indistrial low cost residues, various lignocellulosic materials are under trials. One of the such cellulosic materials under consideration by various workers for conversion to biofuel is sugarcane bagasse, a waste by product of sugar industry.

8. Sugarcane bagasse-a resource rather than a waste

Sugarcane bagasse is an important, renewable, abundant and cheap or even having negative value agricultural waste in many countries (Bustos *et al.,* 2003; Molina Junior *et al.,* 1995; Rodrigues *et al.,* 2001; Van Haandel, 2005). Composition of the fibrous residue may vary based on its different verities, age of cane at the time of harvesting and efficiency of milling operation for extracting the juice

Besides the compositional analysis, different fractions of bagasse can be separated employing suitable techniques. For example, Bustos *et al.* (2003), while describing sugarcane bagasse hydrolysis with HCl have mentioned 128°C, 2% HCl and 51.1 minutes as optimal conditions. At these conditions they obtained 22.6 g xylose, 3.31 g arabinose, 3.77g glucose, 3.59 g acetic acid and 1.54 g furfural/L.

Concerning the bioconversion of the substrate, products such as alcohol, alkaloids, mushrooms, protein enriched animal feed, enzymes L-glutamic acid, fruity aroma, and xylitol have been reported to be obtained from the waste sugarcane bagasse (Alonso *et al.,* 2007; Christen *et al.,* 1994; Liu *et al.,* 2006, 2007; Martinez *et al.,* 2000; Pereira *et al.,* 2007; Sasaki *et al.,* 2003; Van Haandel, 2005). Besides the above mentioned diverse and usually bench scale utilities of the substrate Meunchang *et al.* (2005) have rightly commented that one of the under utilized sources of organic materials, is the sugarcane industry. Global sugar production from sugarcane releases large amounts of sugar mill by-products as filter cake and bagasse. It had been reported that in Brazil at the end of last century during the ethanol production season more than $60x10^6$ tons of sugarcane bagasse containing 50% moisture were produced annually.

Like any other lignocellulosic material the sugarcane bagasse is a complex and stable substrate. Any significant and efficient utilization would require first its hydrolysis. Following acid, enzymatic or microbial hydrolysis of the sugarcane bagasse the monosaccharides yield can find many applications in different bioconversion processes. One consideration is their conversion into biofuel, the ethanol. Sugarcane bagasse has relatively earlier been considered a source of fermentable carbohydrates (Du Toit *et al.,* 1984). However, pretreatment of the bagasse has been found useful for the microbial attack, which may results into its saccharification, fermentation or the both processes simultaneously. Chemical as well as microbial enzymatic pre-treatments have been described by various workers (Chaudhary & Qazi, 2006a; Dominguez *et al.,* 1996; Laser *et al.,* 2002; Lavarack *et al.,* 2002; Martin *et al.,* 2002; Zheng *et al.,* 2002). Martin *et al.* (2002) have described that sugarcane bagasse is a potential lignocellulosic feedstock for ethanol production, since it is cheap, readily available and has a high carbohydrate content. These workers performed different pretratments of the substrate at 205°C for 10 minutes followed by its hydrolysis using cellulolytic enzymes. They found highest yield of xylose (16.2 g/100g dry bagasse), arabinose (1.5 g/100g) and total sugar (59.9 g/100g) in the hydrolysis of the SO_2-impregnated bagasse. The H_2SO_4 impregnated bagasse gave highest glucose yield (35.0 g/100g) but the lowest total sugar yield (42.3 g/100g). Sulfuric acid impregnation led to a three-fold increase in the concentration of the fermentation inhibitors, the furfural and 5-hydroxymethyl furfural and a two fold increase in the concentration of inhibitory aliphatic acids (formic, acetic and levulinic acids) compared to the without any impregnation and sulfur dioxide impregnation yields. They found no major differences in the content of inhibitors in the hydrozylates obtained from SO_2-impregnated and non-impregnated bagasse. When Martin and colleagues studied fermentability of the three hydrolyzates with a xylose utilizing *Saccharomyces cerevisiae* with and without nutrient supplementation they found that the H_2SO_4 impregnated bagasse fermented considerably poorer than the situations found in the other two categories of the bagasse. Cheng *et al.* (2007) have reported the ethanologenic fermentation of sugarcane bagasse hemicellulose hydrolyzates, pretreated by over-liming as well as electrodialysis and supplemented with nutrient materials employing *Pachysolen tannophilus* DW 06. These workers found that compared with detoxification by over-liming, detoxification by electrodialysis decreased the loss of sugars and increased the acetic acid removal. This lead to better fermentability and the Cheng's team found that a batch culture employing electrodialytically pretreated hydrolyzate substrate gave 21g ethanol L^{-1} with a yield of 0.35 g L^{-1} sugar and productivity of 0.59 g L^{-1} h^{-1}. For better yield of the produce ethanol from sugarcane hydrolyzates, the above described studies highlight two important notions. That is detoxification of inhibitory substances, that

may emerge within the hydrolyzates, of different nature and varying levels depending upon the specific pre-treatment employed. Secondly bagasse hydrolyzate would mainly consists of carbohydrates content, its supplementation with a suitable nutritive material is likely to enhance the growth and/or fermentative potential of the microorganism(s).

Fermentation inhibitors can be tackled at two levels i.e., their removal/detoxification or employing the inhibitors' resistant fermentative microorganisms. Martinez *et al.* (2000) reported that hemicellulose syrups from dilute sulfuric acid hydrolyzates of hemicellulose contain inhibitors that prevent efficient fermentation by yeast and bacteria. These workers have optimized overliming treatments for sugarcane bagasse hydrolyzates and found a substantial reduction in furfural, hydroxymethyl furfural and three un-identified high-performance liquid chromatography peaks. They further demonstrated that the extent of furan reduction correlated with increasing fermentability, although furan reduction was not found to be the sole cause of reduced toxicity. Rodrigues *et al.* (2001) studied the influence of pH, temperature and drgree of hydrolyzate concentration on the removal of volatile and non-volatile compounds from sugarcane bagasse hemicellulosic hydrolyzate treated with activated charcoal before and after the vacuum evaporation process. They found that furfural and 5-hydroxymethyl furfural were almost totally removed irrespective of pH, temperature and whether the charcoal was added before or after the vacuum evaporation process. Adding activated charcoal before the vacuum evaporation process favoured the removal of phenolic compounds for all values of pH. Acetic acid was most effectively removed when the activated charcoal was added after the vacuum evaporation process at an acid pH (0.92).

Regarding the use of fermentation inhibitory products' resistant microorganisms, Morita & Silva (2000) reported the fermentation of precipitated sugarcane bagasse hemicellulosic hydrolyzate containing acetic acid, employing *Candida guilliermondii* FT 120037 under different operational conditions for the production of xylose. At pH 7.0 and Kla of 35/h (4.5 vvm), the acetic acid was rapidly consumed and that the acetic inhibition was not important. They concluded that the acetic acid assimilation by the yeast inidicates the ability of this strain to ferment a partially detoxified medium and makes possible the utilization of the sugarcane bagasse hydrolyzate in this manner.

For simultaneous bioconversion of cellulose and hemicellulose to ethanol, need of xylose fermenting microorganisms has been established (Chandrakant & Bisari, 1998; Sedlak & Ho, 2004; Toivari *et al.,* 2001; Yang *et al.,* 1997). De-Castro *et al.* (2003) have described a new approach for the utilization of hemicellulosic hydrolyzates from sugarcane bagasse. They diluted the conventional feedstock, sugarcane juice; by the bagasse hydrozylate to the usual sugar concentration of 150 gm per liter that is employed for industrial production of ethanol. These workers used a pentose fermenting yeast strain, and achieved ethanol productivity of about 11.0 gm per liter per h and overall sugar conversion of more than 95%. Katzen and Fowler *et al.* (1994) reported first commercial application of unique fermenting organism capable of converting five carbon sugars and oligmers of cellulose directly to ethanol. These worker described conversion of hemicellulose content of sugarcane bagasse to the five-carbon sugar by mild acid prehydrolysis, followed by fermentation of the 5-carbon sugar extract with recombinant *Escherichia coli*. The process also recovered the majority of sucrose normally lost with the bagasse fibers to ethanol. Sun & Cheng (2002) have described the benefits of simultaneous saccharification and fermentation that it effectively removed glucose, which is an inhibitor to cellulase activity thus increasing the yield and rate of cellulose hydrolysis.

Various workers have reported different protocols and models for fermenting cellulosic biomass to ethanol and considered it the cleanest liquid fuel alternatives to fossil fuels (Gray *et al.*, 2006; Lawford & Rousseau, 2003; Lin & Tanaka, 2006; Sun & Cheng, 2002). From above cited literature it appears that relatively recently considered renewable resource, the sugarcane bagasse process much potential for the bioethanol production. In Brazil sugarcane cultivtion and its dependent sugar industry is well developed. Consequently, a huge amount of bagasse is generated. It consists mainly of 37% cellulose, 28% hemicellulose and 21% lignin (Bon, 1996). A reasonable number of cellulose saccharifying and/or ethanologenic bacteria as well yeast have been isolated and characterized in our laboratory (Chaudhary & Qazi, 2006a; Saeed, 2005).

Above referred studies suffice to highlight different achievements and areas that require more research concerning the developments of bioprocesses to utilize sugarcane bagasse lignocellulosic material for obtaining bioethanol at economically feasible levels. As in all such bioprocess developments subsidiary supports are very important. Benefits derived from appropriate utilization of auxiliary products/often process wastes, have an influential bearings on the main process economics. In this regard Pandey *et al.* (2000) pointed out an important aspect. Accordingly, developing associated or complimentary technologies, during the fuel ethanol production from sugarcane bagasse which could produce other value-added by-products would improve the overall economy of ethanol production. It is pertinent here to mention that the non-fermentable residues of variously processed sugarcane bagasse would contain the microorganisms employed for the saccharification and/or fermentation of the substrate. Thus the residue may attain the levels of protein (due to single cell protein) that may render them to the status of animal feed / supplement. This may bring additional support to the process economics. Following is a brief review of single cell protein in connection with microbiological utilization of lignocellulosic materials including sugarcane bagasse.

9. Single Cell Protein (SCP) from agro-industrial wastes; Sugarcane bagasse

Growth of microbial cells both bacterial and yeast on any material means that the substrate ingredients are being transferred or altered to proteins along with synthesis and accumulation of other contents of protoplasm. Upgradation of a large number of agro-industrial wastes, which after being fortified with S.C.P. may find their useful application in preparing or supplementing animal feed. The S.C.P. from various agro-industrial wastes has been well documented from several laboratories (Chaudhary & Sharma, 2005; Dimmling & Seipenbusch, 1978; Hongpattarakere & Kittikum, 1995; Kamel, 1979). Stabnikova *et al.* (2005) used extracts of cabbage, watermelon, a mixture of residual biomass of green salads and tropical fruits for yeast cultivation and concluded that the yield was comparable with the yield of yeast biomass grown in potato dextrose broth. These workers commented that the yeast biomass can be considered as protein source. Single cell protein production from sugarcane bagasse has relatively earlier been reported by various workers (Molina *et al.*, 1984; Sindhu & Sandhu, 1980). Molina and colleagues treated sugarcane bagasse pith with 1% NaOH solution at room temperature, at a NaOH/pith ratio of 10%. They used different contact times and found that the shortest period required for maximum protein production was 24h at 25°C. These workers used mixed culture of *Cellulomonas* sp. and *Bacillus subtilis*. Rodriguez *et al.* (1993) reported optimal production of *Cellulomonas* with 1% (w/v) bagasse pith pre-treated with either 0.2M NaOH for 1h at 80°C or 0.4M NaOH for 40h at 28°C to

30°C. With these milder pretreatments they obtained growth comparable to the one found for the substrate prepared with a more severe treatment. Growth was also comparable with other reports for cellulolytic bacteria cultivated on pre-treated bagasse pith. Rodriguez & Gallardo (1993) studied association of *Cellulomonas* sp. with an isolate of *Pseudomons* sp. for S.C.P. production from bagasse pith. They found a mutualistic symbiotic relationship during their mixed growth on bagasse pith, the *Cellulomonas* supplying carbon source (glucose produced from bagasse) to the *Pseudomonas* and the latter producing the vitamin supplements necessary for *Cellulomonas* growth. The metabolic symbiosis allowed the growth of the mixed culture in a minimal medium, without any growth factor supplement. Fed-batch cultivation of the mixed culture yielded high biomass production (19.4 g/L). Perez *et al.* (2002) while reporting use of sugarcane bagasse complemented with a mineral medium and inoculated with *Candida utilis* as bio-filter for ethanol concluded that 57% of the carbon from ethanol was converted to CO_2 and 8.7% into biomass. They found final yeast population of $7x10^9$ cells/g of dry matter corresponding to 56 mg protein/g dry matter. Perez *et al.* (2002) concluded that this much protein offers potential for using the protein enriched bagasse as feed too. The above described studies clearly indicate that the sugarcane bagasse or its pith can be upgraded with the generation of S.C.P. by employing the suitable microorganisms on untreated as well as pretreated substrates.

As has been introduced earlier, that being an agriculture country, sugarcane is cultivated at large commercial scale in Pakistan. The produce is largely used for obtaining sucrose. The bagasse is a waste of the sugar industries. Instead of other lignocellulosic material, its usage as substrate for biofuel ethanol production has two advantages. Tackling of a waste and presence of some amounts of soluble sugars that may be assimilated quickly by the inoculated microorganisms meant for saccharification and/or ethanol fermentation of the substrate. Moreover, the fermented residual material enriched with microbial cells may find its application as animal feed or its supplement there of. The latter notion is likely to bring support to the economic constrains regarding the process developments for obtaining ethanol from lignocellulosic materials in general and specifically from sugarcane bagasse. In our lab. Ahlam (2005) and Chaudhry (2008) conducted studies on the same lines and reported isolation, characterization and optimization of microorganisms both prokaryotic and eukaryotic, which are useful for saccharifying and fermenting fruits and vegetables' wastes and the sugarcane bagasse,respectively. Following maximum yield extraction, the fermented residue is likely to find its application to supplement animal feed with S.C.P.

10. Biohydrogen; Another potential for biofuel provision

Regarding the provision of the clean and sustainably available fuel, hydrogen gas (H_2) has been claimed as an alternative source of energy due to non-emission of pollutants (Das & veziroglu, 2001; Gest *et al*., 1950; Prince & Kheshgi, 2005; Valdez-vazquez *et al*., 2005). It is plentiful element in universe (Bockris, 1981; Levin *et al*., 2004; Suzuki, 1982) and has a wide range of uses (Czuppon *et al*., 1996; Kalia *et al*., 2003; Ramachandran & Menon, 1998). Das & Veziroglu (2001) have summarized the uses of H_2 as reactant in hydrogenation processes, O_2 scavenger, fuel in rocket engines and coolant in electrical generators etc. Thus it is expected that commercial and domestic uses of hydrogen gas will increase in the coming next years. And there are signs that hydrogen may finally become an important component of the energy balance of a global economy (Benemann, 1996; Gregoire-Padro, 1998; Kalia *et al*.,

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

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