Ethanol Production from Xylose by Using Locally Isolated Bacteria

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Certificate

This is to certify that work in this thesis has been carried out by **Mr. Faisal Mahmood** and completed under my supervision in Institute of Microbiology, University of Agriculture Faisalabad and Bio-fuels labs, CAS-EN, National University of Sciences and Technology, H-12, Islamabad, Pakistan.

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Dedication

I dedicate this effort to my parents.

Abstract

The purpose of this study is to improve the conversion efficiency of lignocellulose to ethanol. This was achieved by converting the hemi cellulosic sugar xylose to ethanol. In this research a xylose fermenting bacteria was isolated from local soil sample. By performing different biochemical tests the isolated bacteria was found to be identical to Bacillus subtilis. The optimum fermentation conditions at 150 rpm for 48 h in a shake flask are as follows: pH 7.0, reducing sugar 78 gL^{-1}, inoculation amount 3% and temperature 37°C. The results indicated that a xylose to ethanol yield coefficient of 24% was achieved. At the same time the effect of different sugar concentrations was studied by making different mixtures of xylose and glucose. As a result, the bacteria's ability to ferment xylose was found to be greater than to ferment glucose. The isolation, preservation and usage of isolated Bacillus subtilis can help improve the conversion process of hemicellulose sugar such as xylose to produce ethanol

Key words: xylose, B*acillus subtilis*, fermentation, ethanol

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*Attached as annex 1

**Attached as annex 2

List of the Abbreviations

EtOH	=	Bioethanol
B. Subtillis	=	*Bacillus Subtillis*
RPM	=	Revolutions per Minute
F.E	=	Fermentation efficiency
TRS	=	Total Reducing Sugars
OD	=	Optical Density

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Chapter 1
Introduction

1.1 Recent Energy Panorama

With the emergence of mechanization, man has been enforced to opt for multifarious forms of transport. The fuel shortage has now been the subject of huge debate in all over world, reason, hence investing in alternative energies, which are renewable and environmentally sustainable [1].

The unwelcoming increase in gasoline prices means higher transportation and food costs. Yet we have the solution to high gasoline prices right under our noses, in the form of clean, renewable ethanol, which is an alternative fuel that not only delivers higher octane to motor fuel but also sells at a buck less a gallon than gasoline on the commodity exchange [2]. Thus high blends of ethanol into motor fuel can reduce overall prices at the pump. Hence Ethanol is not a "someday" solution to our current energy crisis. It is here today [3].

1.2 Biofuels the Major Player in Energy Scenario

Biofuel is a type of fuel, derived from biomass, which has been provided about 1.8% of the world's transport fuel in 2008. This phrase covers most of the solid biomasses, liquid fuels and biogases. The reason of Biofuels of gaining public and scientific attention is justified by the factors such as the debates over the greenhouse, the continuous increase in the petroleum prices, the strategic importance in the field of energy security, gas emissions from fossil fuels, and the incentives by government through subsidies. Investment into biofuels production capacity exceeded $4 billion worldwide in 2007 and kept growing [4].

According to the report by International Energy Agency, biofuels have the capacity to meet more than a quarter of world oil demand for transportation fuels by 2050 which is enough to justify its importance in the near future [5].

Bioethanol and Biodiesel are the two most vital biofuels that are presently picking up consideration. Bioethanol is liquor made by maturing the sugar segments of plant materials. A few yields can be utilized to create ethanol [6]. For instance, Brazil centered its generation from sugar cane molasses while US uses corn to get the same result[4]. With cutting edge innovation and the impetus made by biotechnology, cellulosic biomass, for example, trees and grasses are additionally utilized as feedstocks for ethanol creation. The generation depends of a few variables such area, temperature, soil etc. There are different social, monetary, ecological and specialized issues with biofuel generation and use, which include: the impact of directing oil costs, the "food versus fuel" wrangle about, neediness lessening potential, carbon emanations levels, practical biofuel generation, deforestation and soil disintegration, loss of biodiversity, effect on water assets, and in addition vitality equalization and proficiency [5].

Following are fuel properties of ethanol as compared to gasoline and diesel:

Table 1 Fuel properties of ethanol as compared to gasoline and diesel [7]

Chemical Formula	C_2H_5OH	C_4 to C_{12}	C_3 to C_{25}
Molecular Weight	46.07	100–105	≈200
Carbon	52.2	85–88	84–87
Hydrogen	13.1	12–15	33–16
Oxygen	34.7	0	0
Specific gravity, 60° F/60° F	0.796	0.72–0.78	0.81–0.89
Density, lb/gal @ 60° F	6.61	6.0–6.5	6.7–7.4
Boiling temperature, °F	172	80–437	370–650
Reid vapor pressure, psi	2.3	8–15	0.2
Research octane no.	108	90–100	--
Motor octane no.	92	81–90	--
Cetane no.	99.15	5–20	40–55
Fuel in water (vol %)	100	Negligible	Negligible
Water in fuel (vol %)	100	Negligible	Negligible
Freezing point(°F)	-173.2	-40	-40–30[a]
Flash point(°F)	55	-45	165
Auto ignition temperature, °F	793	495	≈600
Btu/gal at 60° F	2,378	≈900	≈700

1.3 Bioethanol - A Fuel of Tomorrow

Following properties of bioethanol that excels it as a fuel of future

(i) Exhaust gasses of ethanol are much more clean, it combust all the more neatly (more finish ignition).

(ii) The utilization of ethanol-mixed fuels, for example, E85 (85% ethanol and 15% fuel) can lessen the net discharges of greenhouse gasses by as much as 37.1%, which is a huge sum [8].

(iii) Positive balance of energy – Depending on a raw stock it can change from 1.24 to 8. The energy output is greater than its input.

(iv) Any plant can be utilized for generation of bioethanol, it just needs to contain sugar and starch. The best decision is sugar stick, yet you can likewise utilize potatoes, grain, and wheat and so on.

(v) It is carbon unbiased i.e. the carbon dioxide discharged in the bioethanol generation procedure is the same sum as the one the harvests beforehand devoured amid photosynthesis.

(vi) Ethanol-mixed fuel as E10 (10% ethanol and 90% gas) lessens nursery gasses by up to 3.9% [8].

The net impact of ethanol use results in a general decline in ozone arrangement which is an essential ecological issue.

1.4 Lignocellulosic Biomass

Lignocellulosic are now being used in the business for bioethanol creation. They are prime plausibility for second era of biofuels, while original biofuels required direct usage of molasses for this reason. They utilized plant biomass that majorly involves cellulose along with lignin and hemicellulose, dependent on physiological characteristics of the plant, accordingly the term lignocellulosic substrate [9]. The agricultural crops mostly used are of wheat, rice, corn, sugarcane, weeds and grasses which have no food regard yet can serve as substrates for fermentation. Lignocellulose has been seen as a choice biomass, yet difficulties stay in isolating the material and completely maturing it. One vital prevention is the vicinity of xylose and other pentose sugars which are not effortlessly fermented by the standard microorganism utilized, S. cerevisiae [10]. This endeavor

considered a modified strain of yeast and its ability to ferment xylose which is a noteworthy pentose sugar. It was figured out that the modified yeast is more appropriate for using xylose. The yeast performed in a perfectly when more than half of the substrate used was glucose [11]. Moreover, it was found that genetically modified yeast has been produced at an expansive rate of progress than that of wild yeast.

1.5 Fermentable and Non-Fermentable Sugars

Pentose's such as xylose and arabinose, have been termed non-fermentable sugars. In a general sense this is true, as the majority of microorganisms, yeasts and bacteria, cannot utilize the pentose's however, there are certain forms which possess the ability to break down these sugars [12]. Apparently these pentose fermenting bacteria are widely distributed and no doubt play an important role in the economy of nature. The fermentation products of the hexoses usually differ from those of the pentoses. A common product, such as lactic acid, is generally produced from both types of sugar, but the difference usually lies in the other major products. The consistency with which lactic acid is formed from such different compounds as glucose and xylose indicates that three of the carbon atoms contained in the sugar molecules appear as lactic acid, while the residuum is converted into a two- or three-carbon compound depending upon the kind of sugar and the type of organism attacking it. In some cases this residuum is transformed into such products as acetic acid, ethyl alcohol, and carbon dioxide, singly or in pairs [13].

1.6 Hemicelluloses vs Celluloses

Hemicelluloses, unlike cellulose, are composed of a cocktail of different pentoses and hexoses namely D-mannose, D-arabinose, D-galactose and D-glucose, with a majority being D-xylose. The pentoses and hexoses are arranged in the chains which are branching out after a few residues forming cross-links between adjacent chains [12]. The hemicelluloses are also arranged in fibers that span the cellulose micro fibrils and link them to lignin, creating a mesh surface that gives added sturdiness to the overall plant cell wall. While cellulose is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base as well as myriad hemicellulose enzyme[14]s. Most of the hemicelluloses contain

xylose with D-xylose as a major sugar constituent in it. so hemicelluloses are the noteworthy for pentoses release [15].

1.7 Xylose Fermentation Limitations and Outcomes

Hemicellulose component of lignocellulosic material contain xylose as a major constituent of monosaccharides. But unfortunately conventional yeast is considered to be incapable to ferment xylose into ethanol as it can't grows on xylose but it does produce a limited amount of xylitol otherwise most of the xylose was sent to the costly waste disposal or burned as biofuel.one of the main factor for this non-conversion is limitations of cofactor [15]. Moreover the transport of xylose is less efficient as compared to glucose transport which is 4 times slower than glucose in aerobic condition and twice as slow in anaerobic conditions. The yeast fermentation is also susceptible to inhibitors present in hydrolysates and thus require detoxification in order to undergo successful fermentation [10].

Recently different strains of yeast, bacteria and fungi has been genetically modified to ferment xylose to xylulose and so that it could be easily fermented to ethanol The fermentation of xylose would affect overall economics of the fermentation process [16].

1.8 Types of Fermentation

1.8.1 Solid State Fermentation

This type of fermentation involves cultivation of the microorganism on the substrate surface where the moisture content is adjusted to 75-85% and the fermentation can be carried out at ambient temperatures for longer periods of time. Traditionally the method has been used in the food industry where fermentations can last from weeks to months. In bioethanol production, solid state fermentation has only been performed as lab scale experiments due to the fact that it is not suitable for large volumetric production over shorter periods of time. Currently, solid state fermentation is being restricted to growth of fungal strains for production of enzymes such as celluloses, xylanases, lignin degrading enzymes etc. but it is being pursued for lab scale production of bioethanol [17][18].

1.8.2 Submerged Fermentation

Since solid state fermentation was only applicable for a few production processes, submerged fermentation rose to the challenge of production of valuable products at large

volumes in shorter times. In this case substrate is immersed in media and inoculated with the selected microorganism, given optimum conditions for a definite time frame, and after product concentration reached required levels, the process is terminated and fermented broth harvested. Ethanol production is majorly produced using this type of fermentation where pretreated lignocellulosic biomass, hydrolysate from scarification is fermented in liquid medium. Over the years, more variables in production process have surfaced with changes in phases of product formation, which called for modifications in the submerged fermentation process. This gave birth to the different operation modes in fermentation that cater to variable concentrations of product complying with the individual production phase requirements [19].

1.9 Operational Modes of Fermentation

1.9.1 Batch Fermentation

The process of batch fermentation involves progression of fermentation in sterile media supplemented with nutrients and vitamins over a defined time frame. Growth of the microorganism requires nutrients which may be circulated by mixing (in case of anaerobic fermentation) or spurge using air. The nutrient composition of the medium varies throughout the fermentation process. Nutrient levels are closely monitored and the complete consumption of nutrients and consistency in product level signals termination of the process, where the bioreactor is drained and products harvested. The batch fermentation has several advantages over other modes of fermentation [20]. There is a relatively low chance of contamination or mutation due to the controlled timeframe. High conversion levels of substrate are also expected due to control of external parameters throughout the process. The application of the batch process limited to processes that result in small amounts of product. It is also implemented where production of multiple substances can be done in a single bioreactor.

1.9.2 Semi continuous Fermentation

An intermediate between batch and continuous fermentation was developed and termed as semi continuous fermentation. The process is started by running the fermentation as a batch to allow consumption of a growth limiting substrate. Once the substrate is used up, fresh medium is added by replacing same quantity of inoculated medium. In this mode fermentation, productivity is maintained throughout the process due to the addition of

fresh medium which supplied the nutrients to the microbes throughout the fermentation process. Semi continuous processes are economically feasible when batch process is failed to produce desirable outcome [21].

1.9.3 Continuous Fermentation

A rare mode of operation in the industry is the continuous process. Sterile media is fed into the bioreactor and inoculated with microorganism. After the growth phase, sterile media alone or with fresh inoculum is fed at a specific rate in continuous fashion into the bioreactor to maintain a steady state, harvesting the product as it forms along the process. This fermentation has also been dubbed as 'chemo stat'. When product is harvested, it is assured that the outgoing stream and incoming stream have the same composition to maintain continuous production levels over a longer period [22]. The continuous process has multiple advantages over other operation modes, one of them being consistent product quality due to constant reaction conditions. The process has immense capabilities of being automated reducing capital spent on labor. The drawbacks feature emphasis on quality of the soluble and insoluble raw materials which are to be replenished at a calculated rate to maintain culture conditions. The continuous process is applicable to products involving use of soluble solid substrates or liquids and for mutation-stable microorganisms.

1.10 Aims and Objectives

Following are the objectives of the project:

(i) The objective of the project was to increase the production of ethanol by fermenting xylose.
(ii) Optimization of fermentation process by using nutrients.
(iii) Revival of soil bacteria strain by optimizing temperature and pH conditions.
(iv) To establish a culture for bioethanol production by using soil bacteria.
(v) Optimizing the fermentation of xylose in SSL by using soil bacteria.
(vi) Identification of bacterial strain present in soil.

Provide cost effective solution of xylose fermentation.

1.11 Organization of Thesis

This thesis is organized in five Chapters. Chapter 1 covers the Introduction portion of thesis. This Chapter presents the research problem statement, outlines the research objectives, and presents the research methodology, significance and limitation of this research. Chapter 2 documents the comprehensive review of the literature on fermentation. It also deals with micro-organisms used for fermentation. Chapter 3 presents the Relevant Methodologies Available. Chapter 4 is about the Experimental set up. Chapter 5 presents the Experimentation Results and Discussion. Conclusion and recommendations are present at the end of the Thesis; it also presents the future research direction for interested researchers.

Summary

Pakistan, even though with the presence of extensive natural resources is still dealing crucially with its energy needs. Pakistan's woes have been further inflamed by its excessive reliance on power plants, using furnace oil and importing crude oil ads up to another financial burden. Keeping in view the worldwide prospective where Brazil is using 100% ethanol in cars since 25 years, Pakistan is lagging behind in technological advancement. But has unremitting options to overcome this catastrophic situation using biofuels because of its favorable climatic conditions. Since Pakistan is an agricultural country so lots of crop residues specially rice husk are being generated every year after harvesting rice crops .This waste of today has an ability to turn into fuel of tomorrow. Bioethanol not only reduces energy import bills but also helps in earning foreign exchange. The fermentation technology is quite mature but in another side, it has challenges with different climate and parametric conditions. From last few years, bacteria took some attention in the fermentation technology. Although bacteria has not been commercially used. Bacteria has some advantages over yeast in many aspects. In this study a bacteria was isolated from local soil and identified using various physiochemical verification tests. Afterwards, fermentation experiments were performed under the parameters of pH, temperature, sugar content and type of sugar. By performing these experiments suitable xylose fermentation conditions were determined using the locally isolated bacteria.

References

[1] M. O. S. Dias, M. P. Cunha, C. D. F. Jesus, G. J. M. Rocha, J. G. C. Pradella, C. E. V Rossell, R. M. Filho, and A. Bonomi, "Second generation ethanol in Brazil: can it compete with electricity production?," *Bioresour. Technol.*, vol. 102, no. 19, pp. 8964–71, Oct. 2011.

[2] M. Economics, "Explorative research on the transport biofuel market," no. September, 2009.

[3] K. Harijan, M. Memon, M. a. Uqaili, and U. K. Mirza, "Potential contribution of ethanol fuel to the transport sector of Pakistan," *Renew. Sustain. Energy Rev.*, vol. 13, no. 1, pp. 291–295, Jan. 2009.

[4] J. Goldemberg, "The Brazilian biofuels industry.," *Biotechnol. Biofuels*, vol. 1, no. 1, p. 6, Jan. 2008.

[5] M. Gulati, K. Kohlmann, M. R. Ladisch, R. Hespell, and R. J. Bothast, "ASSESSMENT OF ETHANOL PRODUCTION OPTIONS FOR," vol. 58, no. 1996, pp. 253–264, 1997.

[6] A. C. Hansen, Q. Zhang, and P. W. L. Lyne, "Ethanol-diesel fuel blends -- a review.," *Bioresour. Technol.*, vol. 96, no. 3, pp. 277–85, Feb. 2005.

[7] J. D. Z. Mary-Louise R Rusek, *Renewable engine fuel.* .

[8] Bio-Nett, "Bioethanol Fact Sheet," p. 4, 2010.

[9] C. E. Wyman, "Ethanol from lignocellulosic biomass: Technology, economics, and opportunities," *Bioresour. Technol.*, vol. 50, no. 1, pp. 3–15, Jan. 1994.

[10] W. Zhang and A. Geng, "Improved ethanol production by a xylose-fermenting recombinant yeast strain constructed through a modified genome shuffling method," *Biotechnol. Biofuels*, vol. 5, no. 1, p. 46, 2012.

[11] M. Wayman, S. Chen, R. S. Parekh, S. R. Parekh, and T. Canada, "Comparative Performance of Zymomonas mobilis and Saccharomyces cerevisiae in Alcoholic

Fermentation of Saccharified Wheat Starch B in a Continuous Dynamic Immobilized Biocatalyst Bioreactor Solids control," vol. 40, pp. 2–7, 1988.

[12] C. E. Wyman, S. R. Decker, M. E. Himmel, J. W. Brady, and C. E. Skopec, "Hydrolysis of Cellulose and Hemicellulose," 2005.

[13] N. D. Hinman, J. D. Wright, W. Hogland, and C. E. Wyman, "Xylose fermentation - An economic analysis," *Appl. Biochem. Biotechnol.*, vol. 20–21, no. 1, pp. 391–401, 1989.

[14] T.-C. Hsu, G.-L. Guo, W.-H. Chen, and W.-S. Hwang, "Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis.," *Bioresour. Technol.*, vol. 101, no. 13, pp. 4907–4913, 2010.

[15] O. Kim, S. Ole, H. A. Thomas, R. Birgitte, K. Olofsson, O. Sibbesen, T. H. Andersen, and B. Rønnow, "Rapid xylose and glucose fermentation by engineered S. cerevisiae for commercial production of cellulosic ethanol," p. 1, 2012.

[16] H. K. Asif, A. Ehsan, Z. Kashaf, a A. Abeera, N. Azra, and Q. Muneeb, "Comparative study of bioethanol production from sugarcane molasses by using Zymomonas mobilis and Saccharomyces cerevisiae," *African J. Biotechnol.*, vol. 14, no. 31, pp. 2455–2462, 2015.

[17] J. Barrios-Gonzáles, a. Tomasini, G. Viniegra-Gonzáles, and J. López, "Penicillin Production By Solid State Fermentation," *Biotechnol. Lett.*, vol. 10, no. 11, pp. 793–798, 1988.

[18] C. M. Drapcho and D. Ph, *Biofuels Engineering Process Technology*. .

[19] Z. Khokhar, Q.-A. Syed, S. Baig, M. Nadeem, M. Irfan, and A. M. Athar, "Study on filtrate obtained from batch-wise pretreatment of wheat straw at different concentrations of H2SO4 and autoclave time.," *World Appl. Sci. J.*, vol. 10, no. 9, pp. 1047–1055, 2010.

[20] Y. Lin and S. Tanaka, "Ethanol fermentation from biomass resources: current

state and prospects.," *Appl. Microbiol. Biotechnol.*, vol. 69, no. 6, pp. 627–42, Feb. 2006.

[21] W.-C. Lee and C.-T. Huang, "Modeling of ethanol fermentation using Zymomonas mobilis ATCC 10988 grown on the media containing glucose and fructose," *Biochem. Eng. J.*, vol. 4, no. 3, pp. 217–227, Feb. 2000.

[22] P. Gunasekaran and K. Chandra Raj, "Ethanol fermentation technology – Zymomonas mobilis." .

Chapter 2
Literature Review

2.1 Bioethanol Production

The bioethanol production process can be separated into four parts: pretreatment, hydrolysis, fermentation, and product purification. Prior to the processing, the substrate is of course harvested. The time of harvest is important since mature feedstocks show increased lignification, whereas, immature commodities lack in sufficient carbohydrates. For example, switchgrass has developed 90% of its dry matter by August [1], [2].The enzymatic hydrolysis and fermentation are combined in a mode known as simultaneous scarification and fermentation (SSF). The pretreatment starts with mechanical size reduction by a hammer mill to increase the surface area, so that enzymatic and microbial attack is enhanced. A particle size of 0.2 to 2 mm can be achieved after milling [3]. The power required for this process increases with decreasing final particle size and may be a limiting factor. Product purification is usually done by distillation together with molecular sieves.

2.2 Pretreatment of Biomass

Lignocellulosic material needs pretreatment prior to enzymatic hydrolysis because of its structure. The carbohydrates necessary for fermentation are cellulose and hemicelluloses. Unfortunately, these polysaccharides are surrounded by a lignin structure that is highly resistant to microbial and/or enzymatic attack and is not fermentable [4], [5]. Pretreatment methods disrupt and pull apart the lignin structure to make cellulose and hemicellulose available for enzymatic hydrolysis. The hemicellulose is typically dissolved in the liquid fraction, called prehydrolyzate, during pretreatment. The prehydrolyzate can also be used for fermentation. Mosier [5] illustrated the effect of pretreatment on biomass. Due to an enhanced convertibility, process efficiency can be increased and costs decrease [5]–[9].

2.2.1 Auto Hydrolysis

Compressed hot water (pressure above saturation point) is used in auto hydrolysis. The operation is carried out in temperature ranging from 150°C to 230°C [10], depending on

the temperature time of reaction may differ from seconds up to hours. The ratio of liquid-to-solid (LSR), may differ from 2 to 100 (w/w) [10], [11], whereas most values occur around 10. The higher LSR are normally related to continuous reactors. The mechanism of auto hydrolysis is similar to that of dilute-acid hydrolysis. Both of these processes are hydronium ion catalyzed processes. Hydronium ions involved in auto hydrolysis are formed in situ when using water auto-ionization and acetic acid and are also formed by from acetyl substituents of hemicelluloses, the process mentioned later has a greater influence on the hydrolysis[12], [13]. The formation of hydronium ions may also be done by uronic acids [13] but the role of these hydronium ions in hydrolysis is not fully understood.

By the process of auto-hydrolysis 55-84% of hemicellulose has been recovered, including inhibitory by products in low amounts [3], [13]–[16]. In auto hydrolysis cellulose and lignin are affected, liquid fractions with a comparative low concentration of potential fermentation inhibitors along with cellulose and lignin rich solid phase are yielded. The issues such as acid recycling and precipitates elimination are avoided as well as the issues of corrosion are reduced as the process occurs at mild pH. Auto hydrolysis has the environmental benefit over other hydrolytic technologies due to which both capital and operational costs have been reduced. When using the liquid stream in the process of ethanol fermentation a drawback that occurs is that the solubilized hemicellulose are formed in oligomeric form.

2.2.2 Steam Explosion

The process in which breakdown of structural constituents is achieved by the help of heat in the form of steam (thermo), shear forces as a result of expansion of moisture (mechano), along with hydrolysis of gylcosidic bonds (chemical) once it has been (self)-catalyzed (e.g., by acetic acid derived from biomass and also by the addition of catalysts) is described as a thermos-mechano-chemical process and is called steam explosion [17]. In this process the material is heated for a few seconds under high pressure steam (preferably to temperatures below 240°C). Due to the high pressure the steam condenses "wetting" the material, which is "exploded" when the reactor pressure is rapidly released. Inter and intra molecular linkages are broken down as well the desegregation of lingo-cellulosic matrix caused by the forces which result from decompression [18].

The pretreatment of LCM by steam explosion and its effectiveness has been carried out in various studies. Furthermore, pretreatments of kraft pulps bleaching in paper pulp industry have been implemented at pilot and industrial using the steam explosion process. The most commonly known digester for kraft pulp bleaching is the STAKE continuous steam explosion digester [19].

High hemicellulose solubility (producing oligosaccharides as a majority) can be achieved by most steam treatments along with the production of slight lignin solubilization. In various studies sugar recoveries in the range of 45-69% have been reported [20]–[23]. It is a common practice to saturate biomass by using acid catalysts such as H_2SO_4 or SO_2. For example, in case of olive tree pruning its pentose yield can be increased to about 30% by using 1% of H_2SO_4 [24]. Similarly a maximum overall sugar yield of 85% can be obtained from wheat straw by the action of 0.9% of H_2SO_4 [25]. Lower treatment temperatures and less reaction time can help in increasing hemicellulose recovery along with the reduction in formation of sugar degradation products can also be achieved as the using catalysts is crucial for softwood (less acetylated).

2.2.3 Wet Oxidation

In case of wet oxidation, auto hydrolysis can be carried out by using oxygen or air as catalysts [26], by which the reactor can operate at low temperatures in short reaction time. Due to the capital costs related to the pressure equipment used, pretreatments are more costly. Instead of oxygen, Na_2CO_3 [27] is a commonly used catalyst in wet oxidation pretreatments. When using an alkaline medium, the rates of biomass-to-monosaccharides are high and can be obtained under moderate conditions, less reaction times and less generation of furan aldehydes [27], [28] and phenolic aldehydes, but no aliphatic acids will be formed [28], [29]. The occurrence of significant delignification [27], [28], is linked with increase in carboxylic acids [28] during the process of wet oxidation.

2.2.4 Alkaline Treatment

Pre-treatments are considered as two major groups of alkaline pre-treatments, that use alkaline/alkaline earth based agents (usually calcium, potassium or sodium) or those which use ammonia. For the purpose of lignin solubilization alkaline pre-treatments as compared to acid or hydrothermal processes are more effective and show small amounts of cellulose along with some higher amounts of hemicellulose solubilization which are

not described in this review with two exemptions: ammonia based and wet oxidation treatments.

The oxidation and decomposition of lignin can be promoted to CO_2, H_2O and carboxylic acids as wet oxidation is used for the pre-treatment of LCM involving oxygen and water at high temperatures and pressures [27], [28]. High rates of hemicellulose solubilization for 82% xylan from sugar bagasse can be achieved by the combination of alkaline agents (particularly Na_2CO_2) [27]. The hmeicellulosic sugars formed are mainly in oligomeric form, a significant amount of carboxylic acid is formed even though a less amount of furan-aldehyde is formed [22], [27], [28]. The process of ammonia recycling percolation (ARP) can be employed by using aqueous ammonia in a flow through mode at elevated temperatures (around 170^{o}C) [30]. Between 40-60% of hemicelluloses can be solubilized by this treatment, the obtained hemicelluloses are in oligomeric form. Only a minor amount of cellulose fraction is solubilized (<10%), but yields closer to theoretical can be obtained by following the cellulose hydrolysis steps [30]–[34]. High values of delignification in the range of 60-85% are obtained but the selectivity is again low [30], [32], [33]. Positive results of applying ARP on hardwood have been obtained [32], [34] as well as on corn stover [30], [33], [35], [36]. Above 93% of cellulose digestibility results from the treated biomass whereas with wastepaper and softwood pulp mill sludge the efficiency was low [31]. The mechanisms of aqueous ammonia reaction and that of lime and NaOH-catalyzed pretreatments are similar to each other, in the cases of breaking the ether and ester bonds in lignin carbohydrate complex and in swelling of biomass. The cost ammonia recovery, safety issues concerned with practically using ammonia and the high solubilization of lignin with the help of suitable detoxification processes are accounted for the economic constraints of ARP. Whereas, the high recovery of sugars without the formation sugar degradation products has a positive influence on the economy of the process [5].

The ammonia fiber explosion/expansion process (AFEX) has also been studied as an alternative [37]. Recently, simultaneous slobulization of cellulose and hemicellulose of corn stover has been successfully achieved with yields over 85%, as well as the amount of sugar degradation products was low by the process of AFEX [38]. Cellulose and hemicellulose are obtained in monomeric and oliogomeric form, more than 35% of soluble

hemicellulose was recovered after enzymatic hydrolysis with xylo-oligosaccharides (XOS) [38]. At 99% the ammonia was recycled and the fermentation step can be performed by using residual ammonia with no other supplement.

2.2.5 Ionic Liquids

In the fractionation processes ILs can serve as a substitute for the conventional media. IL's are organic salts having melting points normally below 100°C. these ionic liquids are sometimes called "green solvents" due to their high thermal stability [39], [40] and very low vapor pressure [41], [42]. One of the advantages claimed regarding ILs is that by employing them on wood there is a chance of complete dissolution of wood even presents in its original form, due to which new possibilities have been opened to fractionate, derivative and process LCM.

Cellulose can be dissolved by the application of some ILs such as 1-allyl-3-methylimidazolium chloride [amim][Cl] and 1-butyl-3-methylimidazolium [bmim][Cl] [43]. Up to 25% of cellulose can be dissolved forming high viscous solutions by the action of some imidazolium ILs, this has been reported by Rogers and co-workers in 2002 [44]. They also reported that dissolution of cellulose occurs by the breakage of extensive hydrogen bonding network by the action of anion present in IL. Water content was also reported by them as another crucial factor due to which the solubility of carbohydrate can be decreased by increasing the re-aggregation of the polymer's chains by the hydrogen bonding. If further modification is required then the influence of water is crucial as accessibility is decreased by aggregation which leads to less reactivity of the polymer. Whereas, re-aggregation can be used for easy regeneration of the dissolved carbohydrates as a result of which a solution is formed, by employing alcohol or acetone or the simply adding water [44]. The solubilization of cellulose is very effective by ILs [45], whereas the solubility of hemicellulose and lignin has been not rarely been reported [46] and requires more investigation. There are two methods for the fractionation of wood. In the first method the complete dissolution can be achieved which is followed selective precipitation of various components [47]–[50]. In the second method selective dissolution of one or more components can be carried out as it has been reported for the dissolution of (hemi)cellulose and lignin [51], [52].

There is less data regarding the toxicity and biodegradability of ILs and is considered as a drawback of the process. Even though, this field is still in the beginning phase still a progressive increase has been noticed [53].

2.3 Effect of Inhibitors on Fermentation Organisms

The choice of pretreatment depends on process costs, speed of reaction, and inhibitor formation. Furfural, hydroxyl-methyl-furfural, and acetic acid, as well as phenolics commonly found in biomass, have inhibitory effects on yeast growth and ethanol formation. In physico-chemical treatments, xylose can be converted to furfural. High temperature and low pH enhance this process. It was shown that furfural inhibits certain key enzymes required for ethanol production. These are hexokinase, triosephosphate dehydrogenase and alcohol dehydrogenase [54], as well as aldehyde dehydrogenase and pyruvate dehydrogenase [55]. The citric acid cycle and ethanol production are blocked, and acetaldehyde accumulates to toxic levels. Triosephosphate dehydrogenase was the enzyme most affected by furfural. A furfural concentration of 2mg/ml leads to complete inhibition of triosephosphate dehydrogenase activity and probably leads to an inhibition of glycolysis. This result was confirmed by Sanchez and Bautista (1988) who tested the effect of furfural and 5- hydroxymethalfurfural on *S. cerevisiae* and *Candida guilliermondii*. Cell growth is also affected by furfural, which results in an extended lag phase. Cell growth is slowed because the yeast needs time to express the necessary enzymes for furfural degradation [56]. Yeast was able to convert furfural to furfuryl alcohol, which is less toxic. This behavior can also be used to adapt yeast to mildly toxic conditions produced by furfural before use in fermentation [57]. It was also shown that inhibition occurs to a greater extent when toxic compounds are added in combination rather than individually. In other words, the sum of inhibition of the single compounds is less than the inhibition of the compounds in combination. Oliva [58] tested the effects of combinations of acetic acid, furfural and catechol on K. marxianus using glucose. The compounds significantly affected growth and ethanol fermentation. The lag phase was increased and no growth was observed until furfural was converted to furfuryl alcohol, but the interaction of all three components strongly affected the ethanol and biomass yield. A cumulative effect of toxic compounds was also described by Lohmeier-Vogel *et al.* [59] with furfural, 5-hydroxymethylfurfural, and acetic acid as inhibitors of P. stipites.

2.4 Fermentation

After the polysaccharides are hydrolyzed to monomers, yeast can start the fermentation process. Fermentation is the anaerobic conversion of carbohydrates (sugars) to ethanol, carbon dioxide, and other minor metabolites. While glucose can be utilized by all yeast, xylose can only be fermented by some species, *P. stipitis* for instance. The net reaction equation for glucose fermentation is:

Equation 1 Glucose fermentation

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Fermentation occurs under exclusion of oxygen (anaerobic). Glucose enters the glycolysis pathway and is in a sequence of chemical reactions converts to pyruvate. Pyruvate is further converted to ethanol by two nicotinamide adenine dinucleotide (NADH) that were produced in glycolysis. Under anaerobic fermentation only 1/18 of the chemical energy (in the form of ATP) is produced compared to aerobic conditions [60]. The optimal fermentation temperature depends on the yeast strain used. It can vary between 20°C with S. cerevisiae [61] to 45°C with K. marxianus [62]. Yeast growing at temperatures between 20°C and 45°C are classified as mesophilic, whereas, yeast growing between 45°C and 70°C are classified as thermophilic [63]. Xylose fermentation is explained in more detail in a later section.

2.4.1 SSF versus SHF

Enzymatic hydrolysis, also known as saccharification, and fermentation can be executed in two different modes. These are SSF and SHF. Both methods show advantages and disadvantages depending on yeast strain, location, annual average temperature, ethanol tolerance and temperature optima of yeast and enzymes. Usually, the enzymes used have thermophilic temperature optima (40°C to 50°C), whereas, yeast often have mesophilic temperature optima (20°C to 30°C). This favors SHF. In each step the optimal temperature is used and high yields can be obtained. However, the high sugar concentration after enzymatic hydrolysis can cause product inhibition to the enzyme. The use of two separate reactors also increases capital costs. In this case, SSF would be favorable because the yeast is immediately converting the hydrolyzed sugar and no inhibiting concentration of sugar can be built up [64]. Various tests with wood, grasses and agricultural residues in SSF

mode have been done [5], [65]–[67]. A test on herbaceous feedstock with S. cerevisiae showed a higher ethanol yield by using SSF instead of the conventional method of separating saccharification and fermentation. Blotkamp *et al.* (1978), Szczodrak and Targonski (1988) and Spindler *et al.* (1988) [67]–[70] are looking for thermophilic yeast strains that can ferment at temperatures up to 50°C to have optimal temperatures for both enzymes and yeast with the advantage of avoiding product inhibition.

2.4.2 Xylose Fermentation

Xylose, as a pentose sugar, cannot be utilized by all yeast. Yeast in the genera *Kluyveromyces, Pichia, Brettanomyces, Candida, Clavispora, Pachysolen and Schizosaccharomyces* have been studied for fermenting xylose [71]. The enzymes essential for xylose fermentation, xylose reductase (XR) and xylitol dehydrogenase (XDH), must be triggered by the presence of xylose. XR and XDH are not available during glucose fermentation [72]. Xylose is reduced to xylitol, catalyzed by XR, and further oxidized to xylulose, catalyzed by XDH. Xylulose enters the pentose phosphate cycle and is finally converted to ethanol [63], [73], [74]. Figure 1 show the pentose phosphate cycle. The overall reaction equation for xylose fermentation is:

Equation 2 Overall reaction for xylose fermentation

$$C_5H_{10}O_5 \rightarrow 5\ C_2H_5OH + 5\ CO_2$$

2.4.3 Oxygen requirement for xylose fermentation

The role of oxygen in xylose fermentation has been reported often. This is caused by different co-factor regeneration systems. Figure 1 shows the pathway from xylose to xylitol and further to xylulose. It is known that yeast which produce ethanol, like P. stipitis, can use NADH as a co-factor for xylose reductase, whereas, xylitol producers mostly use NADPH [75]. Xylitol dehydrogenase uses NAD+ as a co-factor in all xylose fermenting yeast [75]. In case of ethanol producing yeast, a regeneration cycle for NADH is established and xylulose formation is favored. In xylitol producing yeast, NADPH is regenerated with the subsequent pentose phosphate cycle, but NADH accumulates and xylitol dehydrogenase is inhibited which results in xylitol accumulation. It only occurs under anaerobic or micro aerobic conditions since NAD+ is regenerated by oxygen [75].

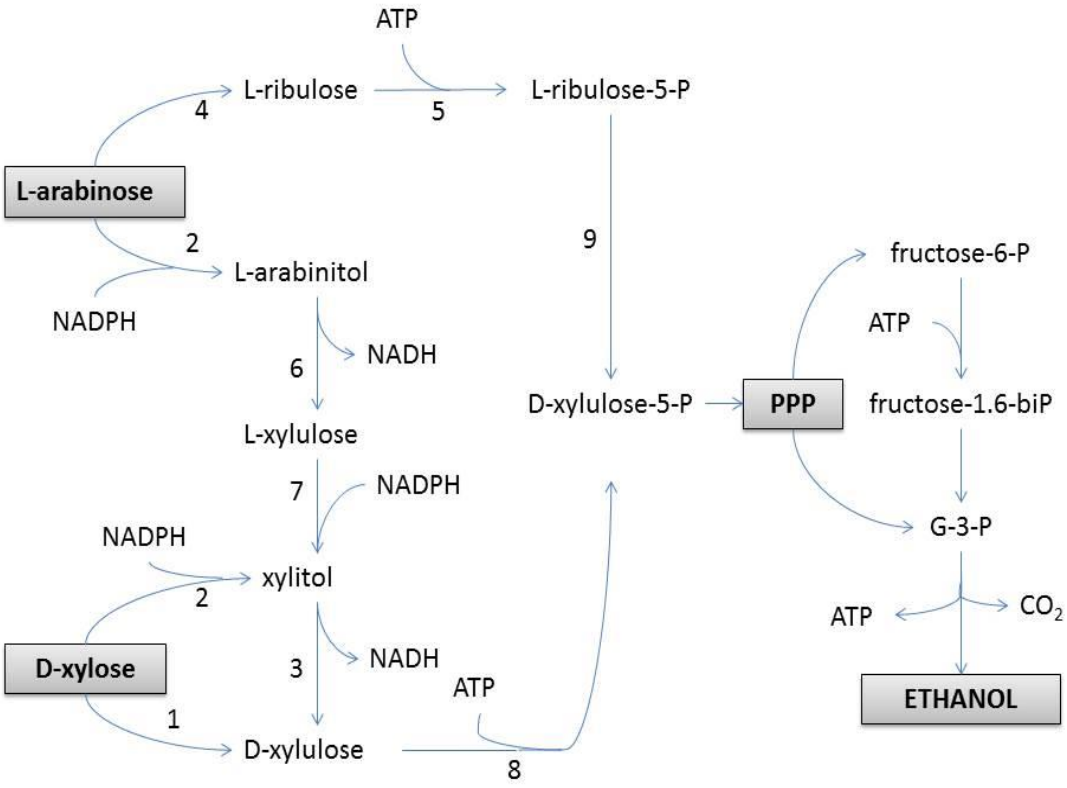

Figure 1 D-xylose and L-arabinose catabolism of engineered S. cerevisiae strains, Numbers represent enzymes involved in the catabolism steps; 1: xylose isomerase, 2: aldose/xylose reductase, 3: xylitol dehydrogenase, 4: L-arabinose isomerase, 5; L-ribulokinase

2.5 Micro-organisms

Bacillus subtilis cells are naturally found in soil and vegetation and are rod-shaped and Gram-positive bacteria. They grow in the mesophilic temperature range and the optimal temperature is 25-35 degrees Celsius [76]. Stress and starvation are common in this environment; therefore, *Bacillus subtilis* has developed a set of strategies that allow survival under these tough conditions. For example, the formation of stress-resistant endospores is one strategy.

Long ago it was considered that *Bacillus subtilis* was unable to grow in the absence of molecular oxygen as a terminal electron acceptor. However, as in the case of other members of the genus *Bacillus*, the capability of *B. subtilis* to use nitrate as an alternative

electron acceptor has been described by several groups [77]–[80]. It has been demonstrated by several groups that *B. subtilis* can be grown anaerobically in the absence of terminal electron acceptors on minimal media.

Summary

Fermentation does not require oxygen. If oxygen is present, some species of yeast oxidize pyruvate completely to carbon dioxide and water. This process is called cellular respiration. But these species of yeast will produce ethanol only in an anaerobic environment (not cellular respiration).

The byproducts formed as a result of xylose fermentation have been discussed in this chapter. To improve the efficiency of xylose fermentation literature related inhibitors was also studied in this chapter. A pathway in Figure 1 is also discussed, which lets us know what enzymes are involved in the conversion of xylose to ethanol. The stages at where these enzymes become active have also been shown with the help of Figure 1. A brief discussion on *Bacillus subtilis* was also done in this chapter.

References

[1] R. Gettle and J. George, "Frost-seeding legumes into established switchgrass: forage yield and botanical composition of the stratified canopy," *Agron. ...*, 1996.

[2] P. Koshi and J. Stubbendieck, "Switchgrasses: Forage yield, forage quality and water-use efficiency," *J. range ...*, 1982.

[3] Y. Sun and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresour. Technol.*, vol. 83, pp. 1–11, 2002.

[4] T. G. Hsu TA, Ladisch MR, "Alcohol from cellulose," *Chem. Technol.*, vol. 10, pp. 315–319, 1980.

[5] N. Mosier, C. Wyman, B. Dale, and R. Elander, "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresour. ...*, vol. 96, no. 6, pp. 673–686, 2005.

[6] K. Kohlmann, A. Sarikaya, and P. Westgate, "Enhanced enzyme activities on hydrated lignocellulosic substrates," 1995.

[7] D. Lee, H. Alex, K. Wong, and J. Saddler, "Evaluation of the enzymatic susceptibility of cellulosic substrates using specific hydrolysis rates and enzyme adsorption," *Appl. Biochem. ...*, 1994.

[8] L. Lynd, R. Elamder, and C. Wyman, "Likely features and costs of mature biomass ethanol technology," *Appl. Biochem. ...*, 1996.

[9] N. Mosier, R. Hendrickson, and N. Ho, "Optimization of pH controlled liquid hot water pretreatment of corn stover," *Bioresour. ...*, 2005.

[10] Y. H. P. Zhang, Z. Zhu, J. Rollin, and N. Sathitsuksanoh, "Advances in cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF)," in *ACS Symposium Series*, 2010, vol. 1033, pp. 365–379.

[11] Y.-H. P. Zhang, S.-Y. Ding, J. R. Mielenz, J.-B. Cui, R. T. Elander, M. Laser, M. E. Himmel, J. R. McMillan, and L. R. Lynd, "Fractionating recalcitrant lignocellulose at modest reaction conditions.," *Biotechnol. Bioeng.*, vol. 97, no. 2, pp. 214–23, 2007.

[12] R. P. Swatloski, S. K. Spear, J. D. Holbrey, and R. D. Rogers, "Dissolution of cellulose with ionic liquids," *J. Am. Chem. Soc.*, vol. 124, no. 18, pp. 4974–4975, 2002.

[13] P. Mäki-Arvela, I. Anugwom, P. Virtanen, R. Sjöholm, and J. P. Mikkola, "Dissolution of lignocellulosic materials and its constituents using ionic liquids-A review," *Industrial Crops and Products*, vol. 32. pp. 175–201, 2010.

[14] R. Rinaldi, R. Palkovits, and F. Schüth, "Depolymerization of cellulose using solid catalysts in ionic liquids," ... *Chemie Int. Ed.*, 2008.

[15] L. Olsson and B. Hahn-Hägerdal, "Fermentation of lignocellulosic hydrolysates for ethanol production," *Enzyme Microb. Technol.*, 1996.

[16] C. Hamelinck, G. Van Hooijdonk, and A. Faaij, "Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long-term," *Biomass and bioenergy*, vol. 28, pp. 384– 410, 2005.

[17] E. Chornet and R. Overend, "Phenomenological kinetics and reaction engineering aspects of steam/aqueous treatments," *Steam Explos. Tech. ...*, pp. 21–58, 1991.

[18] F. Carrasco, "Fundamentos del Fraccionamiento de la Biomasa," *Afinidad*, vol. 46, pp. 425–429, 1989.

[19] B. V. Kokta, "Steam explosion pulping," in *Steam Explosion Techniques: Fundamentals and Industrial Applications : Proceedings of the International Workshop on Steam Explosion Techniques: Fundamentals and Industrial Applications*, CRC Press, 1991, pp. 163–206.

[20] M. Ballesteros, I., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, "Enzymic hydrolysis of steam exploded herbaceous agricultural waste (Brassica carinata) at different particule sizes," ... *Biochem.*, vol. 38, no. 2, pp. 187–192, 2002.

[21] M. Heitz, E. Capek-Menard, and P. Koeberle, "Fractionation of Populus tremuloides at the pilot plant scale: optimization of steam pretreatment conditions using the STAKE II technology," ... *Technol.*, vol. 35, no. 1, pp. 23–32, 1991.

[22] C. Martin, M. Marcet, and A. Thomsen, "Comparison between wet oxidation and steam explosion as pretreatment methods for enzymatic hydrolysis of sugarcane bagasse," *BioResources*, vol. 3, no. 3, pp. 670–683, 2008.

[23] E. Ruiz, E., Cara, C., Manzanares, P., Ballesteros, M., Castro, "Evaluation of steam explosion pre-treatment for enzymatic hydrolysis of sunflower stalks," ... *Microb. Technol.*, vol. 42, no. 2, pp. 160–166, 2008.

[24] E. Cara, C., Ruiz, E., Ballesteros, M., Manzanares, P., Negro, M.J., Castro, "Production of fuel ethanol from steam-explosion pretreated olive tree pruning," *Fuel*, vol. 87, no. 6, pp. 692–700, 2008.

[25] I. Ballesteros, M. Negro, and J. Oliva, "Ethanol production from steam-explosion pretreated wheat straw," in ... -*Seventh Symposium on* ..., 2006.

[26] G. McGinnis, "Biomass pretreatment with water and high-pressure oxygen. The wet-oxidation process," ... *Eng. Chem.* ..., vol. 22, pp. 352–357, 1983.

[27] A. Bjerre and A. Olesen, "Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose," *Biotechnol.* ..., vol. 49, no. 5, pp. 568–577, 1996.

[28] H. Klinke, B. Ahring, A. Schmidt, and A. Thomsen, "Characterization of degradation products from alkaline wet oxidation of wheat straw," *Bioresour. Technol.*, vol. 82, no. 1, pp. 15–26, 2002.

[29] H. Klinke, A. Thomsen, and B. Ahring, "Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by Thermoanaerobacter mathranii," *Appl. Microbiol. Biotechnol.*, vol. 57, pp. 631–638, 2001.

[30] T. Kim and Y. Lee, "Pretreatment and fractionation of corn stover by ammonia recycle percolation process," *Bioresour. Technol.*, vol. 96, no. 18, pp. 2007–2013, 2005.

[31] J. Kim, Y. Lee, and S. Park, "Pretreatment of wastepaper and pulp mill sludge by aqueous ammonia and hydrogen peroxide," *Twenty-First Symp. Biotechnol. ...*, vol. 84, no. 6, pp. 129–139, 2000.

[32] J. Kim, H. Kim, J. Lee, J. Lee, and S. Park, "Pretreatment characteristics of waste oak wood by ammonia percolation," *Biotechnol. Fuels ...*, vol. 148, no. 1–3, pp. 15–22, 2008.

[33] T. Kim, J. Kim, C. Sunwoo, and Y. Lee, "Pretreatment of corn stover by aqueous ammonia," *Bioresour. Technol.*, vol. 90, no. 1, pp. 39–47, 2003.

[34] H. Yoon, Z. Wu, and Y. Lee, "Ammonia-recycled percolation process for pretreatment of biomass feedstock," *Appl. Biochem. Biotechnol.*, vol. 51, no. 2, pp. 5–19, 1995.

[35] T. Kim and Y. Lee, "Fractionation of corn stover by hot-water and aqueous ammonia treatment," *Bioresour. Technol.*, vol. 97, no. 2, pp. 224–232, 2006.

[36] Y. Zhu, T. Kim, Y. Lee, R. Chen, and R. Elander, "Enzymatic production of xylooligosaccharides from corn stover and corn cobs treated with aqueous ammonia," *... Biotechnol. Fuels ...*, pp. 586–598, 2006.

[37] G. Huber, S. Iborra, and A. Corma, "Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering," *Chem. Rev.*, vol. 106, no. 9, pp. 4044–4098, 2006.

[38] M. W. Lau and B. E. Dale, "Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST).," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 5, pp. 1368–73, Feb. 2009.

[39] U. Domanska and R. Bogel-Lukasik, "Physicochemical properties and solubility of alkyl-(2-hydroxyethyl)-dimethylammonium bromide," *... J. Phys. Chem. B*, vol. 109, no. 24, pp. 12124–12132, 2005.

[40] H. Ngo, K. LeCompte, L. Hargens, and A. McEwen, "Thermal properties of imidazolium ionic liquids," *Thermochim. Acta*, vol. 357, pp. 97–102, 2000.

[41] J. W. Earle, M.J., Esperança, J.M.S.S., Gilea, M.A., Lopes, J.N.C., Rebelo, L.P.N., Magee and J. A. Seddon, K.R., Widegren, "The distillation and volatility of ionic liquids," *Nature*, vol. 439, no. 7078, pp. 831–834, 2006.

[42] Y. Paulechka and G. Kabo, "Thermodynamic properties of 1-butyl-3-methylimidazolium hexafluorophosphate in the ideal gas state," *J. Chem. ...*, vol. 48, no. 3, pp. 457–462, 2003.

[43] A. P. Dadi, C. A. Schall, and S. Varanasi, "Mitigation of cellulose recalcitrance to enzymatic hydrolysis by ionic liquid pretreatment.," *Appl. Biochem. Biotechnol.*, vol. 137–140, no. 1–12, pp. 407–21, Apr. 2007.

[44] R. Swatloski and S. Spear, "Dissolution of cellose with ionic liquids," *... Chem. Soc.*, vol. 124, no. 18, pp. 4974–4975, 2002.

[45] H. Ohno and Y. Fukaya, "Task specific ionic liquids for cellulose technology," *Chem. Lett.*, vol. 38, no. 1, pp. 2–7, 2009.

[46] M. Zavrel, D. Bross, M. Funke, J. Büchs, and A. Spiess, "High-throughput screening for ionic liquids dissolving (ligno-) cellulose," *Bioresour. Technol.*, vol. 100, no. 9, pp. 2580–2587, 2009.

[47] L. Edye and W. Doherty, "Fractionation of a lignocellulosic material," *US Pat. App. 12/526,474*, 2008.

[48] R. D. Fort, D.A., Remsing, R.C., Swatloski, R.P., Moyna, P., Moyna, G., Rogers, "Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-n-butyl-3-methylimidazolium chloride," *Green ...*, vol. 9, no. 1, pp. 63–69, 2007.

[49] J. Ren, R. Sun, C. Liu, Z. Cao, and W. Luo, "Acetylation of wheat straw hemicelluloses in ionic liquid using iodine as a catalyst," *Carbohydr. Polym.*, vol. 70, no. 4, pp. 406–414, 2007.

[50] N. Sun, M. Rahman, Y. Qin, and M. Maxim, "Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate," *Green ...*, vol. 11, no. 5, pp. 646–655, 2009.

[51] J. S. Lee, S.H., Doherty, T.V., Linhardt, R.J., Dordick, "Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis," *... Bioeng.*, vol. 102, no. 5, pp. 1368–1376, 2009.

[52] J. Sievers, C., Valenzuela-Olarte, M.B., Marzialetti, T., Musin, D., Agrawal, P.K. and C.W., "Ionic-liquid-phase hydrolysis of pine wood," *... Eng. ...*, vol. 48, no. 3, pp. 1277–1286, 2009.

[53] and B. J. J. Ranke,* S. Stolte, R. Sto¨rmann, J. Arning, "Design of sustainable chemical products the example of ionic liquids," *Chem. ...*, vol. 107, no. 6, pp. 2183–2206, 2007.

[54] N. Banerjee, R. Bhatnagar, and L. Viswanathan, "Inhibition of glycolysis by furfural in Saccharomyces cerevisiae," *... J. Appl. Microbiol. ...*, 1981.

[55] T. Modig, G. Lidén, and M. Taherzadeh, "Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase," *Biochem. J*, 2002.

[56] L. Boyer, J. Vega, and K. Klasson, "The effects of furfural on ethanol production by saccharomyces cereyisiae in batch culture," *Biomass ...*, 1992.

[57] Z. Liu, P. Slininger, and B. Dien, "Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2, 5-bis-hydroxymethylfuran," *J. Ind. ...*, 2004.

[58] J. Oliva, M. Negro, and F. Saez, "Effects of acetic acid, furfural and catechol combinations on ethanol fermentation of Kluyveromyces marxianus," *Process ...*, 2006.

[59] E. Lohmeier-Vogel, C. Sopher, and H. Lee, "Intracellular acidification as a mechanism for the inhibition by acid hydrolysis-derived inhibitors of xylose fermentation by yeasts," *J. Ind. Microbiol. ...*, 1998.

[60] M. Nelson, David, Cox, *Lehninger Biochemie*. 2005.

[61] M. Madigan, J. Martinko, and T. Brock, "Brock Mikrobiologie," 2009.

[62] I. Banat, P. Nigam, and R. Marchant, "Isolation of thermotolerant, fermentative yeasts growing at 52 C and producing ethanol at 45 C and 50 C," *World J. Microbiol. ...*, 1992.

[63] F. W, *Mikrobiologie*. 2002.

[64] J. Savarese and S. Young, "Combined enzyme hydrolysis of cellulose and yeast fermentation," *Biotechnol. Bioeng.*, 1978.

[65] V. Chang and M. Nagwani, "Oxidative lime pretreatment of high-lignin biomass," *Appl. Biochem. ...*, 2001.

[66] K. Grohmann, "Simultaneous saccharification and fermentation of cellulosic substrates to ethanol," *Biotechnol. Agric.*, 1993.

[67] C. Wyman, D. Spindler, and K. Grohmann, "Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol," *Biomass and Bioenergy*, 1992.

[68] P. Blotkamp, M. Takagi, M. Pemberton, and G. Emert, "Enzymatic hydrolysis of cellulose and simultaneous fermentation to alcohol," 1978.

[69] J. Szczodrak and Z. Targoński, "Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose," *Biotechnol. Bioeng.*, 1988.

[70] D. Spindler and C. Wyman, "Thermotolerant yeast for simultaneous saccharification and fermentation of cellulose to ethanol," *Appl. Biochem. ...*, 1988.

[71] K. Skoog and B. Hahn-Hägerdal, "Xylose fermentation," *Enzyme Microb. Technol.*, vol. 10, no. 2, pp. 66–80, 1988.

[72] K. Smiley and P. Bolen, "Demonstration of D-xylose reductase and D-xylitol dehydrogenase in Pachysolen tannophilus," *Biotechnol. Lett.*, 1982.

[73] C. Chiang and S. Knight, "Metabolism of D-xylose by moulds," 1960.

[74] M. Ligthelm and B. Prior, "An investigation of D-{1-13C} xylose metabolism in Pichia stipitis under aerobic and anaerobic conditions," *Appl. Microbiol. ...*, 1988.

[75] E. Yablochkova and O. Bolotnikova, "The activity of key enzymes in xylose-assimilating yeasts at different rates of oxygen transfer to the fermentation medium," *Microbiology*, 2004.

[76] E. Belda, A. Sekowska, F. Le Fèvre, A. Morgat, D. Mornico, C. Ouzounis, D. Vallenet, C. Médigue, and A. Danchin, "An updated metabolic view of the

Bacillus subtilis 168 genome.," *Microbiology*, vol. 159, no. Pt 4, pp. 757–70, Apr. 2013.

[77] P. Glaser, A. Danchin, and F. Kunst, "Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of Bacillus subtilis.," *J. ...*, 1995.

[78] T. Hoffmann and B. Troup, "The anaerobic life of Bacillus subtilis: cloning of the genes encoding the respiratory nitrate reductase system," *FEMS ...*, 1995.

[79] H. Mach, M. Hecker, and F. Mach, "Physiological studies on cAMP synthesis in Bacillus subtilis," *FEMS Microbiol. Lett.*, 1988.

[80] J. Schirawski and G. Unden, "Anaerobic respiration of Bacillus macerans with fumarate, TMAO, nitrate and nitrite and regulation of the pathways by oxygen and nitrate," *Arch. Microbiol.*, 1995.

[81] H. Ramos, L. Boursier, and I. Moszer, "Anaerobic transcription activation in Bacillus subtilis: identification of distinct FNR-dependent and-independent regulatory mechanisms.," *EMBO ...*, 1995.

[82] T. Hoffmann and N. Frankenberg, "Ammonification in Bacillus subtilisUtilizing Dissimilatory Nitrite Reductase Is Dependent onresDE," *... Bacteriol.*, 1998.

[83] M. Nakano and P. Zuber, "Anaerobic growth of a 'strict aerobe'(Bacillus subtilis)," *Annu. Rev. Microbiol.*, 1998.

Chapter 3
Review of Available Methodologies

3.1 Hemicellulose Estimation Method

Hemicellulose is a non-cellulosic, non-peptic cell wall polysaccharide. Georing [1] described a method of hemicellulose estimation by refluxing the sample material with neutral detergent solution. The left out material is weighted after filtration and expressed as neutral detergent fiber (NDF). Van Soest's [2] described another method used for estimating hemicellulose content in substrate by following formula:

Equation 3 Estimation of hemicellulose content in substrate

$$\%\text{Hemicellulose} = \%\text{NDF} - \%\text{ADF}$$

3.2 Batch Fermentation Method

3.2.1 Identification of carbohydrates - Color reactions

Benedict's qualitative reagent, Bardfoed's reagents and Fehling tests are used for qualitative test of carbohydrates. These reactions; change in color indicates presence of carbohydrates. Another simple and adoptable method during large numbers of samples is Dinitrosalicyclic acid (DNS) reagent by Miller [3].Reducing sugar in sample is calculated by drawing standard graph. For determination of Glucose, a method suggested by [4], which is glucose oxidase method as glucose is widely distributed simple sugar with an active aldehyde group, this method gives true glucose concentration eliminating the interference of other reducing sugars.in this method glucose is oxidase catalysis the oxidation of alpha –D-glucose to D-glucono-1, 5 lactone with the formation of hydrogen peroxide and red colored product is formed. Moreover, phenol sulphuric acid method by Dubois [5] is used for calculation of total carbohydrates, in this method, in hot acidic medium, glucose is dehydrated to HMF. This form a green colored product with phenol and had absorption maximum at 490 nm. For hand held calculation, Refractometer is used for measurement of total sugars.

3.3 Fermentation Efficiency

The fermentation efficiency was calculated by the given formula:

Equation 4 Fermentation Efficiency

$$FE = (\text{actual yield} / \text{theoretical yield}) \times 100$$

Where FE = fermentation efficiency.

3.4 Enzyme Growth

Growth cells were taken into the sonication process and enzymes produced for batch fermentation using sonication for the rupturing the cells [6]. Rupturing of cells has been repeated for the better output of enzymes.

3.5 Identification of Bioethanol

There are quality methods to identify bio-ethanol, one such method is also known as potassium Di-chromate test. Bioethanol concentration in a sample can be determined by back titration with acidified potassium dichromate. Reacting the sample with an excess of potassium dichromate, all bioethanol is oxidized to acetic acid. Another chemical test was applied to identify the ethanol potassium permanganate test. The positive result will make the color change of sample from purple to green.

The other analytical methods use in modern era for the identification of bioethanol were, High-performance liquid chromatography (HPLC). HPLC and GC are working on the basic principle of boiling point difference with the reference. These methods are quite accurate for the identification; the only issue for HPLC and GC is to maintain the units. FTIR is another method used for qualitative analysis of bioethanol by matching peak with standard.

3.6 Determination of Bioethanol Concentration and pH

The bioethanol concentrations were determined by Ebulliometer which was approved in distilleries by US department of measurement and Distilleries Associations all around the globe. It has been also determined by HPLC, GC and Spectrophotometry reported in many studies [7]–[9]. The pH was determined by pH meter handy.

An ebulliometer is an instrument used for determination of the alcohol content of water-alcohol solutions by determining the difference in boiling points between pure water and the solution. Based on the comparison, the percentage alcohol (v/v) can be determined by referring to tables or using the calculating dial.

Gas chromatography and high performance liquid chromatography were being reported as most significant and accurate instruments used for the quantification of bioethanol. GC is user friendly and is considered as one of the best instruments used in fermentation process on the other hand HPLC lags in reliability and maintenance. Both HPLC and GC are separation methods for the identification og compounds from a mixture. Whereas HPLC applies to constituents that are fluids, GC is used when the compounds are gaseous or can be vaporized during the separation process. Both have the same fundamental principle of heavy molecules flowing slower than lighter ones.

3.7 Acidity Test

This test method measures acidity in fermentation yield. Very dilute aqueous solutions of low molecular mass organic acids, such as acetic acid, lactic acids may be present in the fermented sample. Due to the variation in pH, the harmful microorganism such as *lactobacillus*, which convert sugar into acids. This test makes sure the sugar was converted into bioethanol rather than acid. Titration method was adopted for the acidity test [10].

Summary

Methodologies, which were adopted for different analysis, were discussed in the previous chapter. The methods which were adopted by different researchers for different experiments were reported. Media preparation method adopted as mentioned in articles bacteria. Acidity test for acid formation in fermentation, which is also used method to test the ethanol concentration. Other methods can be possible for the better analysis subject to the availability of equipment's. In this chapter the discussion about the available methods for different analysis for current study were discussed. Some of the important methods have been discussed in the current chapter.

References

[1] A. Kerr and D. Goring, "Ultrastructural arrangement of the wood cell wall," *Cellul. Chem. Technol.*, 1975.

[2] P. Van Soest and J. Robertson, "Chemical and physical properties of dietary fibre," *Diet. Fibre; Proc. Miles ...*, 1976.

[3] G. Miller, "Use of DNS reagent for the determination of glucose," *Anal chem*, 1972.

[4] C. Malik and M. Singh, "Plant enzymology and histo-enzymology," 1980.

[5] M. Dubois, K. Gilles, and J. Hamilton, "Colorimetric method for determination of sugars and related substances," *Anal. ...*, 1956.

[6] E. Joyce, S. Phull, J. Lorimer, and T. Mason, "The development and evaluation of ultrasound for the treatment of bacterial suspensions. A study of frequency, power and sonication time on cultured Bacillus species," *Ultrason. Sonochem.*, 2003.

[7] Å. Haraldson and C. Rosén, "Studies on continuous ethanol fermentation of sugar cane molasses," *Eur. J. Appl. Microbiol. ...*, 1982.

[8] P. Mathias, *The Brewing Industry in England, 1700-1830*. 1959.

[9] P. Rogers and M. Skotnicki, "Recent developments in the Zymomonas process for ethanol production," *Crit. Rev. ...*, 1983.

[10] S. Vaz Jr, "Analytical techniques for the chemical analysis of plant biomass and biomass products," *Anal. Methods*, vol. 6, no. 20, pp. 8094–8105, Jun. 2014.

Chapter 4
Experimentation

4.1 Place of Work

The collection of soil sample and isolation of the bacteria was done at Bio-fuels lab at CAS-EN NUST, while the remaining of the research was carried out at the Institute of Microbiology, University of Agriculture Faisalabad.

4.2 Substrate Used

Xylose along with other chemicals used for the research was purchased from the market by a local dealer of the company Sigma Tech here in Islamabad.

Figure 2 Xylose as substrate

4.3 Instruments

The instruments used for the experiments are:

1. Spectrophotometer (Hitachi U-1100)
2. Electrical balance (AR3130–USA)
3. Autoclave (HV-50-Hirayam japan)
4. Laminar Flow
5. Shaker
6. pH meter (LIDA PH5-3CW Microprocessor pH/mV Meter)
7. Electric oven (ANC 371-England)
8. Incubator (Syno Japan)
9. Muffle furnance (KE-671)

4.4 Sample Collection and Bacteria Identification

4.4.1 Soil Sample

The fermenting strain was isolated from local soil sample taken from soil under a shrub located at Islamabad campus of NUST, Pakistan. Approximately 5 gm of soil sample was collected by using a clean and dry polythene bag by the help of a sterilized spatula.

Figure 3 Soil sample collected

4.4.2 Incubation

The sample was then transferred under sterile conditions to lab where nutrient broth was prepared. The sample collected was added to the broth and then kept for incubation at 35^{o}C and for a time period of 24 hours.

4.4.3 Streaking

After the 24 hours of the incubation period, supernatant of 0.1 mL from the tube containing the soil sample was inoculated on to plates for the streaking process at 30^{o}C for another 24 hours. The plate consisted of nutrient agar and xylose which served as the sole carbon source. After incubation the plate was then examined and suspected colonies were chosen for the Gram staining method. Gram positive, rod shaped and the spore forming bacilli was selected for further identification tests.

4.4.4 Identification of Locally Isolated Bacteria

Identification of the microorganism was done by performing some common physiological and biochemical tests. The basis of the performed conventional techniques was based on Bergey's manual for the identification of bacteria [1]. By the results obtained the bacteria

was identified as *Bacillus subtilis*. A summary of the tests performed and the results obtained are as follows:

Table 2 Tests performed for the identification of locally isolated bacteria

Test Performed	Result Obtained
Gram Staining	+
Spore Forming	+
Strict Anaerobes	-
Starch Hydrolysis (Amylase)	+
VP Test	+
Cell Diameter $\geq 1\mu m$ (width)	-
Citrate Test	+
NaCl Growth	+

After identification of the locally isolated bacteria the fermentation of xylose under conditions of various pH mediums and at different temperatures were measured and ethanol yield was analyzed to determine optimum fermentation conditions.

4.4.4.1 Gram Staining

Gram staining, also known as Gram's Method, is a method used to categorize bacterial species into two main groups; i.e., Gram-positive and Gram-negative. By detecting peptidoglycan, which is present in a thick layer in Gram-positive bacteria, bacteria is differentiated by Gram staining by the chemical and physical properties of their cell walls [1]. In the identification of a bacterial organism, Gram stain is almost always the first step. The process of gram staining is as under:

1. A heat fixed smear is flooded with crystal violet for 60s. All cells are stained purple.
2. The Gram's iodine solution is added for 3 minutes which forms an insoluble crystal violet-iodine complex inside the cell.
3. Alcohol is used to decolorize the cells for 20s. In Gram-positive bacteria which stain blue/purple, the cell walls become dehydrated, closing cell walls, and preventing escape of the crystal violet-iodine complex. In Gram-negative bacteria, the crystal violet-iodine complex is washed away, leaving the cells colorless.

The cells are counterstained with safranin for 1 to 2 minutes. Gram-negative cells are pink to red whereas Gram-positive cells are purple.

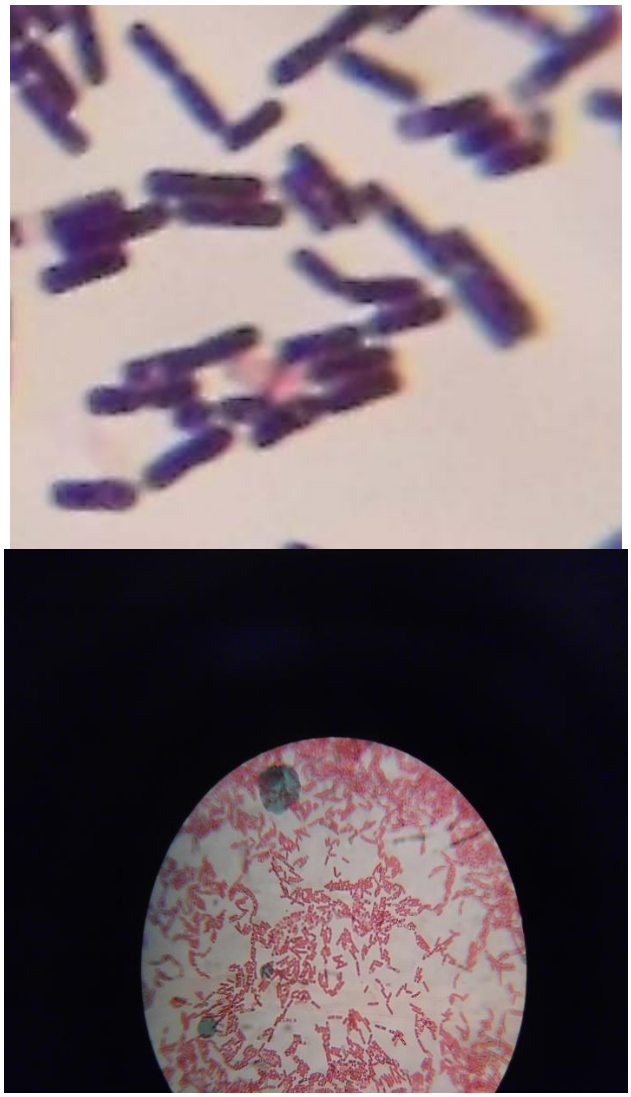

Figure 4 Gram stain results' showing purple color indicating that the bacteria is gram positive

4.4.4.2 Spore Forming

The endospore stain is a differential stain as the spore and the vegetative section of spore-forming bacteria are differentiated. Spores are resistant to chemicals, heat and biological stains. However, under extreme heat application, the stain can be lead to the spore, and the vegetative portion can be de-colorized while the endospore remains colored. The laboratory method used, the Schaeffer-Fulton method, stains the spores with Malachite green, rinsed, and the vegetative section is counterstained with safranin. In case, the unknown contains spores, they will stain green.

4.4.4.3 Anaerobe Bacteria

The isolated bacterium from the soil sample was grown in the absence of free oxygen. After a time interval of 48 hrs. it was shown by the results that the bacteria was unable to be grown under such conditions. This means that the isolated bacterium was different from the category of strict anaerobes.

4.4.4.4 Starch Hydrolysis (Amylase) Test

The purpose of this test is to see if starch which is a complex carbohydrate made from glucose, can be consumed by the microbe as a source of carbon and energy for growth. An enzyme called alpha-amylase accomplishes the use of starch. A medium containing starch is used. After inoculation and overnight incubation, iodine reagent is added to detect the presence of starch. Iodine reagent makes a complex with starch to form a blue-black color in the culture medium. Clear halos surrounding colonies is indicative of their ability to digest the starch in the medium due to the presence of alpha-amylase.

Figure 5 Amylase test showing positive result

4.4.4.5 VP (Voges–Proskauer) Test

Acetoin in a bacterial broth culture is detected by a test called VP. The test is carried out by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which has been inoculated with bacteria. A positive result is indicated by a cherry red color, while a negative result is indicated by a yellow-brown color. The procedure for the test is to first add the alpha-naphthol; and then, add the potassium hydroxide. A reversal in the order of the reagents being added may cause a weak-positive or false-negative reaction.

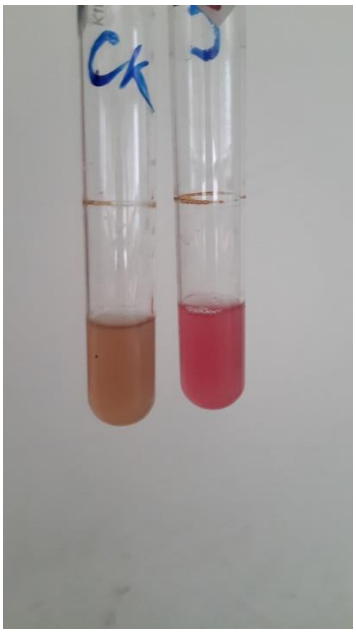

Figure 6 VP Test Performed - Left Test Tube -ve Result and Right Test Tube +ve result

4.4.4.6 Citrate Test

The ability of bacteria to consume sodium citrate as its only carbon source and inorganic ($NH_4H_2PO_4$) is the sole fixed nitrogen source is determined by the Citrate utilization test. Using an organic acid such as citrate as a carbon and energy source, produces alkaline carbonates and bicarbonates ultimately. In addition, ammonium hydroxide is produced when the ammonium salts in the medium are used as the sole nitrogen source.

Figure 7 Citrate test showing positive result

4.4.4.7 6.5% Salt Tolerance Test

Tryptic Soy Broth with added sodium chloride (regular table salt) is used to perform the salt tolerance test to create an overall salt concentration of 6.5%. The capability of an organism to survive in a salt-rich environment is tested by this selective medium. Most organisms cannot survive in such an environment.

4.5 Physical Treatment of Substrate

Xylose was dissolved in distilled water. The water was obtained from the distillation assembly present in Bio-fuel lab. The dissolved sugar was then used to carry out the fermentation experiments under various conditions.

4.6 Analytical

4.6.1 Fermentation Media and Strain

The bacteria used for the fermentation experiments was isolated from the by students at Bio-fuel Lab CAS-EN NUST.

Figure 8 Isolated Bacillus subtilis

4.6.2 Preparation of Bacteria (*B. subtilis*) Media

Media suggested by DSMZ Germany for bacteria consists of bacto-peptone 10 g/L, yeast extract 10 g/L, 15 g/L agar for agar plates and glucose 20 g/L. after taking measured amount of each chemical they were dissolved in distilled water and the flask was filled to mark up to 1000 ml with a pH 7.00. The media was then autoclaved at 121°C for 45 min, later streaking was done on agar plates in Desktop Laminar Flow, the streaked plates were incubated (Syno Japan) for overnight. The growth of cells was analyzed by

Hemocytometer and were inoculated into test tubes for scaling up and stored in glycerol stock for additional.

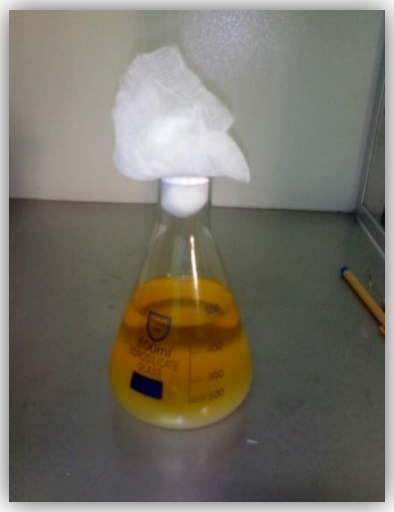

Figure 9 Media for isolated bacteria

4.6.3 Residual Sugar (Xylose) & Xylose Standard Curve

The standard curve of xylose was prepared using the colorimetric method reported by [2]. In this method the reagent was prepared by mixing 0.5 g of phloroglucinol, 100 ml of glacial acetic acid then them into 10 ml of concentrated hydrochloric acid. the standard solutions of xylose were prepared by dissolving xylose in water and making solutions of known concentrations from 1 gm/L to 10 gm/L. The prepared serum was then added in small amount to the standard xylose solution in a disposable test tube. The test tube was then placed in a heat block (Lab Line Instruments, Inc. Melrose Park IL 60559) which was set at a temperature of 105°C. The test tube was placed in the heat block for a time of 4 min as reported by [2]. After heating the test tube it was allowed to cool down to room temperature after, which its optical density (OD) was examined by a spectrophotometer (Hitachi U-1100) set at 554 nm.

The percentage of residual sugar (xylose) and ethanol produced was determined by using the standard curves. Whereas, sugar consumption in xylose fermentation experiments was determined by the following formula:

Equation 5 Sugar consumption

$$Sp = (S1 - S2)/S1$$

Where,

S_p = percentage of sugar consumption

S_1 = sugar concentration (gL^{-1}) before fermentation

S_2 = sugar concentration (gL^{-1}) after fermentation.

The color change of standard solutions of xylose having different concentrations can be seen in the following figure:

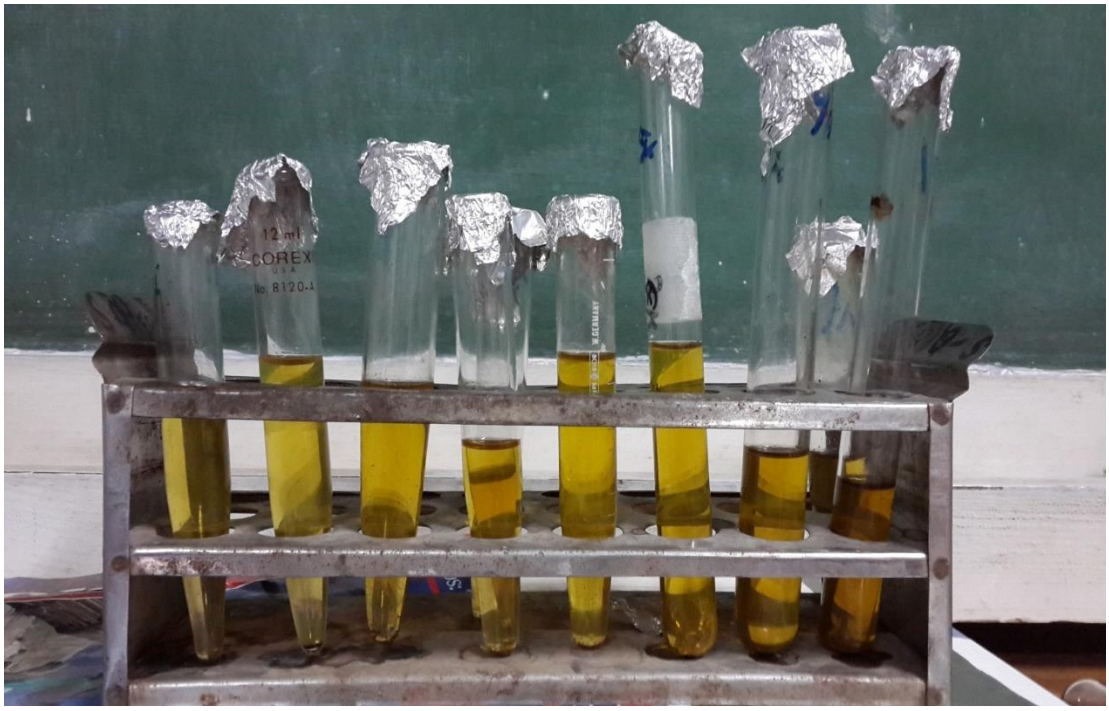

Figure 10 Reagent added to xylose standard solutions

As the solutions on the left side have less amount of xylose present their color is lighter than the solutions on the right side that have more concentrations of xylose. The OD of other standard solutions were also determined and tabulated by following the same procedure as mentioned above. The absorbance values obtained from the spectrophotometer for standard xylose solutions are as follows:

Table 3 Absorbance values for xylose solutions

Xylose Conc. (%)	Absorbance (nm)
10	0.258
20	0.268
30	0.275
40	0.289
50	0.301

60	0.312
70	0.323
80	0.331
90	0.345
100	0.357

The graph obtained from the above values is as follows:

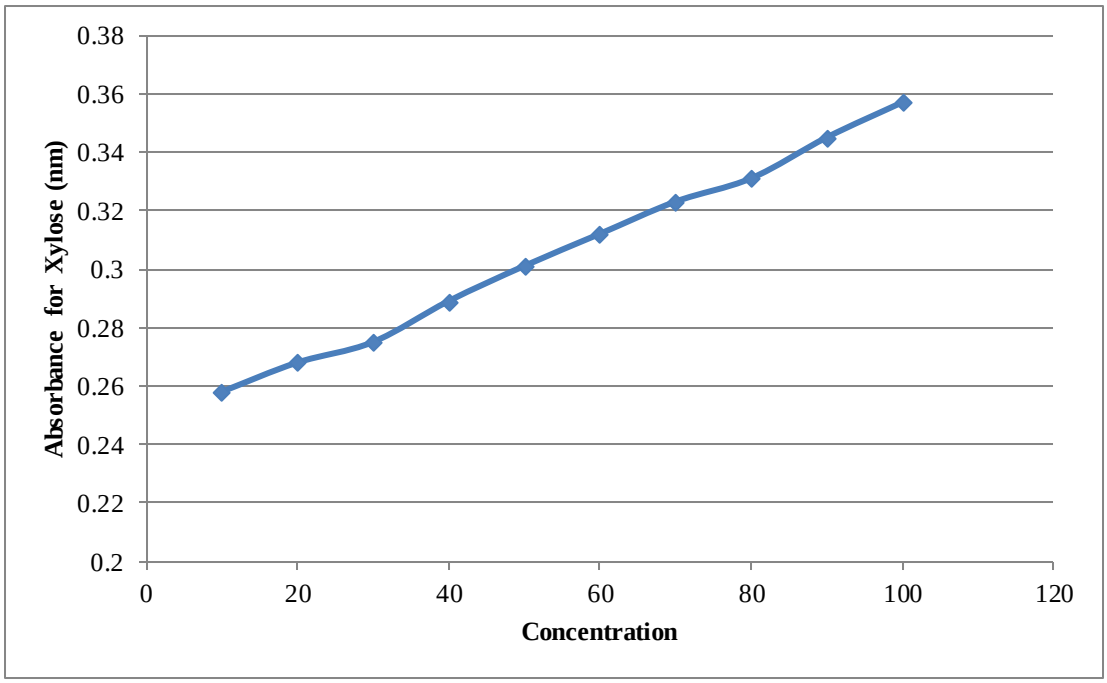

Figure 11 Standard xylose curve

4.6.4 Ethanol Standard Curve

The standard curve of ethanol was prepared by using the method described by [3]. Following this procedure three different reagents was prepared.

4.6.4.1 Preparation of Sodium Dichromate Solution

The first reagent was made by dry heating 4 g of sodium dichromate at a temperature of 120^{o}C for a time period of 3 hrs. After heating the sodium dichromate is it dissolved in water to make a solution.

4.6.4.2 Preparation of Acetic Buffer (pH 4.3)

The acetate buffer with a pH of 4.3 was prepared by following the procedure specified by US pharmaceutical.

4.6.4.3 Preparation of Sulfuric Acid

A sulfuric acid solution of 1N was prepared by measuring carefully 54 mL and dissolving it in 1 L of water.

After the preparation of the above mentioned three reagents, they were mixed together in a specific quantity. To form a standard reagent 5 mg/mL of sodium dichromate, 5 mL of acetate buffer with a pH of 4.3 and 25 mL of 1N of sulfuric acid were added to a 50 mL flask. This mixture was added to standard solution of ethanol; it was then shaken and allowed to stand for a time period of 2 hr at room temperature. As a result of this the reagent changed to light greenish blue color product. The OD was then measured by using a spectrophotometer (Hitachi U-1100) at 578 nm.

Figure 12 Reagent added to ethanol standard solutions

As the concentrations of standard ethanol solutions changes from 1% to 10% the color changes from yellow to greenish blue color. The OD of other standard solutions were also determined and tabulated by following the same procedure as mentioned above. The absorbance values obtained from the standard solutions of ethanol are as under:

Table 4 Absorbance values for standard ethanol solutions

Ethanol Conc. (%)	Absorbance (nm)
10	0.084
20	0.105
30	0.163
40	0.184
50	0.191
60	0.196
70	0.212
80	0.268
90	0.311
100	0.34

The graph made from the above obtained values is as:

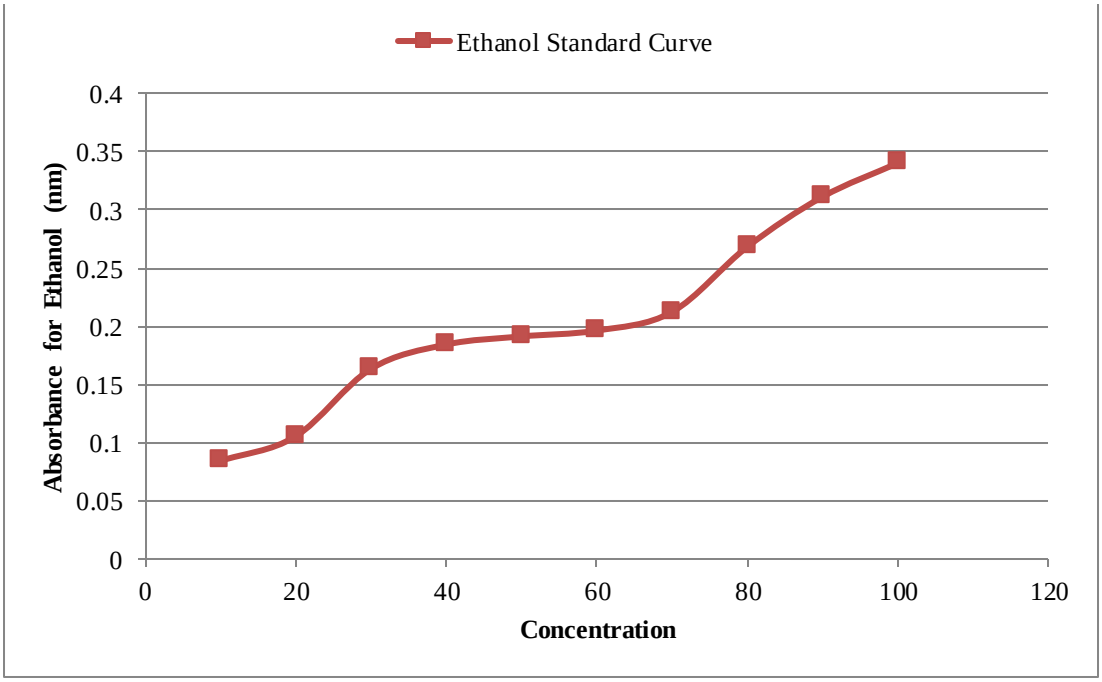

Figure 13 Ethanol standard curve

4.7 Detection of Ethanol

The fermented media was distilled by using rotary evaporator as shown in the figure below. Distillation was done under vacuum to achieve ethanol at a lower boiling point of 51-55°C instead of 70°C.

Figure 14 Rotary Evaporator apparatus for distillation of ethanol

Five (5) ml fermented sample was taken and pinch of Potassium dichromate reagent and a few drops of concentrated H_2SO_4 were added. The brownish color of sample was changed into greenish blue which indicated the presence of bioethanol.

Summary

Experiments were carried out for different analysis for parametric study of bioethanol production from xylose using locally isolated bacteria, which was identified to be *Bacillus subtilis* by performing different physiochemical and biochemical tests. Media were prepared for bacteria. Growth study was conducted, and this growth strain was used for further pre-fermentation. Batch fermentation were conducted and analyzed for the optimum production of bioethanol using *Bacillus subtilis*. Effects of the leading parameters were analyzed. Different experiments have been carried out for the analysis for fermented samples. Sugar consumption test was conducted for all fermented samples. Acidity test was one of the important experiments to make sure the acid production instead of bioethanol.

Furthermore, bioethanol detection test was conducted by a colorimetric method. Quantification of bioethanol was analyzed by using photo spectrophotometer.

Fermentation temperature, pH, sugar concentration, type of sugar were the key factors for the process optimization for bioethanol production from xylose.

References

[1] D. Bacteriology, G. Negative, F. A. Gram, F. Enterobacteriaceae, G. C. Key, E. Gram, N. Gram, N. Gram-positive, W. G. Nonsporlating, A. Fast, and S. Rods, "Gram Stain & Morphological Flowchart," *Differentiation*, pp. 1–8.

[2] T. J. Eberts, R. H. B. Sample, M. R. Glick, and G. H. Ellis, "A simplified, colorimetric micromethod for xylose in serum or urine, with phloroglucinol," *Clin. Chem.*, vol. 25, no. 8, pp. 1440–1443, 1979.

[3] S. Sumbhate, S. Nayak, D. Goupale, A. Tiwari, and R. S. Jadon, "Colorimetric Method for the Estimation of Ethanol in Alcoholic-Drinks," vol. 1, pp. 1–6, 2012.

Chapter 5
Results & Discussions

5.1 Statistical Analysis

All experiments were performed thrice and all measured values are average of the triple measurements ± standard deviation.

5.2 Effect of pH on Xylose Fermentation to Ethanol

The growth of microorganism in the process is greatly influenced by pH of the solution, as pH may be a reason for the change in charge of the cell membrane, as a result of which permeability of the membrane changes. At the same time, ionic substances required for the growth of microorganisms are affected by the change in pH as it may lead to a change in the degree of ionization. As a result of this, both absorption of nutrients and the growth of microorganism will be affected. Also, the enzymes involved in the microbial physiological and biochemical activity are also affected by the change in pH of the intracellular environment. Therefore, pH of the medium plays a crucial role in the bacterial growth.

Ethanol yield and the fermentation rate of the process were considerably influenced by change of pH of the medium. For the optimization of pH in the fermentation process, effect of pH on the ethanol concentration can be seen in Fig. 15. First the amount of residual sugar (xylose) was measured by the above mentioned colorimetric method. Then amount of residual sugar and ethanol concentration was measured after the fermentation process at 48 h. Whereas, fermentation conditions for the process were as: inoculation 2%, temperature for the process was maintained at 35°C with a rotating speed of 150 rpm and an initial sugar concentration of 100 gL^{-1} was taken. The amount of sugar consumed was calculated by subtracting the amount of residual sugar from total amount of sugar before the fermentation process.

Table 5 Effect of pH on absorbance and amount of sugar consumed

pH	Absorbance (nm)	Xylose Intake (%)	Xylose Left (%)
3	0.315	36	64
4	0.304	46	54
5	0.296	53	47
6	0.282	66	34
7	0.269	78	22
8	0.277	71	29

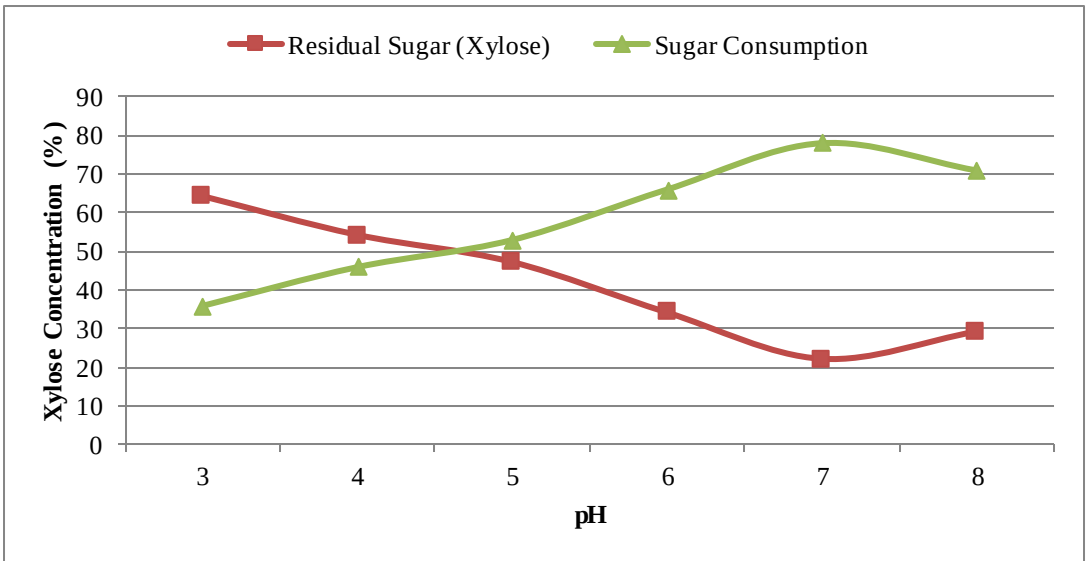

Figure 15 Effect of pH on sugar intake and residual sugar

The pH varied from 3 to 8, by looking at Fig. 15 a pH of 7 was selected to be a suitable pH value for the fermentation process. At a pH of 7 highest yield could be achieved as at this pH consumption of sugar was the highest. At lower pH levels the concentration of acids will inhibit cell fermentation. Whereas, higher pH levels will help in the senescence of microorganism cells and the fermentation rate would eventually decrease. At the same time, the chances of contamination at higher pH levels increased [1]. The values of the absorbance for ethanol produced obtained from experiments performed at various pH are tabulated as follows:

Table 6 Effect on absorbance and amount of ethanol produced

pH	Absorbance (nm)	Ethanol Produced (%)
3	0.331	10.29
4	0.413	13.38
5	0.514	17.23
6	0.584	19.87
7	0.688	23.79
8	0.623	21.34

The graph made from the above table is as:

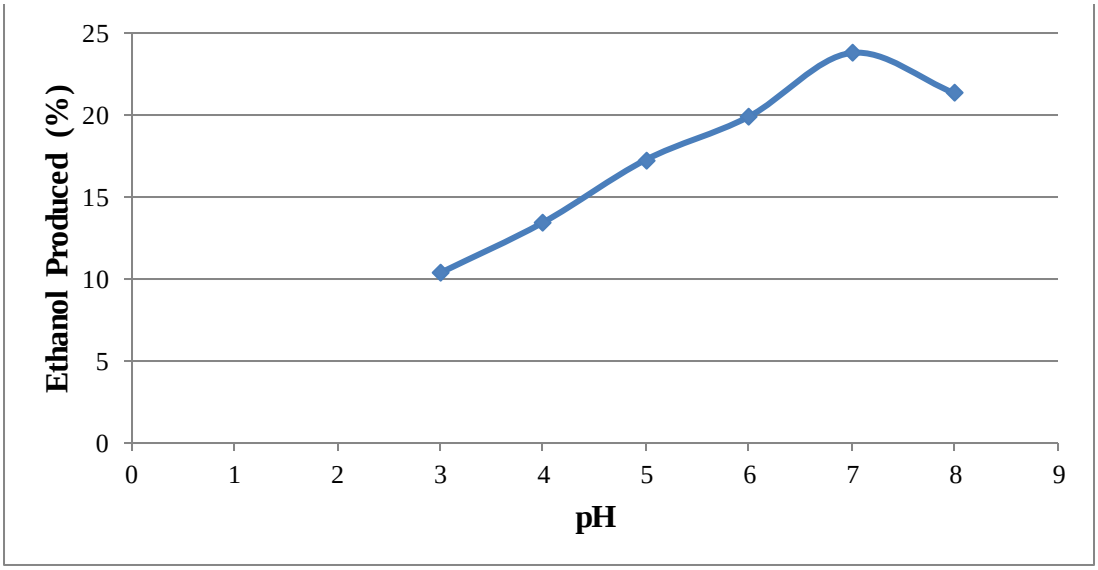

Figure 16 Effect of pH on ethanol produced

From Fig. 16, it can be seen that the maximum amount of ethanol produced/achieved was at a pH of 7. Fermentation media having a pH of 7 can said to be a optimum media for the fermentation of xylose using *Bacillus subtlilis*. As the pH was lower than 7 the microorganism (*Bacillus subtlilis)* was not able to ferment xylose as compared to pH 7. As the pH of the fermenting media was increased from 7 to 8 the amount of ethanol produced dropped to nearly 20% from 23%. These experiments regarding the pH of the fermenting media show that a pH of 7 is optimum for *Bacillus subtlilis* to ferment xylose as the sole carbon source.

5.3 Effect of Temperature on Xylose Fermentation to Ethanol

For the survival of microorganisms temperature is an important factor. The effect of temperature on the isolated *Bacillus subtilis* was analyzed. Temperature was varied from 25°C to 45°C. The fermentation conditions are as: amount of inoculation 2%, pH 7, a rotation speed of 150 rpm was maintained and an initial sugar concentration of 100 gL^{-1}.

Table 7 Effect of temperature on absorbance and sugar intake

Temperature (°C)	Absorbance (nm)	Xylose left (%)	Xylose Intake (%)
25	0.286	38	63
30	0.281	33	67
35	0.279	31	69
37	0.271	24	76
40	0.288	39	61
45	0.291	42	58

The above table gives us the following graph:

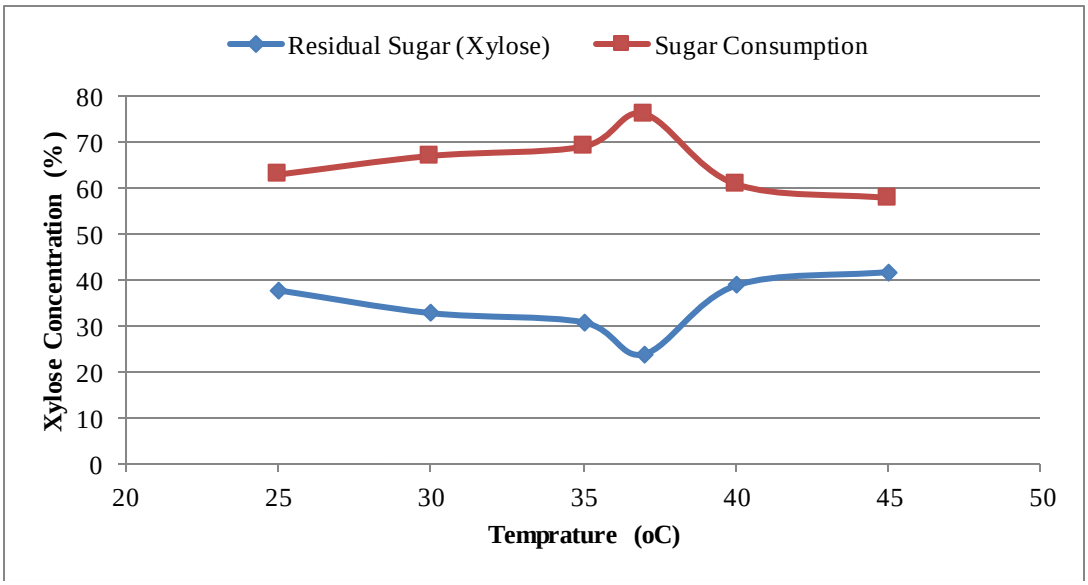

Figure 17 Effect of temperature on sugar intake and residual sugar

After a time period of 48 h residual sugar concentration and concentration of ethanol produced were determined. Fig. 17 shows the effect of temperature on amount of residual sugar (xylose) and the amount of ethanol produced after the fermentation process. The amount of sugar consumed was calculated by subtracting the amount of residual sugar from total amount of sugar before the fermentation process.

Table 8 Effect of temperature on absorbance and ethanol produced

Temperature (ºC)	Absorbance (nm)	Ethanol Produced (%)
25	0.539	18.16
30	0.597	20.34
35	0.661	22.76
37	0.699	24.21
40	0.664	22.91
45	0.488	16.23

The graph obtained from the above tabulated values is as follows:

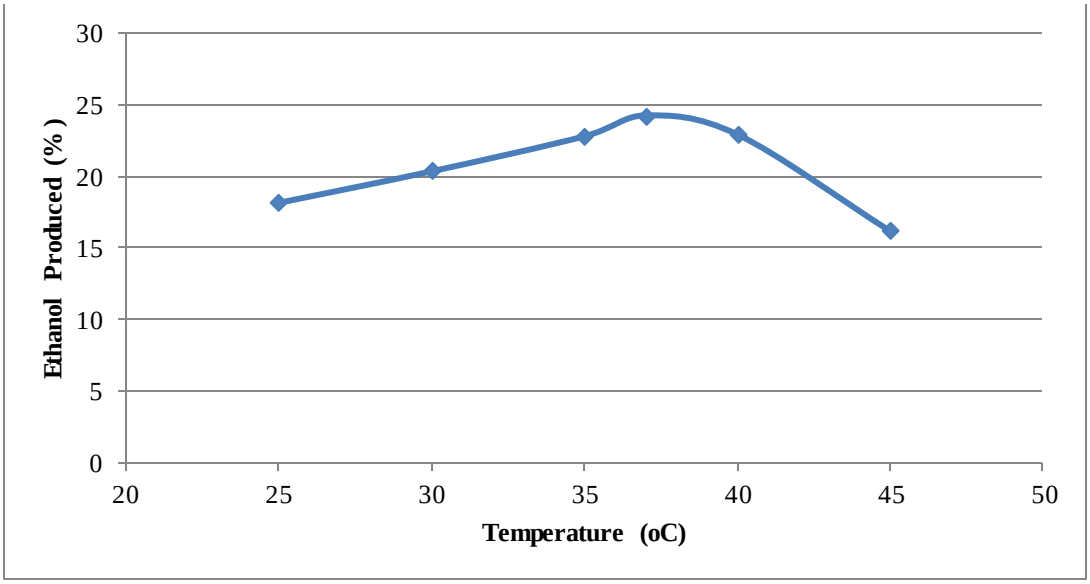

Figure 18 Effect of temperature on ethanol produced

The rate of enzymatic reaction is changed by change of temperature, which also affects the activity of enzymes. Enzymatic reaction rate will be increased and as a result of which metabolism rate and rate of growth of the microorganism will increase within a proper temperature range. At a suitable temperature, growth and reproduction of microorganisms is the fastest. Microorganisms can be divided into groups such as mesophilic bacteria, psychrophilic bacteria and thermophilic bacteria according to the optimum temperature required for their growth. Transport of substance and fluidity of cell membrane are also affected by temperature and as a result of which it has an effect on the absorption of nutrients as well as on the secretion of metabolites. In this research study, at a temperature of 37oC the sugar to ethanol conversion was the highest up to 24%. According to study

reports, the ability of bacteria to produce more ethanol can be improved at higher temperature, which can be observed from Lacis's research [2].

5.4 Sugar Content and Type of Sugar

Ethanol production is crucially affected by sugar content. Fermentation mediums consisting of different concentrations of sugar were made and fermentation process was carried out to produce ethanol. The amount of residual sugars left and the amount of ethanol were noted after a time period of 48h. Following are the fermentation conditions used: 3% of inoculation, pH 7, a rotating speed of 150 rpm was maintained and a temperature of 37°C was set. The concentration of the different media's ranged from 20 to 100 gL^{-1}.

Table 9 Effect of sugar content on absorbance and sugar consumption

Sugar Content (%)	Absorbance (nm)	Sugar Left (g/L)	Sugar Consumption (%)
20	0.245	7.6	62
40	0.259	13.2	67
60	0.262	15.6	74
80	0.266	19.2	76
100	0.302	52	48

Then trend for the above tabulated data is as follows:

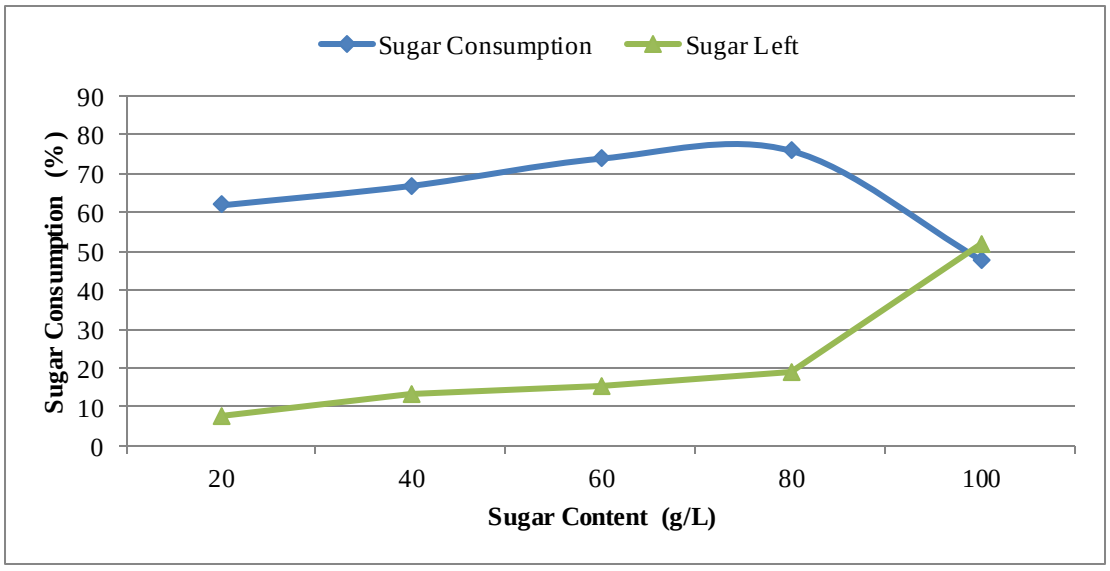

Figure 19 Effect of sugar content on amount of sugar consumed

The amount of ethanol produced from the experiments carried out on sugar content can be seen from Figure 20, the data noted for these experiments are in Table 10:

Table 10 Effect of sugar content on ethanol produced

Sugar Content (%)	Absorbance (nm)	Ethanol Produced (%)
20	0.398	12.79
40	0.56	18.93
60	0.644	22.11
80	0.691	23.89
100	0.472	15.67

As seen in the Fig. 20 below, after the maximum concentration of ethanol the next value decreased. The optimum value of sugar content for the fermentation process was approximately 78 gL^{-1}.

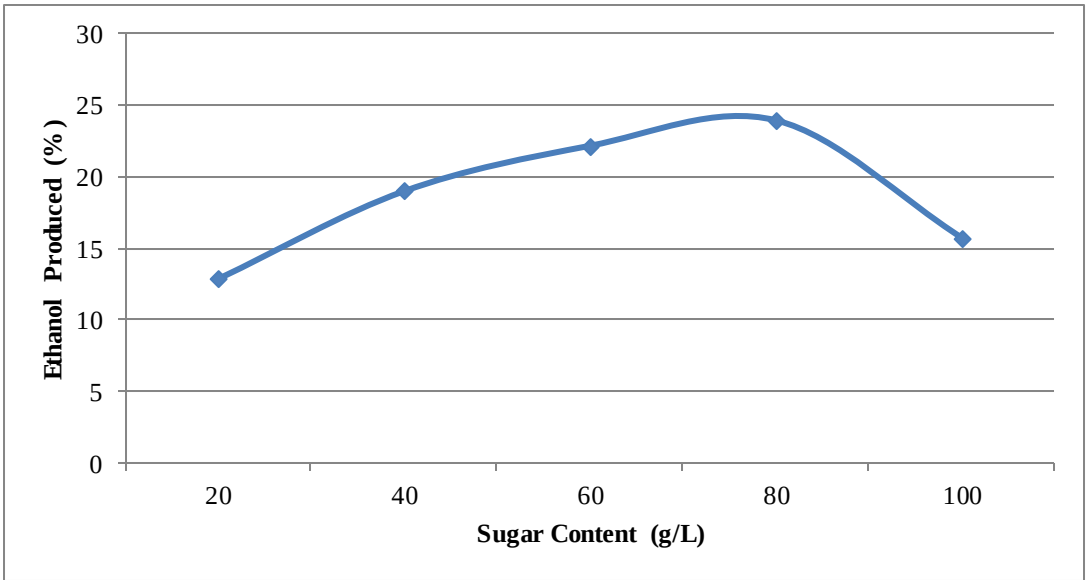

Figure 20 Effect of sugar content on ethanol produced

After the experiments of sugar content were carried out, experiments related to the type of sugar were performed as well. Five (5) types of sugar solutions were prepared to see the effect of types of solutions on the fermentation of xylose by the locally isolated *Bacillus subtilis*. Five types of sugar combinations prepared are which constituted monosaccharide and fermentation media. The different fermentation media's are as follows:

A- Fermentation solution consisted of 50 gL^{-1} of xylose.

B- Fermentation solution consisted of 50 gL^{-1} of glucose.

C- Fermentation consisted of 25 gL^{-1} of each xylose and glucose.

D- Fermentation solution consisted of 40 gL^{-1} of xylose and 10 gL^{-1} of glucose.

E- Fermentation solution consisted of 10 gL^{-1} of xylose and 40 gL^{-1} of glucose.

The following fermentation conditions were used for the above mentioned fermentation solutions: temperature 37°C, 3% of inoculation, a rotating speed of 150 rpm was maintained and a pH of 7.

Table 11 Effect of different type of sugar solution on ethanol produced

Solution Type	**Ethanol Produced (%)**
A	23.89
B	13.2
C	18.73
D	21.38
E	15.78

The above tabulated results in Table 5.7 show that the amount of ethanol fermentation was highest for the fermentation solution A (xylose 50 gL^{-1}). The trend for the above mentioned results can be seen in Fig. 7. By these experiments it was found that fermentation ability of the isolated Bacillus subtilis was better for xylose than its ability to ferment glucose.

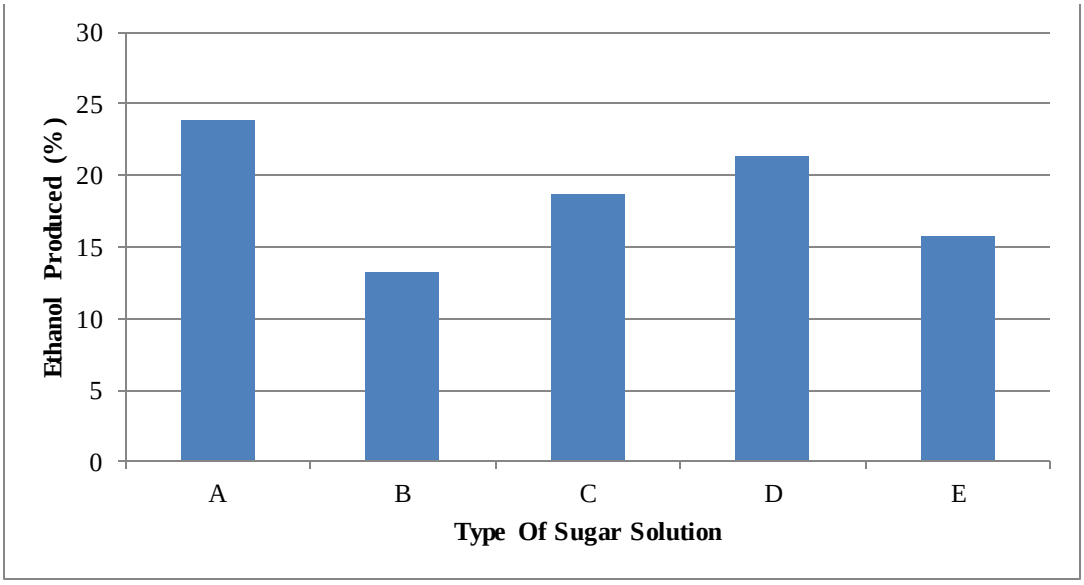

Figure 21 Effect of different type of sugar solution on ethanol produced

The maximum amount of ethanol produced was obtained from sugar solution A, this sugar solution consisted of 50 gL^{-1} of xylose. Upon completion of fermentation process 23.89% of ethanol was produced. This was the maximum amount of ethanol achieved from the five solutions, which were prepared for fermentation. Least amount of ethanol was produced from fermenting solution B, which had glucose as the only carbon source. This result shows that the locally isolated *Bacillus subtilis* possess only those enzymes, which are suitable for the fermentation of xylose instead of glucose. After solution A, solution D produced 21.38% of ethanol produced which is greater than remaining of the four solutions. As solution D consists of 40 gL^{-1} of xylose and 10 gL^{-1} of glucose, which is also an indication that the isolated *B. subtilis* is suitable for fermentation.

Summary

Parametric study was done by analyzing the leading parameters, primarily responsible in batch fermentation for bioethanol production. Optimization of pH, sugar concentration, fermentation temperature, fermentation time and type of sugar were studied and recorded in current chapter. Bacteria was investigated for the optimum production of bioethanol using xylose as the sole carbon source. Bacteria produced better yield at the optimum conditions mentioned in this current chapter by consuming higher sugar content.

As far as the incubation time is concerned, bacteria took 48 hours to complete the fermentation process. The isolated *Bacillus subtilis* follows the PPT pathway to convert the xylose into ethanol in which furfural is produced as the by product. As the process is carries on sugar is consumed and the ethanol is produced up to a specific amount, after achieving a limited amount of ethanol the bacteria become inactive as they are affected by ethanol produced. To achieve or to improve the amount of ethanol a mechanism is to applied to the fermentation set up, which will remove the produced ethanol so that the bacteria is not effected by ethanol produced.

Intracellular enzymes were used for the batch fermentation and done also the bioethanol analysis as in previous method. Results were achieved but not so effective. Enzymatic fermentation needs more innovation and mature technology to improve the efficiency of hemicellulose sugar (xylose) to ethanol, only then this fermentation process may be feasible for commercialization.

References

[1] J. Domínguez and N. Cao, "Ethanol production from xylose with the yeast Pichia stipitis and simultaneous product recovery by gas stripping using a gas-lift loop fermentor with attached side-arm," ... *Bioeng.*, 2000.

[2] L. Lacis and H. Lawford, "Effect of growth rate on ethanol production byThermoanaerobacterethanolicus in glucose-or xylose-limited continuous culture," *Biotechnol. Lett.*, 1988.

Conclusion & Recommendation

Xylose is a major part of lignocellulose biomass, which is present in abundance. Conversion of xylose into ethanol is still a key issue. The successful fermentation of xylose to ethanol will result in an increase in the overall efficiency of biomass to ethanol conversion process.

In this research study a xylose fermenting bacteria Bacillus subtilis was isolated from local soil and was identified by various biochemical tests.

The locally isolated *Bacillus subtilis* is capable of fermenting xylose and produces ethanol. By performing experiments it was found that its ability to ferment to xylose was greater than to ferment glucose. This was seen by performing experiments on different fermenting solutions consisting of different concentrations of xylose and glucose.

As a result of the experiments performed optimum fermentation conditions were determined, which are as: pH 7, a temperature of 37^{o}C, a rotating speed of 150 rpm was maintained and a inoculation amount of 3%. The maximum amount of xylose converted to ethanol was found to be approximately 0.24 g/g.

More innovation and mature technology is needed where the proper removal of ethanol can be ensured as the production of ethanol can affect the bacteria's capability to convert sugar to ethanol. Only then this fermentation process may be feasible for commercialization. By doing so then the proper utilization of biomass to fuel can be used in our daily life and overall can reduce the greenhouse effect and can move towards living a better life.

Annexure I

Ethanol Production from Xylose Using Locally Isolated Bacteria

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Abstract

The purpose of this study is to improve the conversion efficiency of lignocellulose to ethanol. This was achieved by converting the hemi cellulosic sugar xylose to ethanol. In this research a xylose fermenting bacteria was isolated from local soil sample. By performing different biochemical tests the isolated bacteria was found to be identical to *Bacillus subtilis*. The optimum fermentation conditions at 150 rpm for 48 h in a shake flask are as follows: pH 7.0, reducing sugar 78 gL^{-1}, inoculation amount 3% and temperature 37°C. The results indicated that a xylose to ethanol yield coefficient of 24% was achieved. At the same time the effect of different sugar concentrations was studied by making different mixtures of xylose and glucose. As a result, the bacteria's ability to ferment xylose was found to be greater than to ferment glucose. The isolation, preservation and usage of isolated *Bacillus subtilis* can help improve the conversion process of hemicellulose sugar such as xylose to produce ethanol.

Keywords: xylose, *Bacillus subtilis*, fermentation, ethanol

1. Introduction

Due to the rising need of fuel to power various sectors of the society, the world is turning its focus to alternatives fuels. These alternative fuels are not only cheap as compared to the present fossil fuels but also are environment friendly. These fuels include bio-ethanol and bio-diesel, which are the ultimate products from biomass. Biomass is an abundant resource, which can be used for the production of various fuels and chemicals. In various countries bioethanol is the leading biofuel being produced [1]. Studies show that emission of greenhouse gases (GHG) can be reduced by using bioethanol [2]. Ethanol is a fuel which can be used as alternative to fossil fuels; it is a clean and renewable energy resource [3]. Lignocelluloses have gained importance as they can be used as raw material for ethanol production [4].

The cost of lignocellulose such as cellulose waste, crop residue and municipal solid waste is less than those of starches. Due to its low price, it is available in large quantity [5]. The utilization of lignocellulose makes use of waste and is easily available throughout the world. By the action of acids and enzymes, lignocellulose materials are hydrolyzed during fermentation process. Sugars can be quickly produced from lignocellulose by dilute acid hydrolysis, which is a common but good method [6]–[8].

The hydrolysis of hemicellulose obtained from many plants produce Xylose as the major product. A plant material such as corn straw, has two main polymers, hemicellulose and cellulose, the conversion of these into fermentable sugars is easy, the hydrolysis of corn straw generate xylose, hexose and other byproduct compounds. The hexoses are easily fermented to ethanol whereas; fermentation of xylose is more problematic. For lignocellulose-ethanol fermentation to be successful the utilization of xylose is critical [9].

Xylose is present in abundance in lignocellulosic biomass such as forest industry waste [10] and corn stover [11]. The efficiency of biomass-ethanol fermentation process can be improved by successfully utilizing xylose. In the past, scientists have made various attempts to obtain a stable microorganism, which is proficient in utilizing xylose. In literature, it has been reported that fermentation strains that can ferment xylose have been separated, such as *Candida shehatae* [12], *Pichia stipites* [13], *Pachysolen tanniphilus* [14] and some genetically modified xylose fermenting yeasts [15].

However, fermentation by these strain microorganisms is poor. The growth and fermentation of these microorganisms was greatly influenced by the acid hydrolysis by products [16]. Xylose can be fermented by using many different bacteria and yeasts, due to formation of by products or slow conversion of xylose permits the economical application for production of ethanol.

Pakistan is generating molasses from 2 to 2.5 million tons each year. About 80% of these molasses are being exported annually overseas [17] and the remaining molasses are locally used for producing bioethanol. According to reports this bioethanol produced can account for 2 to 3% of the transportation fuel used annually [18]. Ethanol is being produced only by utilizing cellulosic sugars in molasses. Hemicellulose sugars such as Xylose, Arabinose etc. are not being considered for producing bioethanol. By utilizing sugars such as Xylose, the amount of ethanol production can be increased many folds.

In Pakistan, research has not been carried out to produce ethanol from xylose using local bacterial strain. In this research study, attempt was made to isolate local bacteria from soil for conversion of xylose to bio-ethanol. The study also aimed at analyzing fermentation conditions under varying pH and temperature levels for identifying the optimum conditions.

2. Materials and Methods

2.1 Soil sample

The fermenting strain was isolated from local soil sample taken from soil under a shrub located at Islamabad campus of NUST, Pakistan. Approximately 5gm of soil sample was collected by using a clean and dry polythene bag by the help of a sterilized spatula.

2.2 Incubation

The sample was then transferred under sterile conditions to lab where nutrient broth was prepared. The sample collected was added to the broth and then kept for incubation at 35^{o}C and for a time period of 24 hours.

2.3 Streaking

After the 24 hours of the incubation period, supernatant of 0.1 mL from the tube containing the soil sample was inoculated on to plates for the streaking process at 30^{o}C for another 24 hours. The plate consisted of nutrient agar and xylose which served as the sole carbon

source. After incubation the plate was then examined and suspected colonies were chosen for the Gram staining method. Gram positive, rod shaped and the spore forming bacilli was selected for further identification tests.

2.4 Identification of Locally Isolated Bacteria

Identification of the microorganism was done by performing some common physiological and biochemical tests. The basis of the performed conventional techniques was based on Bergey's manual for the identification of bacteria [19]. By the results obtained the bacteria was identified as *Bacillus subtilis*. A summary of the tests performed and the results obtained are as follows:

Table 1 Tests performed for the identification of locally isolated bacteria

Test Performed	Result Obtained
Gram Staining	+
Spore Forming	+
Strict Anaerobes	-
Starch Hydrolysis (Amylase)	+
VP Test	+
Cell Diameter $\geq 1\mu m$ (width)	-
Citrate Test	+
NaCl Growth	+

After identification of the locally isolated bacteria the fermentation of xylose under conditions of various pH mediums and at different temperatures were measured and ethanol yield was analyzed to determine optimum fermentation conditions.

3. Fermentation Experiments

The preserved isolated bacteria was activated in a culture medium and then inoculated into sugar solution with a specific amount of xylose which served as the carbon source in shaking flasks. After a time period of 48 h and a rotating speed of 150 rpm, the concentrations of residual sugar and produced ethanol concentrations were measured. The effects of pH (3-8), temperature (25-45^{o}C), sugars concentrations and types of sugar were studied. At the same time, fermentation experiments of glucose were also carried out as a comparative study of xylose and xylose fermentation. The efficiency of xylose fermentation was represented by percentage of sugar consumed and yield of sugar to ethanol conversion.

4. Analytical Methods

4.1 Standard Curves

Colorimetric methods [20], [21] were used to determine both residual sugar (xylose) concentration and the concentration of ethanol yield respectively. For this purpose before experiments, standard curves were developed for both xylose and ethanol using standard concentration solutions. Fig. 1 shows the standard curves of both xylose and ethanol by using standard concentration solutions.

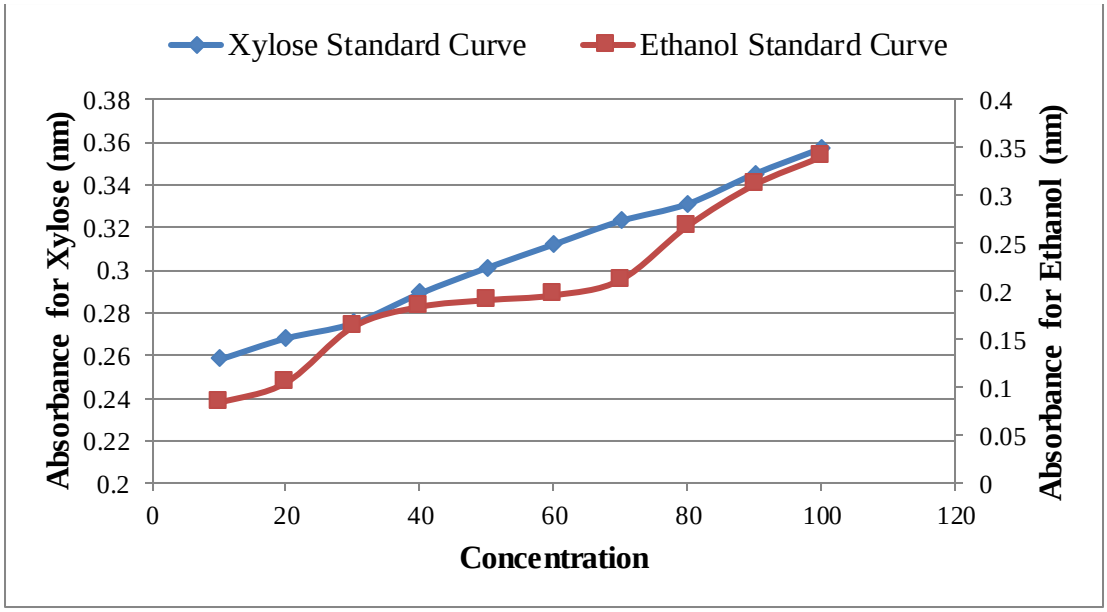

Figure 1 Standard curves for both Xylose Standard curves for both Xylose and Ethanol using standard concentration solutions

4.2 Residual Sugar (Xylose) and Ethanol Produced

Using the colorimetric method [21] ethanol amount (volume, v/v) in the distilled fluid was determined. The concentration of residual sugar (xylose) was also measured by colorimetric method [20]. The wavelengths of both ethanol and xylose solutions obtained from the photo spectrometer were compared with the standard curves of each, which were made by using known standard concentration solutions.

The pH was measured using pH meter (LIDA PH5-3CW Microprocessor pH/mV Meter). The quality of the ethanol produced was the ethanol yield produced by consumption of per gram of sugar. The optical density (OD) and cell growth were noted after regular intervals of time at a wavelength of 600 nm by using a spectrophotometer (Hitachi U-1100).

The percentage of residual sugar (xylose) and ethanol produced was determined by using the standard curves. Whereas, sugar consumption in xylose fermentation experiments was determined by the following formula:

Equation 1 Percentage of residual sugar

$$Sp = (S1 - S2)/S1$$

Where,

S_p = percentage of sugar consumption

S_1 = sugar concentration (gL^{-1}) before fermentation

S_2 = sugar concentration (gL^{-1}) after fermentation.

5. Results and discussions

There are many factors on which the fermentation of xylose depends upon. These factors include pH, temperature, sugar content and types of sugar.

5.1 Effect of pH on Xylose Fermentation to Ethanol

The growth of microorganism in the process is greatly influenced by pH of the solution, as pH may be a reason for the change in charge of the cell membrane, as a result of which permeability of the membrane changes. At the same time, ionic substances required for the growth of microorganisms are affected by the change in pH as it may lead to a change in the degree of ionization. As a result of this, both absorption of nutrients and the growth of microorganism will be affected. Also, the enzymes involved in the microbial physiological and biochemical activity are also affected by the change in pH of the intracellular environment. Therefore, pH of the medium plays a crucial role in the bacterial growth.

Ethanol yield and the fermentation rate of the process were considerably influenced by change of pH of the medium. For the optimization of pH in the fermentation process, effect of pH on the ethanol concentration can be seen in Fig. 2. First the amount of residual sugar (xylose) was measured by the above mentioned colorimetric method. Then amount of residual sugar and ethanol concentration was measured after the fermentation process at 48 h. Whereas, fermentation conditions for the process were as: inoculation 2%, temperature for the process was maintained at 35°C with a rotating speed of 150 rpm and

an initial sugar concentration of 100 gL^{-1} was taken. The amount of sugar consumed was calculated by subtracting the amount of residual sugar from total amount of sugar before the fermentation process.

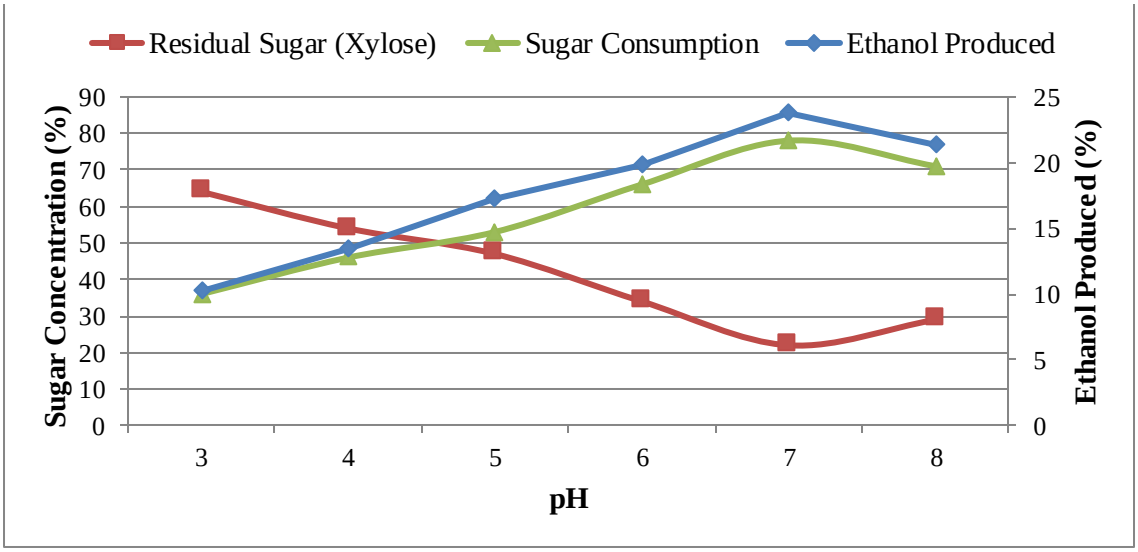

Figure 22 Effect of pH on the residual sugar (Xylose), sugar consumption and on ethanol produced

The pH varied from 3 to 8, by looking at Fig. 2 a pH of 7 was selected to be a suitable pH value for the fermentation process. At a pH of 7 highest yield could be achieved as at this pH consumption of sugar was the highest. At lower pH levels the concentration of acids will inhibit cell fermentation. Whereas, higher pH levels will help in the senescence of microorganism cells and the fermentation rate would eventually decrease. At the same time, the chances of contamination at higher pH levels increased [13], [14].

5.2 Effect of Temperature on Xylose Fermentation to Ethanol

For the survival of microorganisms temperature is an important factor. The effect of temperature on the isolated *Basillus subtilis* was analyzed. Temperature was varied from 25°C to 45°C. The fermentation conditions are as: amount of inoculation 2%, pH 7, a rotation speed of 150 rpm was maintained and an initial sugar concentration of 100 gL^{-1}.

After a time period of 48 h residual sugar concentration and concentration of ethanol produced were determined. Fig. 3 shows the effect of temperature on amount of residual sugar (xylose) and the amount of ethanol produced after the fermentation process. The

amount of sugar consumed was calculated by subtracting the amount of residual sugar from total amount of sugar before the fermentation process.

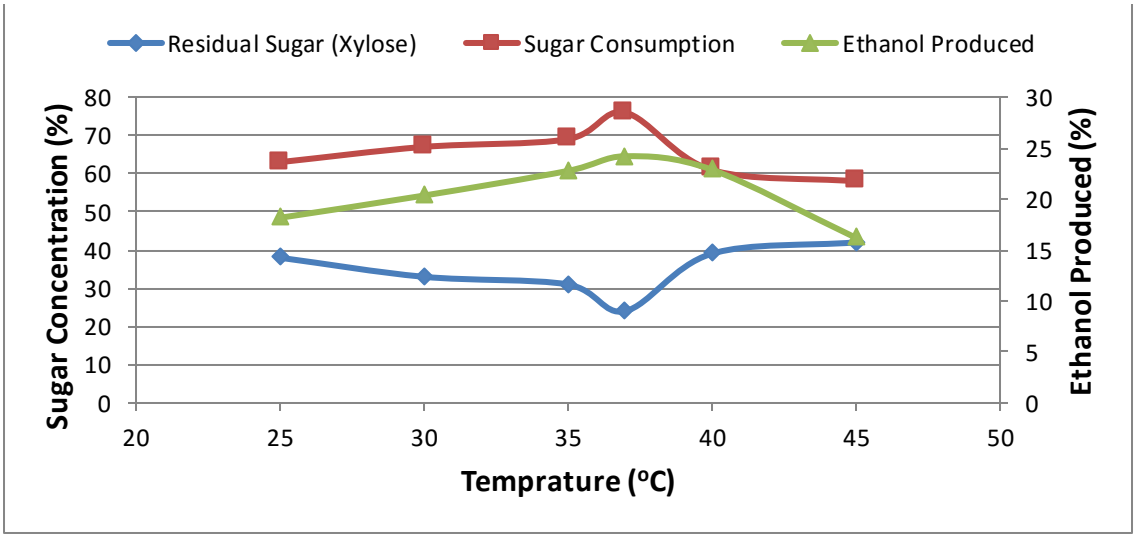

Figure 3 Effect of temperature on residual sugar (xylose), sugar consumption and ethanol produced

The rate of enzymatic reaction is changed by change of temperature, which also affects the activity of enzymes. Enzymatic reaction rate will be increased and as a result of which metabolism rate and rate of growth of the microorganism will increase within a proper temperature range. At a suitable temperature, growth and reproduction of microorganisms is the fastest. Microorganisms can be divided into groups such as mesophilic bacteria, psychrophilic bacteria and thermophilic bacteria according to the optimum temperature required for their growth. Transport of substance and fluidity of cell membrane are also affected by temperature and as a result of which it has an effect on the absorption of nutrients as well as on the secretion of metabolites. In this research study, at a temperature of 37°C the sugar to ethanol conversion was the highest up to 24%. According to study reports, the ability of bacteria to produce more ethanol can be improved at higher temperature, which can be observed from Lacis's research [22].

5.3 Sugar Content and Type of Sugar

Ethanol production is crucially affected by sugar content. Fermentation mediums consisting of different concentrations of sugar were made and fermentation process was carried out to produce ethanol. The amount of residual sugars left and the amount of ethanol were noted after a time period of 48h. Following are the fermentation conditions

used: 3% of inoculation, pH7, a rotating speed of 150 rpm was maintained and a temperature of 37°C was set. The concentration of the different media's ranged from 20 to 100 gL^{-1}. As seen in the Fig. 3 below, after the maximum concentration of ethanol the next value decreased. The optimum value of sugar content for the fermentation process was approximately 78 gL^{-1}.

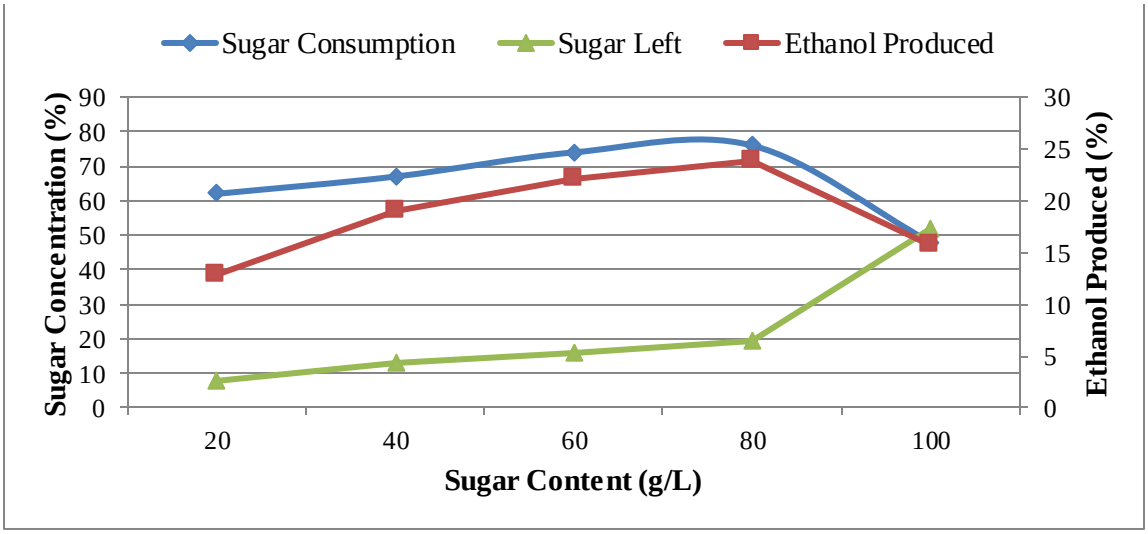

Figure 4 Effect of sugar content on residual sugar (xylose), sugar consumption and ethanol produced

The effect of the types of sugar was also studied in this research. Five types of sugar combinations were prepared, which constituted monosaccharide and fermentation media. The different fermentation media's are as follows:

A- Fermentation solution consisted of 50 gL^{-1} of xylose

B- Fermentation solution consisted of 50 gL^{-1} of glucose

C- Fermentation consisted of 25 gL^{-1} of each xylose and glucose

D- Fermentation solution consisted of 40 gL^{-1} of xylose and 10 gL^{-1} of glucose

E- Fermentation solution consisted of 10 gL^{-1} of xylose and 40 gL^{-1} of glucose

The following fermentation conditions were used for the above mentioned fermentation solutions: temperature 37°C, 3% of inoculation, a rotating speed of 150 rpm was maintained and a pH of 7.

The results in Fig. 5 show that the amount of ethanol fermentation was highest for the fermentation solution A (xylose 50 gL^{-1}). By these experiments it was found that fermentation ability of the isolated *Bacillus subtilis* was better for xylose than its ability to ferment glucose.

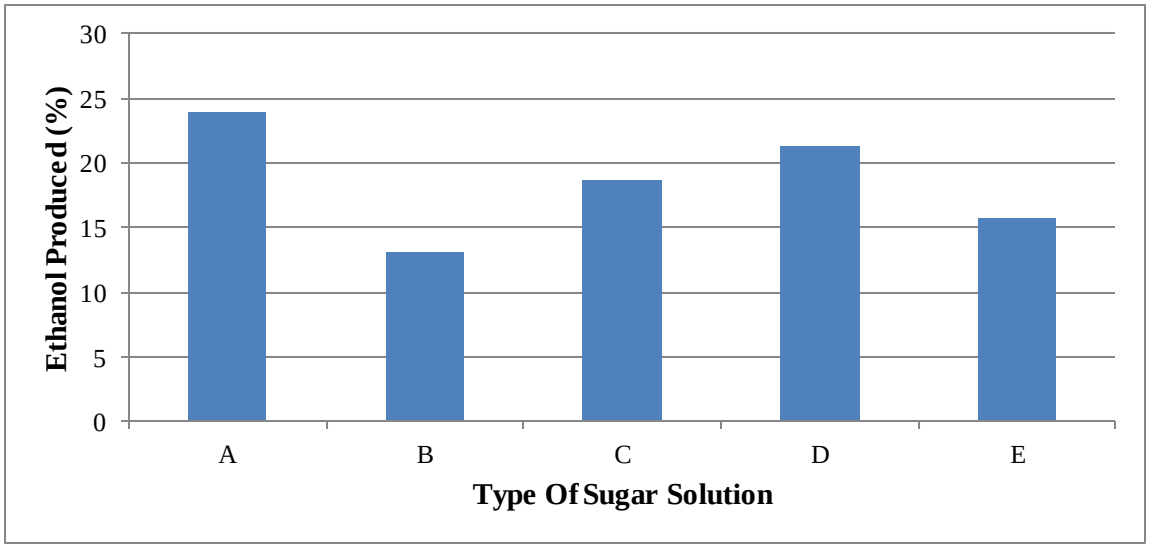

Figure 5 Effect of different type of sugar solution on ethanol produced

6. Conclusions

Xylose is a major part of lignocellulose biomass, which is present in abundance. Conversion of xylose into ethanol is still a key issue. The successful fermentation of xylose to ethanol will result in an increase in the overall efficiency of biomass to ethanol conversion process.

In this research study a xylose fermenting bacteria *Bacillus subtilis* was isolated from local soil and was identified by various biochemical tests.

This isolated bacteria is capable of fermenting xylose and produces ethanol. By performing experiments it was found that its ability to ferment to xylose was greater than to ferment glucose. This was seen by performing experiments on different fermenting solutions consisting of different concentrations of xylose and glucose.

As a result of the experiments performed optimum fermentation conditions were determined, which are as: pH 7, a temperature of 37°C, a rotating speed of 150 rpm was

maintained and an inoculation amount of 3%. The maximum amount of xylose converted to ethanol was found to be approximately 0.24 g/g.

7. Acknowledgments

The authors of this are very thankful to the Institute of Microbiology, University of Agriculture Faisalabad for their support and also for allowing us to use their labs for this research study.

References

[1] D. Mousdale, *Introduction to biofuels*. 2010.

[2] S. Lee and Y. Shah, *Biofuels and bioenergy: processes and technologies*. 2012.

[3] S. Mohana, B. K. Acharya, and D. Madamwar, "Distillery spent wash: Treatment technologies and potential applications," *J. Hazard. Mater.*, vol. 163, no. 1, pp. 12–25, 2009.

[4] O. Sanchez and C. Cardona, "Trends in biotechnological production of fuel ethanol from different feedstocks," *Bioresour. Technol.*, 2008.

[5] P. Renewab and G. Britain, "transformation could be made very , but most of the products are produced in much smaller quantifies than the example given in Table 1 . Table 1 Approximate World Production of Some Bio-technical Products Products Ethanol Tons / year SCP Amino acid Citric," vol. 5, pp. 866–874, 1994.

[6] M. Khiyami, A. Pometto, and R. Brown, "Detoxification of corn stover and corn starch pyrolysis liquors by Pseudomonas putida and Streptomyces setonii suspended cells and plastic compost support biofilms," ... *food Chem.*, 2005.

[7] K. Kadam, L. Forrest, and W. Jacobson, "Rice straw as a lignocellulosic resource: collection, processing, transportation, and environmental aspects," *Biomass and Bioenergy*, 2000.

[8] J. Nigam, "Ethanol production from wheat straw hemicellulose hydrolysate by Pichia stipitis," *J. Biotechnol.*, 2001.

[9] Y. Sun and J. Cheng, "Hydrolysis of lignocellulosic materials for ethanol production: a review," *Bioresour. Technol.*, vol. 83, pp. 1–11, 2002.

[10] H. Sreenath and T. Jeffries, "Production of ethanol from wood hydrolyzate by yeasts," *Bioresour. Technol.*, 2000.

[11] D. Schell, C. Riley, N. Dowe, and J. Farmer, "A bioethanol process development unit: initial operating experiences and results with a corn fiber feedstock," *Bioresour. ...*, 2004.

[12] "Growth, death, and oxygen uptake kinetics of Pichia stipitis on xylose," *Biotechnol. ...*, 1991.

[13] J. Domínguez and N. Cao, "Ethanol production from xylose with the yeast Pichia stipitis and simultaneous product recovery by gas stripping using a gas-lift loop fermentor with attached side-arm," ... *Bioeng.*, 2000.

[14] W. Zhang, A. Bai, X. Chen, and G. Wei, "Ethanol Production from Lignocelluloses Hydrolyzates with Immobilized Multi-microorganisms," *Energy Sources, Part A Recover. Util. Environ. Eff.*, vol. 34, no. 13, pp. 1206–1212, Apr. 2012.

[15] N. Ho, Z. Chen, and A. Brainard, "Genetically engineered Saccharomycesyeast capable of effective cofermentation of glucose and xylose," ... *Environ. Microbiol.*, 1998.

[16] W. Zhang and G. Wei, "The Effects of Lignocellulose Acid-hydrolysis By-products on Ethanol Fermentation of Xylose," *Energy Sources, Part A Recover. Util. Environ. Eff.*, vol. 34, no. 13, pp. 1178–1186, Apr. 2012.

[17] S. Qureshi, A. Faruque, A. Q. Khattak, A. E. Hasham, and C. M. Shakeel, "ANNUAL REPORT 2013 MANAGEMENT OF Mr . Muhammad Khan Saddozai Chairman Vice Chairman Vice Chairman Vice Chairman Member Member Member 24-D , Mezzanine Floor , Rashid Plaza , Jinnah Avenue , Islamabad Fax : Website : www.psmacentre.com ANNUAL REPORT 2013 Mr," 2014.

[18] "Solutions for Energy Crisis in Pakistan i | Ehsan Ali - Academia.edu." [Online]. Available: http://www.academia.edu/5748061/Solutions_for_Energy_Crisis_in_Pakistan_i. [Accessed: 11-Sep-2015].

[19] D. Bacteriology, G. Negative, F. A. Gram, F. Enterobacteriaceae, G. C. Key, E. Gram, N. Gram, N. Gram-positive, W. G. Nonsporlating, A. Fast, and S. Rods, "Gram Stain & Morphological Flowchart," *Differentiation*, pp. 1–8.

[20] T. J. Eberts, R. H. B. Sample, M. R. Glick, and G. H. Ellis, "A simplified, colorimetric micromethod for xylose in serum or urine, with phloroglucinol," *Clin. Chem.*, vol. 25, no. 8, pp. 1440–1443, 1979.

[21] S. Sumbhate, S. Nayak, D. Goupale, A. Tiwari, and R. S. Jadon, "Colorimetric Method for the Estimation of Ethanol in Alcoholic-Drinks," vol. 1, pp. 1–6, 2012.

[22] L. Lacis and H. Lawford, "Effect of growth rate on ethanol production byThermoanaerobacterethanolicus in glucose-or xylose-limited continuous culture," *Biotechnol. Lett.*, 1988.

Annexure II

Algae to Ethanol - A Review

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Abstract

Population outburst together with the increment in mechanization has led to a dramatic boost in the fuel demand. Hence Ethanol proofs to be a promising alternative energy source and is a major chemical used in beverages, chemical industries, a component in fuels, such as reformulated gasoline. This review explores the characteristics of Ethanol as fuel, Zymmomonas as the only potential bacteria for ethanol production and algae as a potential Lignocellulosic biomass and impact of algal biomass pre-treatment for sugars' extraction as an initial step in the production of bioethanol. Pretreatment of lignocellulose materials to remove lignin and hemicellulose can fundamentally improve the hydrolysis of cellulose. The main challenges in the production of ethanol from lignocellulose materials are the high cost and low yield of the hydrolysis process. Extensive research efforts have been made to improve the hydrolysis of lignocellulose materials. The purpose of this study was to investigate the overall process parameters of pretreating algae for ethanol fermentation by Zymomonase mobilis bacteria .In this study, the strategy of algae pretreatment techniques followed by acid-base hydrolysis is focused to improve sugar concentration because the hydrolysates contained glucose, mannose, galactose, and mannitol, among other sugars, at different ratios. After that, the extracted sugar is inoculated with zymomonase bacteria to produce ethanol from local algal biomass as Algae have a tendency to have a much different makeup than does most feed stocks used in ethanol, such as corn and sugar cane. Ethanol from algae is possible by converting starch and cellulose. Some prominent strains of algae that have high carbohydrate contents are promising from ethanol production.

Keywords: Ethanol, Zymomonase, hydrolysate

1. Introduction

1.1 Energy Outlook

At the turn of century, there has been a worldwide rising interest in the use of biofuels in order to replace petroleum [1], every country is ferreting about harnessing renewable energy sources that could not only replace fossil fuels but also help in the mitigation of carbon dioxide in accordance with Kyoto protocol [2] . The imposition of ethanol derived from different biomasses especially for the purpose of blending in gasoline would make nations less reliant on current petroleum sources, this would help underdeveloped and developing nations to save foreign exchange reserves, improve their rural economies by providing job opportunities to the natives in a clean and safe environment. The key drivers for effective commercial ethanol production are cheap raw materials, economic pretreatment technologies, in-house cellulase production with high and efficient titers, high ethanol fermentation rates, downstream recovery of ethanol and utilization of by-products to its maximum [3]. Petroleum is the largest single source of energy in USA(40%) and in world (35%) [4] .Because almost 70% of the global carbon monoxide and 19% of global carbon dioxide emissions are from vehicles that are running on petroleum fuel ,Thus Biomass emerge as one of the most promising renewable source for generating biofuels and contributes almost 13% of global energy supply [5]. The increase in Carbon dioxide emission is another factor that increased the importance of Biofuels as potential carbon dioxide consumers [6]. Bioethanol is one of the most widely used fuel amongst all biofuels as used for transportation worldwide because of its high octane number[7]. Thus production of ethanol can help in mitigating both energy crisis as well as environmental problems [8] since carbon cycle has unavoidable close loop in this case[1]. The potential of alcohol as a fuel remain avoided because of low prices of gasoline but 1970 era of global oil crisis urged its importance once again [1]. For this very purpose, biomass is pretreated which aims at easy access to the three major components of biomass includes cellulose, hemicellulose and lignin [9]. Along these lines, Incrementation of its share in the world matrix will automatically help in prolonging the reserves of fossil fuels , addresses the threats postured by rapid climatic change, and empower better security and vitality of the energy supply on a global level [10].

1.2 Bioethanol is Not New

Bioethanol Is not an innovative venture in today's era .Since in 1908, Model T-Frod in USA was the first car that run on bioethanol and until now, 15 Millions such cars are running on ethanol. UK runs its first car on bioethanol in 1930 named: Cleveland Discol. And Brazil started this technology through Proacool Program [11]. it had made such a wonderful progress by now, that they are able to design flex fuel engines cars that are running on 100% ethanol blend and provide 40% of transportation fuel [12]. In average 73% of the world ethanol produced are used for fuel purposes [13]. 11 Giga liters per year of ethanol is produced in brazil from sugar cane [14].Hence bioethanol production is economical in Brazil because Brazil constitutes 27% of global sugarcane producer [8] .In U.S, 90% of ethanol is derived from corn [15].Thus where India, US, UK and Brazil are using ethanol in their aircraft, cars and tractors, Pakistan ,although an energy deficient country is still far behind in this race. Pakistani state oil has recently developed E10 pilot scale program to enhance ethanol use since its spending around US$6.65 billion per annum for importing oil [16].

1.3 Bioethanol Fuel Properties

Ethanol widely known as ethyl Alcohol or grain alcohol and belongs to the group of Aldehyde [13] with formula CH_3-CH_2-OH .It's an oxygenated fuel with almost 35% oxygen content. Thus it is used as an oxidative agent in fuel. It has a motor octane number of 98 which exceeds that of gasoline having an octane number of 80 [17]. It also reduces NO_x and particulate emission from car engines during combustion. Furthermore, ethanol has higher flame speeds, broader flammability limit and higher heats of vaporization as compared to gasoline. These enhanced properties of ethanol as compared to gasoline leads to higher compression ratio, shorter burn time and leaner burn engine, which brings about the theoretical efficiency advantages over gasoline in an internal combustion engine [2]. The value of its octane number is around 108 and auto ignition temperature of 606 K as compared to methanol as 112 and 737K respectively [18]. Thus the presence of ethanol causes modification in the physio-chemical properties of fuel after being blended in the fuel. These modifications include flash point , low heat content, viscosity, lubricity, pour point [19][20]. While it has some Disadvantages over gasoline which includes lower

energy density than gasoline , corrosiveness, low flame luminosity, difficult in making cold start, miscibility with water, and toxicity to ecosystems [21].

Table 1 Properties of ethanol produced [22]

Properties	**Requirement**
Ethanol Yeild	>90%
Ethanol productivity	>1g/l/h
Ethanol tolerance	>40g/l
Growth requirement	Inexpensive, medium formulation
Culture growth conditions	Acidic PH, high temperatures

2. Bioethanol Feedstock and Substrate

The lignocellulosic biomass would be best utilized for ethanol production because of its narrow range of competition with food production , land use and minimum deforestation [23]. The feedstock used for ethanol production are mostly wheat , corn, barley, rice husk, algae, paper, saw dust, sugarcane beet, paper , saw dust, straw, spent grain, corn steep liquor , bagasse, corn fiber , MSW, cheese whey, agave etc. and substrates are starch hydrolysate (maltose and glucose), inulin hydro lysate (fructose), laminarin, lactose, starch, xylose, arabinose, cellubiose etc. [11].

2.1 Algae as a potential source of bioethanol production

There has been a rising concern about algal mass culture and fuel production these days because the production of biofuels from crop seeds have come under major controversy due to its food vs. fuel competition [24]. According to US DOE report, microalgae have the potential to produce 100 times more oil per acre land than by any other terrestrial plant. Algae is considered to be as one of the oldest form of phytoplankton specie on earth [25]. These are simple aquatic plants ranging from single-celled microalgae to macro-algae as large seaweeds. Cultivation of algae for biofuel production can be done by oil extraction using mechanical tools; and by applying algal biomass to various biomasses to fuel conversion technologies such as fermentation and anaerobic digestion. This algal biomass is a potential source of biofuel production in the light of fact high productivity of algae [26]**.** Fermentation process of microalgae is given as follows:

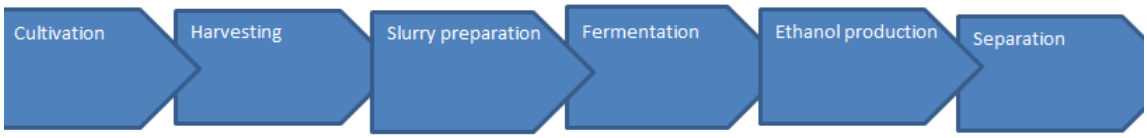

Figure 1 Algae fermentation process [27]

The actual global oil production in 2007–2008 from oilseed crop was 0.592 t ha^{-1} for that year [28]. The 365 t ha^{-1} a^{-1}productivity in the Algae Link bioreactors equates to 153.3 t ha^{-1} A^{-1} oil produced, which is about 259 times better productivity than the actual terrestrial oilseed crops. For open ponds, ~300 t ha^{-1} A^{-1} dry weight producing an algae containing 20–30% oil, production is 100–150 times greater and for continuous high rate ponds, assuming 20–30% oil content for the species that dominate them, 17–25 times. On average therefore the 30 times more productive claim is well justified with regard to oil production [29]. Thus microalgae are the only economic route to biodiesel and ethanol [30]. Since it's easy to disrupt algal cell walls as compared to other lignocellulose materials. The ethanol yield of 11mg is reported from 1 g of dry weight algae [31].

Table 2 Biomass yield of microalgae as compared to other crops [32]

Crops	Yield in ton/ha
Microalgae	1600
Sugarcane	125
Sweet potato	4.0
Crop residues	50
Soyabean	40

2.2 Algae Applications and Determination of its Composition

The first use of algae was by Chinese 2000 years back, who used Nostoc to survive their famine [33]. Chemical composition is a key factor that could be effective in enhancing the yield . Algae have consistent chemical composition [34]. Its biomass will be highly yielding and have low cost .Algae is a high protein biomass which grows mostly on saline lands[35]. The high content of protein results in low C/N ratio [25]. Algae also contains

amino acids. Starch, glucose, sugars and polysaccharides are some of the carbohydrates that are generally found in algae. These carbohydrates have high overall digestibility and have 70% of lipid content .Because of its favorable composition, it is used as a nutrition in human food, animal feed, aquaculture, bio fertilizer, polyunsaturated fatty acids and as recombinant proteins etc. [25]. A typical chemical composition of lignocellulosic materials is given below in Table 3 [36]**.**

Table 12 Chemical composition of lignocellulosic materials

Elements present	**wt.%**
Oxygen	45 wt. %
Carbon	48wt. %
Hydrogen	6wt. %
Inorganic matter	~ 1wt.%

3. Algal Growth conditions and challenges

To commercially employ algae, there is a need to identify different algal strains and optimum conditions which includes Temperature, PH, nutrients and sunlight [37].

3.1 pH

The pH range for most algal species is between 7 and 9 [38]. Continuous checking and maintenance of pH is necessary because during algal growth, many intercellular processes cause disruption of cells or utilization of nutrients which causes pH changes. pH can be maintained by addition of acid or base. In highly dense algal culture, the increased pH can be maintained by the addition of carbon dioxide, which may maintain pH up to 9 as a result of the CO_2/HCO_3- balance.

3.2 Aeration and mixing

Blending or Aeration is carried out with a specific end goal to avoid sedimentation of the algae and to guarantee that all cells of the growing population are equally exposed to the nutrients and illumination as to avoid thermal stratification process [38].

3.3 Temperature

Most micro-algae cultured species are best grown at temperatures between 16 to 27°C [39]. Because lower temperatures hampers the growth process, while increase in temperature could be lethal and proofs deadly for most of the algal species [38] . Subsequently to keep up temperature conditions, algal cultures can be cooled down either by stream of flowing water over the surface of the culture vessel or by controlling the surrounding air temperature with refrigerated air ventilating units.

3.4 Salinity

Marine phytoplankton is extremely resistant to the in salinity. The salinity level which is more appropriate for most of the algal species is at slightly lower than its native saline condition. The reduction in salinity can be acquired by diluting the marine water with the tap water. The optimal salinity range is 20-24 $g.L^{-1}$ [38].

4. Types of Algae Growth Systems

Algal growth systems are of two types which are either open pond systems or closed systems. Open pond systems include raceway pond, Aquariums or in large flasks, tubes, fiber glass, plastic tanks, polyethylene bags supported with inside steel mesh. Etc. Open ponds are shallow and nutrients to algae is provided by nearby and surrounding atmosphere and water is kept in motion by Paddle wheel [32] while Close pond systems include PBR, where algae is densely grown . PBR can be flat PBR, column PBR, tubular PBR etc. which provides control over algal growth conditions including temperature, illumination, contamination etc. Since Scientists are able to grow genetically modified strains of algae so once it is cultured properly, conventional methods are used to harvest algae includes centrifugation, froth floatation, froth fractionation, membrane filtration and ultrasonic separation , but most of these methods have technical and economic challenges such as high cost, toxication and non-feasibility to scale them up [32].

4.1 Economic analysis of algal systems

Determination of cost of algal system plays a very important role in its economical application. The cost factors to be focused more are illumination conditions and mixing. Moreover, costs of photosynthetic efficiency of systems, its medium cost and carbon dioxide costs also play a paramount role in determining overall economy of the system. Under these mentioned factors, the Optimization of production resulted in a price of €

0.68 per kg. Similarly the 3 plants as mentioned below in table have a very different operating economy with respect to mixing and overall plant cost [41].

Table 13 Algal production systems and their cost

Algal production types	**Mixing cost € per kg**	**Cost € per kg**
Open ponds	0.08	4.95
Horizontal tubular photo bioreactors	1.27	4.15
Flat panel photo bioreactors	3.10	5.96

4.2 Algae Culture Management Conditions

The diameter of cultural vessel is important for improving the yield as the cell density decreased with increased diameter of culture vessel for fixed level of illumination. Similarly internally illuminated cultures also help to improve productivity. No doubt they are costly to construct but cheap to operate. The productivity of algae is determined through total number of algal cells counted each day. So the basic culture conditions are almost the same for every strain. But the main difference is the water used for culture medium. The yield must be maintained for long time and have high incubation time to maintain the hatchery output of the fully grown algae [42].

5. Algal fuel conversion technologies

5.1 Pretreatment methods

Like plants, many algal biomasses has rigid cellulosic wall and contains starch as their necessary carbohydrates. Approximately 20-30% of total marine algal biomass contains cellulose or starch [50]. The marine algae approximately contains 70% cell wall polyssaccrides which contains cellulose, mannan hemicellulose and xylan [51]. As cellulose is a linear polymer while lignin which act as a seal , is a complex 3-D polymer, The breaking of the cellulosic and lignin walls in order to extract the sugars from algal biomass is the basic requirement [52]. So the first step is the pretreatment of the biomass to break cell walls and to reduce the crystallanity of biomass. Moreover temperature, incubation time, reagent concentration plays a very important role in improving glucose

release[53]. Following are some of the methods used for the purpose of pretreatment of algae.

Gamma rays irradiation

Gamma irradiation has been considered as a compelling strategy for the depolymerization of complex polysaccharides. In this technique, biomass is illuminated at different irradiation doses of 0, 10, 50, 100, 200 and 500 kGy and then hydrolyzed using sulfuric acid. Microscopic analysis is used to study the impact of gamma irradiation. This analysis gives the data regarding structural changes and reducing sugars [55]

Super critical fluid technology

Near critical water is also used for the pretreatment and partial hydrolysis of lignocelluloses materials. For this purpose; CO_2 is dissolved in water at near critical temperature and pressure as a catalyst. Enzymatic catalysis is done for further hydrolysis after pretreatment. The resulting ethanol after fermentation is processed by multistage supercritical carbon dioxide extraction in a counter-current fashion up-to 99.8 wt. % of ethanol. The residues could also be employed to the second stage hydrolysis to improve yield [56].

Ultrasound assisted lime pretreatment

Pretreatment helps in removal of 64% to 68% of lignin with the good recovery of residue and sugars. This technique exploits ultrasound rays for the pretreatment alongside lime in order to augments the delignification proportion. The range of expected ethanol yield is between 0.32 to 0.43 for most of the scenerios .[57] . GC-MS analysis results have demonstrated that this method not only influenced the chemical compositions of bio-oils but also helps in the improvement of 5-hydroxymethylfurfural (HMF) content in most of the heavy oils.[58]

Acid hydrolysis

For acid hydrolysis, different concentration of acid were tested for sugar extraction including, 2% v/v ,5%v/v, 10%v/v,12%v/v. 15%v/v at different temperature from 50 °C to 121°C for at least 3 hours [59]. Less severe conditions are required to break hemicellulose oligomers to glucose as compared to cellulosic oligomers because cellulose

is a crystalline but hemicellulose is amorphous in nature. Normally increasing acid concentration and temperature helps in improving sugar yield but this is not the case in most of the time.[9] Sometimes, there seems a trade off in maximizing the glucose yield by using severe conditions of acid and temperature as it may lead glucose to degrade into HMF, levulinic acid or formic acid.[60]

Alkaline Hydrolysis

This is done using NaOH, $Mg(OH)_2$, $Ca(OH)_2$, or KOH, of same concentration and temperature conditions for cell disruption. The use of alkali helps in degradation of glycosidic side chains and esters which results in decraystallization of cellulose and structural alteration of lignin [61]Although alkali pretreatment is a harsh method and extracted glucose are tested by Fehling test [62].

Table 14 Ethanol and Glucose yield at different temperature and alkaline pretreatment conditions [63]

Assays	Temperature	NaOH %(w/v)	Pretreatment time(min)	Ethanol yield %(%g ethanol/g algae)	Glucose yield(mg/g)
1	120	0.75	30	26.13	350.13
2	80	2	60	23.75	272.93
3	120	0.75	60	23.37	176.37
4	80	2	30	22.14	186.08
5	80	0.75	60	21.26	285.00
6	140	1	45	20.94	267.06
7	100	3	45	10.94	240.11

Enzymatic Pretreatment

Treating algae with enzymes help in releasing starch which then converted into glucose without undergoing physical pretreatment techniques. Various conditions in the liquefaction and saccharification processes, such as temperature, enzyme concentration, pH, and residence time, have been investigated to obtain an optimum product using orthogonal analysis [64].However the largest obstacles for applying enzyme is the cost

especially to the industrial processes where main goal is to make product as cheaper as it could be [65]

Zymomonas Mobilis Use for Fermentation

Traditionally, Zymomonas mobilis and Saccharomyces cerevisiae are used for ethanol fermentation from biomass. But they are unable to utilize non-fermentable sugars like xylose [7]. The development of efficient and cost effective fermentation process for ethanol production from lignocellulosic hydrolysate will oblige a microorganism that have an ability to convert both hexoses and pentoses to the ethanol. In response to this challenge the recombinant strains of E.coli, S.Cerevisiae, Klebsiella oxytoca and Zymomonas mobilis have been developed. The issues need to be considered for these bacteria and yeast strains are their long term stability , resistance to inhibitors in lignocellulosic hydrolysate and their potential for contamination resistance during extended process [66]. Of the particular recent interest, the properties of Zymomonas mobilis are very suitable for high sugar uptake and high ethanol production [67]. Zymomonas mobilis is a Gram-negative bacterium capable of producing almost a theoretical amount of ethanol from glucose via an Entner–Doudoroff pathway under anaerobic conditions [68]. But culturing it on substrates like glucose, fructose or sucrose, would significantly reduce the yield of ethanol because sucrose-based substrates, reduces the conversion efficiency due to by-products formation which are mainly sorbitol and levan [69]. Zymomonas mobilis's has an ability to convert high sugar uptake of 10-25% to ethanol with theoretical yield of ethanol 97% and under 1.8 to 1.9 moles of ethanol per mole of glucose [70] but Ethanol yield from Zymomonas mobilis is lower for sucrose and theoretical yield is 70% due to formation of sorbitol and levan 11% and 8% respectively as mentioned above [71]. The factors that needs to be controlled for ZM growth are temperature, ethanol and sugar concentration and PH in the anaerobic conditions [72]

Transformation of *E. coli* with genes from Z. mobilis has been successfully carried out to increase ethanol yield as this helps Z. mobilis to utilize sugars other than glucose which may be mannose, xylose, arabinose and lactose [73].

Z. Mobilis Experimentation Conditions

Different strains of *Z. mobilis* are streaked down in either L.B media or semidefined media. The fermentation medium was then agitated at 120 rpm. Temperature should be maintained steady at 30°C and so is pH=5. In order to maintain anaerobic conditions into fermentation broth, nitrogen gas is being purged. However, once the cell growth reached the exponential phase, the nitrogen flow was stopped and culture was kept anaerobic by the CO_2 evolved in the fermentation broth.

6. Ethanol Removal

Once production of ethanol is confirmed , it has to be removed from fermenter or broth by using different techniques which includes ethanol removal by vacuum, membrane, by gas stripping, flashing and by liquid extraction [74]. These processes allow high productivity by ensuring the removal of end product inhibition [75].

6.1 Determination and Analysis

Identification of algal composition is done by first collecting the algae from its cultural medium and drying it. The dried algal biomass was then finely grounded with a blender or with hammer and crusher, and the obtained amount of fraction is then sieved with approximately 35 mesh screen for further use. The determination of Ash content was done by heating the sample in a muffle furnace at 550 °C for 12 h and weighing the obtained samples before and after heating. Micro-Kjeldahl method is used for the determination of protein using a factor of $N = 6.25$ and Soxhlet is used to determine Lipid content [21].

Biomass concentration in the fermenter was determined spectrophotometric ally at 540 nm and the corresponding dry weight concentration of cells was obtained from an established calibration curve of absorbance versus dry weight concentration.

The sugars % in Brix can be measured by using hand held Refractrometer. The hydrolysates are analyzed by HPLC using sugar PAK column and RI detecter. The mobile phase is water containing 50 mM calcium EDTA [76] . Sugar's seperation has also been achieved using gas chromatography (GC) or thin layer chromatography (TLC). HPLC is the most employed among these techniques. Recently, for more accuracy, evaporative light scattering (ELSD), Ultraviolet (UV) spectrometry, fluorescence spectrometry, mass spectrometry (MS) are being coupled with HPLC for the detection. New techniques such as near infrared spectroscopy (NIRS) and enzyme immunosassay (EIA) have also been

used for analysis process [77]. FTIR analysis is done and peaks for ethanol detection can be recorded at 3500-3200 cm^{-1}. Potassium permanganate test could also be employed for ethanol detection and confirmation.

References

[1] A. A. Bazmi, A. W. Bhutto, and M. Ghauri, "ETHANOL FUEL AS FEASIBLE AND DESIRED OPTION IN PAKISTAN," 2007.

[2] N. Sarkar, S. K. Ghosh, S. Bannerjee, and K. Aikat, "Bioethanol production from agricultural wastes: An overview," *Renew. Energy*, vol. 37, no. 1, pp. 19–27, Jan. 2012.

[3] L. V. R. and M. L. N. Anuj K Chandel1, Om V Singh2, Gajula Chandrasekhar3, "No Title," *J. Commer. Biotechnol.*, vol. ISSN: 1462, no. 25 May 2010, pp. 239–257, 2010.

[4] C. E. Wyman, B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch, and Y. Y. Lee, "Coordinated development of leading biomass pretreatment technologies.," *Bioresour. Technol.*, vol. 96, no. 18, pp. 1959–66, Dec. 2005.

[5] S. Kim and B. E. Dale, "Global potential bioethanol production from wasted crops and crop residues," *Biomass and Bioenergy*, vol. 26, no. 4, pp. 361–375, Apr. 2004.

[6] P. Alvira, E. Tomás-Pejó, M. Ballesteros, and M. J. Negro, "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review.," *Bioresour. Technol.*, vol. 101, no. 13, pp. 4851–61, Jul. 2010.

[7] M. Balat, "Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review," *Energy Convers. Manag.*, vol. 52, no. 2, pp. 858–875, Feb. 2011.

[8] M. Balat, H. Balat, and C. Öz, "Progress in bioethanol processing," *Prog. Energy Combust. Sci.*, vol. 34, no. 5, pp. 551–573, Oct. 2008.

[9] D. Chiaramonti, M. Prussi, S. Ferrero, L. Oriani, P. Ottonello, P. Torre, and F. Cherchi, "Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method," *Biomass and Bioenergy*, vol. 46, pp. 25–35, Nov. 2012.

[10] José Goldemberg*, "Ethanol for a Sustainable Energy Future," *Sci. Vol. no. 5813 pp. DOI*, vol. 315, pp. 808–810.

[11] G. Walker, "Bioethanol : challenges and opportunities," 2008.

[12] J. Martines-filho, H. L. Burnquist, and C. E. F. Vian, "Bioenergy and the Rise of Sugarcane-Based Ethanol in Brazil," vol. 21, no. 2, pp. 91–96, 2006.

[13] O. J. Sánchez and C. a Cardona, "Trends in biotechnological production of fuel ethanol from different feedstocks.," *Bioresour. Technol.*, vol. 99, no. 13, pp. 5270–95, Sep. 2008.

[14] C. E. Wyman, "Ethanol from lignocellulosic biomass: Technology, economics, and opportunities," *Bioresour. Technol.*, vol. 50, no. 1, pp. 3–15, Jan. 1994.

[15] M. E. D. D. E. Oliveira, B. E. Vaughan, and E. J. R. Jr, "Ethanol as Fuel: Energy , Carbon Dioxide Balances , and Ecological Footprint," vol. 55, no. 7, pp. 593–602, 2005.

[16] K. Harijan, M. Memon, M. a. Uqaili, and U. K. Mirza, "Potential contribution of ethanol fuel to the transport sector of Pakistan," *Renew. Sustain. Energy Rev.*, vol. 13, no. 1, pp. 291–295, Jan. 2009.

[17] J. Goldemberg, "The Brazilian biofuels industry.," *Biotechnol. Biofuels*, vol. 1, no. 1, p. 6, Jan. 2008.

[18] M. Balat and H. Balat, "Recent trends in global production and utilization of bio-ethanol fuel," *Appl. Energy*, vol. 86, no. 11, pp. 2273–2282, Nov. 2009.

[19] D. Li, H. Zhen, L. Xingcai, Z. Wu-gao, and Y. Jian-guang, "Physico-chemical properties of ethanol–diesel blend fuel and its effect on performance and emissions of diesel engines," *Renew. Energy*, vol. 30, no. 6, pp. 967–976, May 2005.

[20] A. C. Hansen, Q. Zhang, and P. W. L. Lyne, "Ethanol-diesel fuel blends -- a review.," *Bioresour. Technol.*, vol. 96, no. 3, pp. 277–85, Feb. 2005.

[21] H. L. MacLean and L. B. Lave, "Evaluating automobile fuel/propulsion system technologies," *Prog. Energy Combust. Sci.*, vol. 29, no. 1, pp. 1–69, Jan. 2003.

[22] B. S. Dien, M. a Cotta, and T. W. Jeffries, "Bacteria engineered for fuel ethanol production: current status.," *Appl. Microbiol. Biotechnol.*, vol. 63, no. 3, pp. 258–66, Dec. 2003.

[23] M. O. S. Dias, M. P. Cunha, C. D. F. Jesus, G. J. M. Rocha, J. G. C. Pradella, C. E. V Rossell, R. M. Filho, and A. Bonomi, "Second generation ethanol in Brazil: can it compete with electricity production?," *Bioresour. Technol.*, vol. 102, no. 19, pp. 8964–71, Oct. 2011.

[24] P. S. Nigam and A. Singh, "Production of liquid biofuels from renewable resources," *Prog. Energy Combust. Sci.*, vol. 37, no. 1, pp. 52–68, Feb. 2011.

[25] L. Brennan and P. Owende, "Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products," *Renew. Sustain. Energy Rev.*, vol. 14, no. 2, pp. 557–577, Feb. 2010.

[26] A. Singh, P. S. Nigam, and J. D. Murphy, "Mechanism and challenges in commercialisation of algal biofuels.," *Bioresour. Technol.*, vol. 102, no. 1, pp. 26–34, Jan. 2011.

[27] S. Amin, "Review on biofuel oil and gas production processes from microalgae," *Energy Convers. Manag.*, vol. 50, no. 7, pp. 1834–1840, Jul. 2009.

[28] M. Carriquiry, D. J. Hayes, J. Bin, W. H. Meyers, L. Wilcox, and A. W. Womack, "FAPRI 2008 U . S . AND WORLD AGRICULTURAL OUTLOOK," no. January, 2008.

[29] M. Packer, "Algal capture of carbon dioxide; biomass generation as a tool for greenhouse gas mitigation with reference to New Zealand energy strategy and policy," *Energy Policy*, vol. 37, no. 9, pp. 3428–3437, Sep. 2009.

[30] P. M. Schenk, S. R. Thomas-Hall, E. Stephens, U. C. Marx, J. H. Mussgnug, C. Posten, O. Kruse, and B. Hankamer, "Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production," *BioEnergy Res.*, vol. 1, no. 1, pp. 20–43, Mar. 2008.

[31] S. M. and S. N. F. Shirai, K. Kunii, C. Sato, Y. Teramoto, E. Mizuki, "Cultivation of microalgae in the solution from the desalting process of soy sauce waste treatment and utilization of the algal biomass for ethanol fermentation," *World J. Microbiol. Biotechnol. 14*, vol. 14, p. 839±842, 1998.

[32] M. Awasthi and R. K. Singh, "Development of algae for the production of bioethanol, biomethane, biohydrogen and biodiesel," pp. 14–23, 2011.

[33] P. Spolaore, C. Joannis-Cassan, E. Duran, and A. Isambert, "Commercial applications of microalgae.," *J. Biosci. Bioeng.*, vol. 101, no. 2, pp. 87–96, Feb. 2006.

[34] H. B. Feedstocks, "Composition of Herbaceous Biomass Feedstocks," no. June, 2007.

[35] P. R. Boyer, "La Culture de Spirulina en Photobioreacteur."

[36] M. Molina-Sabio and F. Rodríguez-Reinoso, "Role of chemical activation in the development of carbon porosity," *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 241, no. 1–3, pp. 15–25, Jul. 2004.

[37] H. C. Greenwell, L. M. L. Laurens, R. J. Shields, R. W. Lovitt, and K. J. Flynn, "Placing microalgae on the biofuels priority list : a review of the technological challenges Placing microalgae on the biofuels priority list : a review of the technological challenges," 2009.

[38] Mary Mennes Allen, "SIMPLE CONDITIONS FOR GROWTH OF UNICELLULAR BLUE-GREEN ALGAE ON PLATES," vol. Volume 4, no. Issue 1, p. pages 1–4.

[39] E. R. C. andNiels N. Philipp Mayer1,†,*, Jesper Frickmann1, "Influence of growth conditions on the results obtained in algal toxicity tests," vol. Volume 17, no. Issue 6, p. pages 1091–1098,.

[40] S. Nahak, G. Nahak, I. Pradhan, and R. K. Sahu, "Bioethanol from Marine Algae : A Solution to Global Warming Problem," vol. 1, no. 4, pp. 74–80, 2011.

[41] N.-H. Norsker, M. J. Barbosa, M. H. Vermuë, and R. H. Wijffels, "Microalgal production--a close look at the economics.," *Biotechnol. Adv.*, vol. 29, no. 1, pp. 24–7, 2011.

[42] "Hatchery culture of bivalves," *FAO Fish. Tech. Pap.*, p. 175, 2004.

[43] L. Gouveia and A. C. Oliveira, "Microalgae as a raw material for biofuels production.," *J. Ind. Microbiol. Biotechnol.*, vol. 36, no. 2, pp. 269–74, Feb. 2009.

[44] A. R. Zhang Cheng-Wua, b, Odi Zmorac, Reuven Kopela, "An industrial-size flat plate glass reactor for mass production of Nannochloropsis sp. (Eustigmatophyceae)," *Sci. direct*, vol. 195, no. 1–2, pp. 35–49, 2001.

[45] L. J. Pruvost J1, Van Vooren G, Cogne G, "Investigation of biomass and lipids production with Neochloris oleoabundans in photobioreactor.," *Bioresour Technol.*, vol. 23, 2009.

[46] N. L. R. Ben-Arie, P. Sarig, Y. Cohen-Ahdut, Y. Zutkhi, L. Sonego, T. Kapulonov, "CPPU AND GA3 EFFECTS ON PRE- AND POST-HARVEST QUALITY OF SEEDLESS AND SEEDED GRAPES," *ISHS Acta Hortic. 463*, vol. 463, 2004.

[47] Español Français, *Hatchery culture of bivalves*. 2004, p. 175.

[48] "Wikipedia, "Hemocytometer --- Wikipedia{,} The Free Encyclopedia." wikipedia, 2014.

[49] R. G. & michael . S. S. R.L, "counting cells in cultures with light microscope," Frank cynar, Ed. Elsevier acedamic press, pp. 239–251.

[50] J. L. Soojin Lee, Younghoon Oh, Donghyun Kim, Doyeon Kwon, Choulgyun Lee, "Converting Carbohydrates Extracted from Marine Algae into Ethanol Using Various Ethanolic Escherichia coli Strains," *Appl. Biochem. Biotechnol.*, vol. 164, no. 6, pp. 878–888, 2011.

[51] X. Wang, X. Liu, and G. Wang, "Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation.," *J. Integr. Plant Biol.*, vol. 53, no. 3, pp. 246–52, Mar. 2011.

[52] G. T. T. K.W.LIN, M.R.LADISCH, C.H.NOLLER, V.LECHTENBERG, "Review on effect of pretreatment on digestibility of cellulosic materials." 1981.

[53] A. P. Dadi, S. Varanasi, and C. a Schall, "Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step.," *Biotechnol. Bioeng.*, vol. 95, no. 5, pp. 904–10, Dec. 2006.

[54] R. Harun and M. K. Danquah, "Influence of acid pre-treatment on microalgal biomass for bioethanol production," *Process Biochem.*, vol. 46, no. 1, pp. 304–309, Jan. 2011.

[55] D.-H. P. Minchul Yoona, Jong-il Choia, , , Ju-Woon Leea, "Improvement of saccharification process for bioethanol production from Undaria sp. by gamma irradiation," *Radiat. Phys. Chem.*, vol. 81, no. 8, pp. 999–1002, 2012.

[56] G. B. Christian Schacht, Carsten Zetzl, "From plant materials to ethanol by means of supercritical fluid technology."

[57] and K. M. * Soumya Sasmal , Vaibhav V. Goud *, "Ultrasound Assisted Lime Pretreatment of Lignocellulosic Biomass toward Bioethanol Production," *Energy Fuels*, pp. 3777–3784, 2012.

[58] and Y. Z. Wen Shi , Sining Li , Jingfu Jia, "Highly Efficient Conversion of Cellulose to Bio-Oil in Hot-Compressed Water with Ultrasonic Pretreatment," *ACS journals*, pp. 586–593, 2012.

[59] Y. Zheng, Z. Pan, and R. Zhang, "Overview of biomass pretreatment for cellulosic ethanol production," vol. 2, no. 3, pp. 51–68, 2009.

[60] C. E. Wyman, S. R. Decker, M. E. Himmel, J. W. Brady, and C. E. Skopec, "Hydrolysis of Cellulose and Hemicellulose," 2005.

[61] G. Brodeur, E. Yau, K. Badal, J. Collier, K. B. Ramachandran, and S. Ramakrishnan, "Chemical and physicochemical pretreatment of lignocellulosic biomass: a review.," *Enzyme Res.*, vol. 2011, p. 787532, Jan. 2011.

[62] C. C. E Neyens, , J Baeyens, "Alkaline thermal sludge hydrolysis," *elsevier*, vol. 97, no. 1–3, pp. 295–314, 2003.

[63] R. Harun, W. S. Y. Jason, T. Cherrington, and M. K. Danquah, "Exploring alkaline pre-treatment of microalgal biomass for bioethanol production," *Appl. Energy*, vol. 88, no. 10, pp. 3464–3467, Oct. 2011.

[64] S. J. S. Seung Phill Choi, Minh Thu Nguyen, "Enzymatic pretreatment of Chlamydomonas reinhardtii biomass for ethanol production," *Bioresour. Technol.*, vol. 101, no. 14, pp. 5330–5336, 2010.

[65] C. M. Drapcho and D. Ph, *Biofuels Engineering Process Technology*. .

[66] P. L. R. Eva Joachimsthal, Kevin D. Haggett, "Evaluation of recombinent strains of zymmomonas mobilis for ethanol production from glucose/xylose media," *Appl. Biochem. Biotechnol.*, vol. 77–79, p. pp 147–157, 1999.

[67] P. L. R. K. J. Lee, M. L. Skotnicki, D. E. Tribe, "Kinetic studies on a highly productive strain of Zymomonas mobilis," *Biotechnol. Lett.*, vol. Volume 2, no. Issue 8, p. pp 339–344.

[68] Y. Yamashita, A. Kurosumi, C. Sasaki, and Y. Nakamura, "Ethanol production from paper sludge by immobilized Zymomonas mobilis," *Biochem. Eng. J.*, vol. 42, no. 3, pp. 314–319, Dec. 2008.

[69] W.-C. Lee and C.-T. Huang, "Modeling of ethanol fermentation using Zymomonas mobilis ATCC 10988 grown on the media containing glucose and fructose," *Biochem. Eng. J.*, vol. 4, no. 3, pp. 217–227, Feb. 2000.

[70] D. E. T. P. L. Rogers, Kye Joon Lee, "Kinetics of alcohol production by zymomonas mobilis at high sugar concentrations," *Biotechnol. Lett.*, vol. Volume 1, no. , Issue 4, p. pp 165–170.

[71] Liisa Viikari, "Formation of levan and sorbitol from sucrose by Zymomonas mobilis," *pplied Microbiol. Biotechnol.*, vol. Volume 19, no. Issue 4, p. pp 252–255.

[72] Uday verramalu, "Study of fermentataion behavior and formulation of a semidefine," *Biotechnol. Bioeng.*, vol. 31, pp. 770–782, 1987.

[73] P. Gunasekaran and K. Chandra Raj, "Ethanol fermentation technology – Zymomonas mobilis." .

[74] C. a Cardona and O. J. Sánchez, "Fuel ethanol production: process design trends and integration opportunities.," *Bioresour. Technol.*, vol. 98, no. 12, pp. 2415–57, Sep. 2007.

[75] E. F. Processes, "Biotechnology Report," no. X, 1983.

[76] S. Patle and B. Lal, "Ethanol production from hydrolysed agricultural wastes using mixed culture of Zymomonas mobilis and Candida tropicalis.," *Biotechnol. Lett.*, vol. 29, no. 12, pp. 1839–43, Dec. 2007.

[77] Nicola Fuzzati, "Analysis methods of ginsenosides," *J. Chromatogr. B*, vol. 812, no. Issues 1–2, p. Pages 119–133.