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Fermentation process using enzymatic strain enhances the bio fuel Production from lignocellulosic biomass

Devendra Pratap Singh

Department of Applied Chemistry

Dr. Ambedkar Institute of Technology for Handicapped, Kanpur 208024, India

Dr. Rakesh Kumar Trivedi

Professor & Head, Department of Oil and Paint Technology

Harcourt Butler Technological Institute, Kanpur 208002, U.P., India

Abstract: Bio ethanol is an attractive, sustainable energy source to fuel transportation, produced from kinds of biomasses. Among kinds of biomass, lignocellulosic biomass is a very useful feedstock to economically produce environmentally friendly biofuels. The main goal of the research described in this paper is to investigate the process of pretreated biomass, acid or enzymatic hydrolysis, Fermentation and Ethanol separation/ purification. Ethanol can be produced from many feedstock's, including cereal crops, corn (maize), sugar cane, rape straw, wheat straw, corncob etc. Technologies for the production of alternative fuels are receiving increased attention owing to concerns over the rising cost of petrol and global warming. Hexoses are the Primary carbohydrate, which can easily fermented by yeast but yeast has ability to ferment only hexoses but not Pentoses. Xylose is the second most abundant carbohydrate in the lignocellulosic biomass hydrolysate. The fermentation of xylose is essential for the bioconversions of lignocelluloses to fuels. For the complete fermentation of carbohydrates genetically engineered yeast strain is a better way, which enhances the production of ethanol. For the better result improvement in fermentation process, use of yeast strain is an acceptable necessary action. The development of novel yeast strains with increased tolerance toward inhibitors in lignocellulosic hydrolysates is highly desirable for the production of bio-ethanol. This yeast displayed improved tolerance to xylose and ethanol production. The use of bioethanol as a motor fuel has as long a history as the car itself.

Keywords: Bioethanol, Biomass, Ethyl alcohol, Fermentation, Hydrolysis, Yeast Strain.

I. Introduction

Biomass is a renewable energy resource derived from the carbonaceous waste of various human and natural activities. It is derived from numerous sources, including the by-products from the timber industry, agricultural crops, raw material from the forest, major parts of household waste and wood. Biomass is available in different forms in the country, it cannot be defined easily because all things, which are dead and produces energy on combustion is a part of biomass either it may consist different thing. Liquid fuels are prepared by those types of biomass which consist starch, cellulose and another different forms of carbohydrates followed by fermentation in a feasible conditions. Biomass is an important source of energy and the most important fuel worldwide after coal, oil and natural gas. Biofuels are referred to liquid, gas and solid fuels predominantly produced from biomass. Lignocellulosic materials are the world's most widely available low-cost renewable resources to be considered for ethanol production. Biofuel is a renewable energy source produced from natural (biobased) materials, which can be used as a substitute for petroleum fuels. However, bioethanol is a petrol additive/substitute that can be produced from plentiful, domestic, cellulosic biomass resources such as herbaceous and woody plants, agricultural and forestry residues, and a large portion of municipal and industrial solid waste streams. The best biofuels from biomasses are bioethanol and biodiesel. Production of bioethanol from biomass is one way to reduce both the consumption of crude oil and environmental pollution. There is also a growing interest in the use of vegetable oils for making biodiesel, which is less polluting than conventional petroleum diesel fuel ([6]-[8]).

Bioethanol is produced from different resources of biomass by using different process, such as pretreatment, hydrolysis and fermentation. Production of ethanol from agricultural and biodegradable wastes provides a variable solution to multiple environmental problems simultaneously creating sink for waste and renewable energy production as well. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use and greenhouse gas emissions [28]. Alkaline pretreatment of lignocellulosic materials causes swelling, leading to decreased crystallinity, increased internal surface area, disruption of the lignin structure, and separation of structural linkages between lignin and carbohydrates ([10]- [11]). Alkali pretreatment refers to the application of alkaline solutions such as NaOH, Ca(OH)₂ (lime) or ammonia to remove lignin and a part of the hemicellulose, and efficiently increase the accessibility of enzyme to the cellulose. Alkaline pretreatment also removes acetyl and various uronic acid substitutions on hemicellulose that reduce the accessibility of

hemicellulose and cellulose to enzymes [5]. The digestibility of NaOH-treated hardwood was reported to increase from 14% to 55% with a decrease of lignin content from 24-55% to 20%. However, no effect of dilute NaOH pretreatment was observed for Lignocelluloses with lignin content greater than 26% [21]. Dilute NaOH pretreatment was also found to be effective for the hydrolysis of straws with relatively low lignin contents of 10-18% [4].

Several types of acids, concentrated or diluted, can also be used, such as sulphurous, sulphuric, hydrochloric, hydrofluoric, phosphoric, nitric and formic acid for the pretreatment and acid hydrolysis of lignocellulosic biomass. Sulphuric and hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosic biomass [16]. Dilute acid process is the oldest technology for converting cellulose biomass to ethanol. The main step is essentially hemicellulose hydrolysis. In simple terms, acid catalyzes the breakdown of long hemicellulose chains to form shorter chain oligomers and then to sugar monomers that the acid can degrade. However, because hemicellulose is amorphous, less severe conditions are required to release hemicellulose sugars ([29], [30]). The goal of the pretreatment process is to break down the lignin structure and disrupt the crystalline structure of cellulose, so that the acids or enzymes can easily access and hydrolyze the cellulose ([22], [17]- [18]). Pretreatment can be the most expensive process in biomass-to-fuels conversion but it has great potential for improvements in efficiency and lowering of costs through further research and development ([13], [19]).

II. Material and Methods

Lignocellulosic dry samples such as (Sugarcane baggase, wheat straw, rape straw etc.) were taken from agricultural rural areas. All the biomass samples were dried in to oven and cut in to small pieces by milling. The method of producing ethanol from lignocelluloses involves reducing the size of biomass to smaller particles prior and using acid or enzyme treatments to hydrolyze the biomass to sugars. Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. The Nelson-Somogyi method is one of the classical and widely used methods for the quantitative determination of reducing sugars [26].

Microorganisms and preparation for inoculation: Yeast was cultured on PDA slants for 2 days. Liquid medium for the preparation of the yeast was composed of 50 grams glucose, 5 grams yeast extract, 1 gram KH_2PO_4 , 0.3 grams NH_4Cl and 2 grams $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per litre [1]. After inoculation of the yeast from slant in this environment, it was placed in shaker at 41°C and 130 rpm for 24 hours. 2.5% (by volume) of this prepared yeast was inoculated to SSF medium for ethanol production.

Acid Pretreatment: Acid pretreatment is the one of the pretreatment method which is used to degrade the biomass component in to fermentable sugars by further acid hydrolysis or using enzymatic hydrolysis. There are two types dilute and concentrated acid treatment is used for the biomass degradation. The advantage of the acid hydrolysis is that acids can penetrate lignin without any preliminary pre-treatment of biomass, thus breaking down the cellulose and hemicelluloses polymers to form individual sugar molecules. The main advantage of the dilute hydrolysis process is the low amount of acid required (2-5%). However this process is carried out at high temperatures to achieve acceptable rates of cellulose conversion. The high temperature increases the rates of hemicellulose sugars decomposition thus causing the formation of toxic compounds such as furfural and 5-hydroxymethyl-furfural (HMF). The acid concentration used in the concentrated acid hydrolysis process is in the range of 10-30%. The process occurs at low temperatures, producing high hydrolysis yields of cellulose (i.e. 90% of theoretical glucose yield). However, this process requires large amounts of acids causing corrosion problems to the equipments.

For the pretreatment with acid the following steps has been used:- Prepare biomass samples as necessary (grinding, blending, etc.) Weigh out 3g of biomass into a pre weighed aluminium tray (do replicates of this). Be sure to record the weight of the biomass as well as the weight of the tray. Place the trays in a 105°C oven overnight. Prepare 3% sulphuric acid. Once the moisture content is determined, weigh the amount of wet biomass correlating to 1.5g dry mass to a 125ml flask. Apply 45ml of 0.8% sulphuric acid to each sample. Autoclave it at 121°C for 30 min. Centrifuge the obtained at 1500 rpm for 10 minutes. Separate the solid part, wash with distilled water and again make slurry by adding known concentration of acid, autoclaved again at 121°C for 30min. immediately after the cycle is finished, remove the samples and filter it. Wash the biomass with 200ml water using the filter. Maintain pH at 5.0, and dilute it for enzymatic hydrolysis.

Enzyme production: The medium composed of 2 grams KH_2PO_4 , 1.4 grams $(\text{NH}_4)_2\text{SO}_4$, 0.75 grams peptone, 0.4 grams $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 grams urea, 0.3 grams $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 grams yeast extract and 0.005 grams $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 grams COCl_2 , 0.0016 grams $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.0014 grams $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per litre was used to moisten solid bed for enzyme production [24]. In Erlenmeyer flask, 3 grams of milled bagasse was moistened by aforementioned medium plus 1 ml of microbial suspension to achieve 80% moisture. The flask was incubated for 4 days at 30°C . Produced enzymes were extracted by citrate buffer (pH 4-6). After filtering

the bagasse from the mixture, the rest was centrifuged by 1500 rpm for 10 minutes at 4°C in order to separate fungus spores.

Fermentation Experiments: Batch fermentation experiments were carried out in pH-controlled 500 mL flasks under semi anaerobic conditions with working volumes of 350 mL essentially as described by Bothast [3]. Lignocellulosic hydrolyzates were sterilized separately. Inoculum size was 5% (v/v). A 5N KOH/ NaOH solution was used for pH control. Samples were withdrawn periodically to determine cell mass, ethanol content, and residual sugars. Base consumption and pH were also recorded. For separate hydrolysis and fermentation (SHF) experiments, the fermentation was carried out at pH 6 and 35°C using the liquid portion of the hydrolyzate after separating it from the solids. For simultaneous saccharification and fermentation (SSF) experiments, 2 L fermenters with a working volume of 1.5 L were used at pH 6.0 and 35°C and shaking at 150 rpm. The dilute acid pre-treated whole biomass (bagasse) hydrolyzates were added to the fermenter as a substrate after adjusting the pH to 5.0-6.0, with 5 M NaOH before adding enzymes and inoculum.

Analytical Method: Compositional analysis of raw and pretreated sugarcane bagasse was done in a similar way to that introduced by US National Renewable Energy Laboratory with small changes [2]. In the case of pretreated bagasse the procedure was performed without ethanol washing step. The overall cellulase activity was measured as filter paper activity according to Ghose method [27]. To measure carbohydrates and ethanol concentration after enzymatic hydrolysis and SSF, they were sampled and analyzed by HPLC.

III. Result and Discussion

Yeast is the main source of *Saccharomyces cerevisiae*. This enzyme is able to ferment only Hexoses but it cannot able to ferment Pentoses, this is because the major sugars from cellulosic biomass are not just glucose but also xylose with ratio glucose to xylose being approximately 2 or 3 to 1. It is generally agreed that unless both glucose and xylose from the cellulosic biomass can be fermented however nearly all of the fermentative yeasts including *Saccharomyces cerevisiae* are found to be unable to ferment xylose to ethanol or utilize the pentose sugar for growth. In order to solve the above described problem, we had taken not only genetically engineered enzyme to ferment xylose but also to be able for the effective co fermentation of both glucose and xylose simultaneously so that mixed sugar will be fermented as fast as possible. The batch culture produced the maximum ethanol at a single point, but it could not maintain the production due to the depletion of glucose. *Saccharomyces* yeasts the production of ethanol decreases because of no effect upon xylose fermentation, but the amount of glucose consumed completely to produce ethanol as shown in Fig. 1. But in presence of genetically engineered *Saccharomyces* yeasts strain the concentration of ethanol yield becomes more as compared to the ethanol yield concentration by an engineered *Saccharomyces* yeasts. It is clear that, only *Saccharomyces* yeasts are not sufficient to ferment both glucose and xylose as shown in Fig. 1. In Fig. 1 the fermentation of Xylose remains unaffected, while ethanol concentration was found 47g/l in 30 hours of fermentation, while in presence of yeast strain, the maximum ethanol concentration was found 60g/l in 30 hours, as shown in Fig. 2.

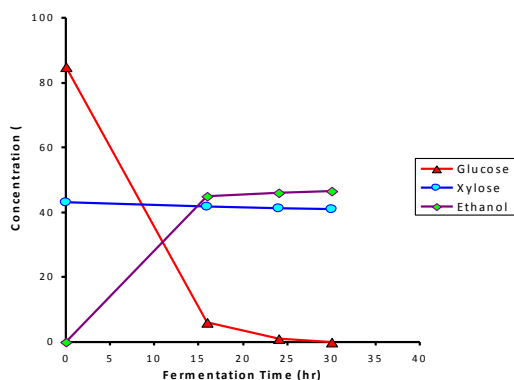


Fig.1- Fermentation of glucose and xylose by the *Saccharomyces* yeasts

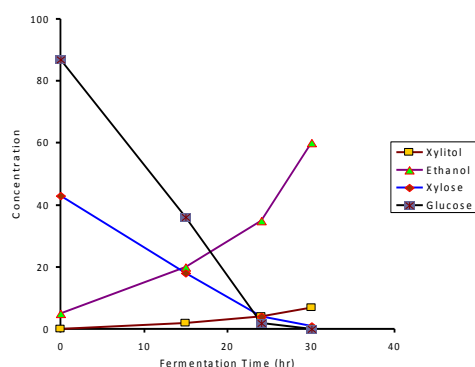


Fig.2- Fermentation of glucose and xylose by the *Saccharomyces* yeasts strain

Medium is an also important factors which effects the rate of fermentation and growth of enzymes. In absence of Carbon the growth of enzymes becomes slower in comparison with YEP medium containing at least one carbon source. Presence of xylose or glucose is the rich source of carbon source in YEP medium. This allows the yeasts to maintain their plasmids in YEPX (YEP with 2% xylose) medium. This also allows engineered *Saccharomyces* yeasts cultured in YEPX during early stages of growth to be cultured in YEPD as concluded by [14]. In case of comparative study between yeast and yeast strain, the maximum ethanol productivity % was measured 62% by yeast strain and 47% by yeast (unengineered) in 30 hours at optimum conditions. And at 24 hours the maximum ethanol was found by strain (48%) against un engineered yeast (30%) as shown in Fig.4.

The growing media culture was also play a effective role for ethanol production, YEPD medium was give best result than YEP medium in 24 hours as well as in 30 hours of enzyme grow is shown in Fig. 3.

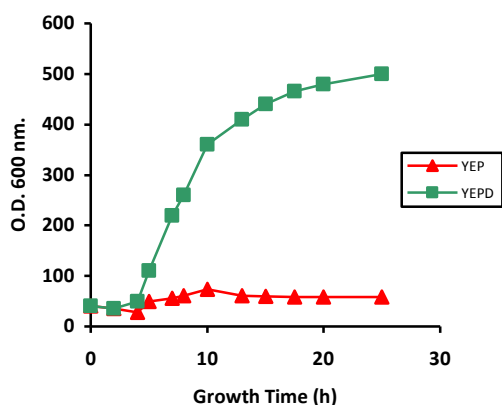


Fig.3- Culturing of *Saccharomyces cerevisiae* strain

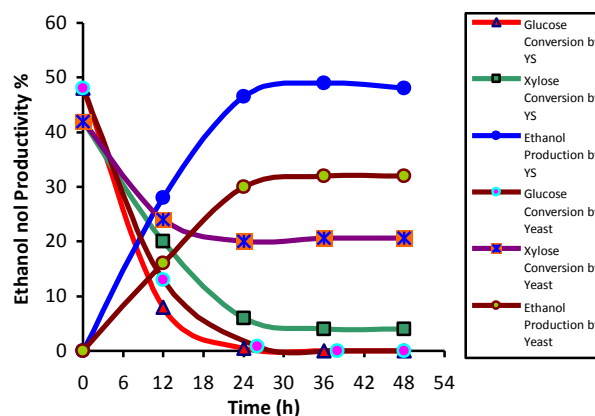


Fig.4- Comparison of co fermentation of glucose and xylose present in the medium under identical conditions by genetically engineered *Saccharomyces* yeasts strain and by yeast extract

On the basis of the above discussion different types of lignocellulosic biomasses were used in the production of ethanol at optimum pH 5-6, optimum temperature of fermentation 28-30°C and in presence of suitable medium. On inoculation of yeast for the fermentation of acid pretreated different kinds of lignocellulosic biomass has been done at pH 5. The result of the fermentation yield of ethanol is shown in Table- 1. Yeast strain is one of the effective parameter which is genetically engineered for higher ethanol yield by fermentation. The fermentation efficiency for bagasse was counted 92.2% theoretically in 24 hours at pH 5 and temperature 28-30°C. While the minimum ethanol concentration was noted 38.22g/l for corncob by utilizing 86.40% of substrate at optimum temp. (28-30°C) and pH 5. The maximum yield of Ethanol was recorded 0.46g/g in bagasse. *Saccharomyces cerevisiae* strain was used for the fermentation of all of the following biomasses. During fermentation the cell yield of the following biomass straw Wheat Straw, Wheat bran, Rape Straw, Rice Straw, Barley Straw were found as 0.014, 0.013, 0.013, 0.012, 0.014 g/g.

Overall Parameters	Sugarcane Baggase	Wheat Straw	Wheat bran	Rape Straw
Amount of Sugar Con. g/l	100	100	100	100
Cellulose (%)	34	30	31	33
Hemicellulose (%)	30	24	26	18
Lignin (%)	29	18	20	15
Residual Sugar Con. g/l	11.42	12.75	14.20	13.47
Final Ethanol Con. g/l	41.60	39.40	38.02	38.54
Final Biomass Con. g/l	1.37	1.30	1.12	1.20
Substrate Utilized (%)	88.80	87.25	82.87	86.53
Ethanol Yield, Y p/s (g/g)	0.46	0.45	0.45	0.44
Cell Yield, Y x/s (g/g)	0.015	0.014	0.013	0.013
Fermentation Efficiency* (% of theoretical)	92.2	88.2	86.3	87.2
Fermentation Time (h)	24	24	24	24

* Theoretical yield based on total sugars is 0.511 gg⁻¹

Table 1- Composition and Fermentation yield of ethanol of different biomass

The amount of cell yield was found approximately same in most of the biomass, which was calculated by final biomass upon substrate utilized. Hence it is clear that maximum the amount of substrate utilized maximum ethanol yield was recorded. Table-1 represented the maximum yield of ethanol at optimum temperature and concentration at a suitable pH 5. The percentage of cellulose and hemicelluloses in bagasse after pretreatment was found 34 and 30%. It was also calculated maximum for corncob (cellulose 34%, hemicelluloses 29%). The biomass composition was calculated after pretreatment by analytical methods and techniques. These results of % composition of biomasses prove as many of the researcher [15].

IV. Conclusion

The biomass pretreatment and the intrinsic structure of the biomass itself are primarily responsible for its subsequent hydrolysis. Pretreatment of biomass is an extremely important step in the synthesis of biofuels from

lignocellulosic biomasses, and there is a critical need to understand the fundamentals of various processes, which can help in making a suitable choice depending on the structure of the biomass substrate and the hydrolysis agent. Pre-treatment's are usually focused on lignin and hemicelluloses removal, cellulose crystallinity reduction and accessible surface area increase. Pretreatment of lignocellulosic and algal biomass can be held in three ways Acid, Alkali and enzymatic pretreatment. Pretreatment with acid autoclaving at 121°C for 30 min, is an effective and cheap parameter in comparison to enzymatic degradation and other methods. Acid pretreatment had a greater influence on the sugar release through enzymatic hydrolysis. An increase in acid severity in terms of concentration resulted in higher sugar releases. By the experimental analysis 3% (w/v) of acid was found optimum. However, although significant progress has been achieved in biomass pretreatment, cellulase production and co-fermentation of the pentose and hexose sugars in recent decades, bio ethanol is still not economically competitive compared with petroleum-based fuels, making cost reduction the biggest challenge. Yeast strain is necessary for the complete fermentation of hexoses and pentoses to enhance the ethanol production in the present scenario.

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