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Challenges in Biochemical Engineering and Biotechnology for Sustainable Environment

Simultaneous Saccharification and Fermentation of Bioethanol from Softwood *Moringa Oleifera* using Thermo-Tolerant Yeast *Kluyveromyces marxianus* MTCC 1388

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Abstract: Research on bio-ethanol production has been promoted since the recent past as the fuel is efficient, biodegradable, environment friendly and cost effective alternate for the existing fossil fuel. Agricultural and forestry raw materials can be utilized to produce renewable energy in the form of liquid/gaseous fuel which has got reduced CO_2 emission on combustion unlike fossil fuels and hence it has an impact on climate variations. Bio-ethanol derived from lignocellulosic material is a potential renewable carbon source and behaves as an alternative fuel to the existing conventional fuel. In this paper, ethanol production by Simultaneous Saccharification and Fermentation (SSF) process from lignocellulosic materials of the soft wood (*Moringa oleifera*) has been performed by using the thermo-tolerant yeast *Kluyveromyces marxianus* MTCC 1338. This soft wood appears to be one of the best sources of renewable energy and it has given the highest yield when compared to other substrates. This paper investigates the production of bio-ethanol and the found optimum conditions were temperature at 42^{o}C, pH 4.6 and substrate concentration of 38 g/L. The results indicate the maximum ethanol concentration of 20.91(g/L) was obtained with the designed fermentation conditions.
Keywords: Lignocellulosic materials, Bio ethanol, *Moringa oleifera*, Simultaneous Saccharification and Fermentation (SSF), *Kluyveromyces marxianus*, Softwood

1. Introduction:

The unavoidable depletion of the world petroleum supply and the increasing problem of green house gases have strengthened the world wide interest in alternative, non-petroleum based sources of energy. The use of bio ethanol as fuel significantly reduces net carbon dioxide emission once it replaces conventional fuel system as fermentation derived renewable fuel is already a part of global carbon cycle. Lignocellulosic materials (LCM) are found to be attractive due to the non food portion of the plant that can be used for bio ethanol production[1]. Merits of LCM include its availability in abundance, independence of geographical

location, neutral carbon cycle and renewable source[2]. When compared to the first generation fuels such as starch based materials, LCM carries a complex heterogeneous structure which makes it difficult to hydrolyse the polysaccharide[3].

The annual production of lignocellulosic material is estimated in 1 X 10^{10} MT world wide which includes feedstock such as forest, agricultural crops and its residues (4).Lignocellulosic material is made up of a matrix of cellulose and hemicelluloses chains bound by lignin structure. Pre-treatment of LCM is carried out to break the cellulose and lignin matrix so that the degree of crystallinity of cellulose is replaced by its amorphous structure thus making it susceptible to quick hydrolysis with increase yield. Apart from various pre-treatment steps, acid and alkali based steps were found to be promising and effective in nature, as it enhances cellulose and hemicelluloses hydrolysis quickly.[5].

Simultaneous Saccharification and Fermentation (SSF) is favoured in most cases unlike Separate Hydrolysis and fermentation (SHF) in terms of higher ethanol yield, lower energy consumption and short processing time. In SSF, end product inhibition of the enzyme is avoided because fermenting organism immediately consumes the released sugars. Also capital investments are low as the total reactor volume is decreased due to higher rate of productivity.[6].

For good ethanol productivity, the optimum temperature for enzymatic hydrolysis is at 40-50˚C, but micro organisms usually does not tolerate this high temperature. In order to overcome this problem, thermo tolerant micro organisms such as *Zymomonas mobilis, Candida lusitaniae* and *Kluyveromyces marxianus* are used.[7] The main objective of using *Kluyveromyces marxianus* as thermo tolerant yeast is higher Saccharification yield, significant decreased risk of contamination and possibility of continuous ethanol removal[8].

2. Materials and Methods

2.1. Microorganism and maintenance:

Kluyveromyces marxianus strain MTCC 1338 was procured from the Microbial Technology culture collection (MTCC), Chandigarh (India). The strain was maintained on agar slant having the following composition: lactose, 20 g L^{-1}; bactopeptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; agar, 20 g L^{-1}. A 24 hr growth of the yeast was preserved at 4^{o}C.

2.2. Analytical methods:

2.2.1. Cellulose determination

The estimation of cellulose was carried out using anthrone method. 10 mL of anthrone reagent prepared by dissolving 200 mg of anthrone in 100 mL conc. sulphuric acid and chilled for 2 hrs before use, was added to 1 ml sample and mixed well. The tubes were heated in boiling water bath for 10 min, cooled and the intensity of color was measured at 630nm in a spectrophotometer[9].

2.2.2. Cell mass

The dry cell mass of sample was estimated by centrifuging the known volume of sample in a pre-dried and pre-weighed centrifuge tube for 20 min. After resuspension in 2 ml of distilled water, the sample was centrifuged and the settled cell mass was dried. The dried cell mass was calculated by reweighing the tube.[9].

2.2.3. Ethanol determination

The ethanol concentrations in the samples were measured using a gas chromatograph (Model 5765, Nucon gas chromatograph, Nucon Eng., India) with a poropak column (1/8 “ID, liquid-10% FFAP, solid-ch-WIHP, 80/100 mesh) and flame ionization detector. Nitrogen was used as the carrier gas. The temperatures of the injection port, oven and the detection port were 250^{o}C, 120^{o}C and 250^{o}C, respectively. For the analysis of liquid samples, 2 mL of liquid sample was withdrawn at various times (12 hr duration). From the fermentation broth, in gas tight syringes and then injected into GC. The standard chart to determine the ethanol concentration is shown in the graph[10].

2.2.4. Simultaneous Saccharification and Fermentation (SSF)

Simultaneous Saccharification and Fermentation (SSF) utilizes both cellulase enzymes and fermenting microbes in a single step which enables sugar production and fermentation into ethanol in one vessel. Simultaneous Saccharification of both carbon polymer, cellulose to glucose; and hemicelluloses to xylose and arabinose; and fermentation will be carried out by the yeast or organism which has the ability to utilize both C5 and C6 sugars. The accumulation of ethanol in the fermenter does not inhibit cellulase action as much as high concentration of glucose. Batch fermentation was performed in Erlenmeyer flasks in an anaerobic shaker at an agitation of 500 rpm. The temperature was controlled at 41^{o}C and the pH was maintained at 4.5 by frequent addition of sterile 6N NaOH. Samples were collected at an interval of 12 hrs. After recording absorbance at 580 nm, the remaining volume of the sample was centrifuged at 4800 rpm for 15 minutes. The supernatant was stored at 4^{o}C for lactose and ethanol estimation.

2.3. Pretreatment methods

Alkali and Acid treatment

20g each of the substrate were taken individually and treated with 320ml of 1% sodium hydroxide and 320 ml of 1% hydrochloric acid separately. Both the samples were kept at room temperature overnight. Then the samples were sterilised to remove any contaminations and thoroughly washed with water to remove traces of alkali and acids present, (if any). The samples were then placed in hot air oven and dried at 100^{o}C for about 2-3 days in order to remove the moisture.

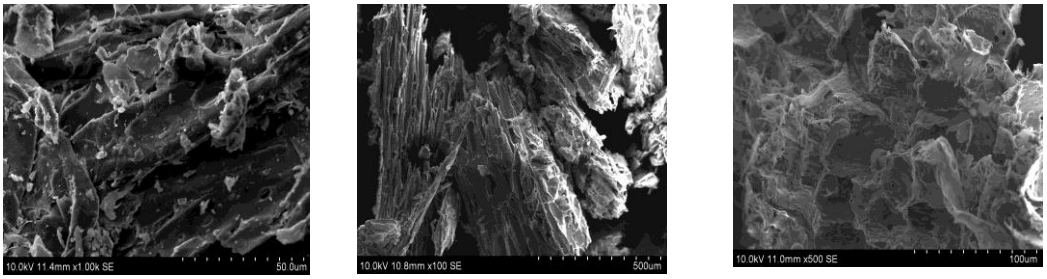
3. Results and Discussion:

3.1 Pretreatment of soft wood *Moringa olifera*

The surface morphologies of original *Moringa oleifera* bark were observed by scanning electron microscope with an accelerating voltage of 0.3-30KV.

The cellulose recovery after acid treatment was slightly higher than that after alkali pre-treatment. Cellulose concentration after treatment with HCl was found to be 60% while that after NaOH treatment was 56%.

The surface morphology (Fig. 1) of acid treated substrate is more porous than untreated and alkali treated substrate. This may be the reason for increased in bioethanol production by *Moringa oleifera* using acid pretreated substrate.



(a) Before pretreatment (b) Pretreated using 1% NaOH (c) Pretreated with 1% HCl
Fig. 1. SEM Images of untreated and pretreated substrate *Moringa oleifera* bark

The temperature, pH, substrate concentration are optimized in this work by monitoring of fermentation parameters at a time interval of 12 hours up to 108 hours and the optimum fermentation conditions are found graphically.

3.2. Effect of temperature:

The effect of temperature on biomass (Fig. 2), product concentration (Fig. 3) and substrate concentration (Fig. 4.) in the production of ethanol was studied.

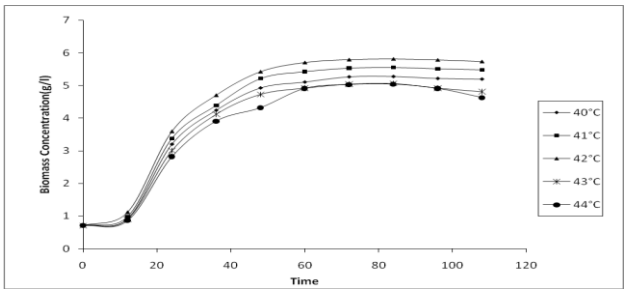


Fig. 2. Effect of temperature on biomass concentration in the production of bioethanol

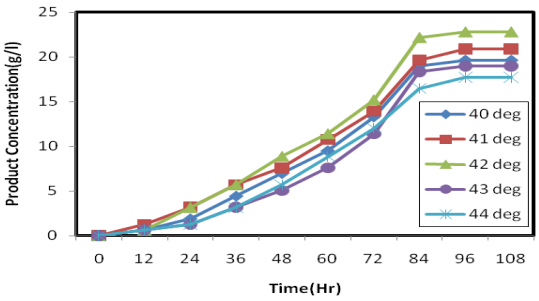


Fig. 3. Effect of temperature on product concentration in the production of bioethanol

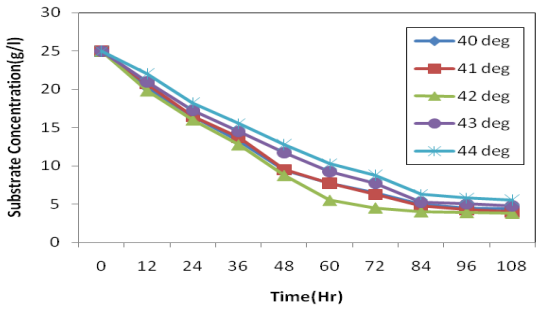


Fig. 4. Effect of temperature on substrate concentration in the production of bioethanol

Temperature is one of many important criteria in fermentation reactions. Only at an optimum temperature there can be maximum production of biomass and product with maximum utilization of the substrate. Thus from the above graphical results, it can be seen that the optimum temperature is 42^{o} C, only at which there is maximum product formation and biomass concentration. Substrate is also maximally utilized at this temperature.

3.3. Effect of pH:

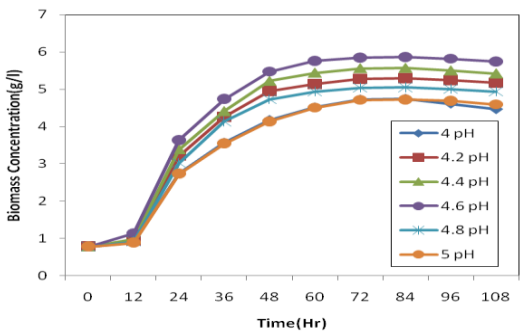


Fig. 5. Effect of pH on biomass concentration in the production of bioethanol

The effect of pH on biomass (Fig. 5), product concentration (Fig. 6.) and substrate concentration (Fig. 7) in the production of ethanol was studied.

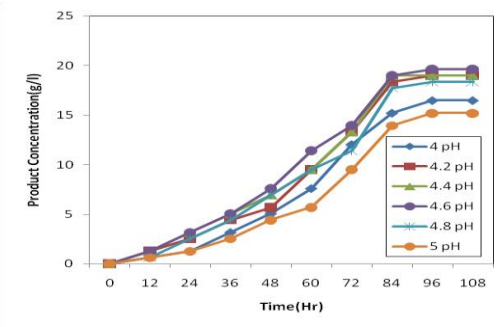


Fig. 6. Effect of pH on product concentration in the production of bioethanol

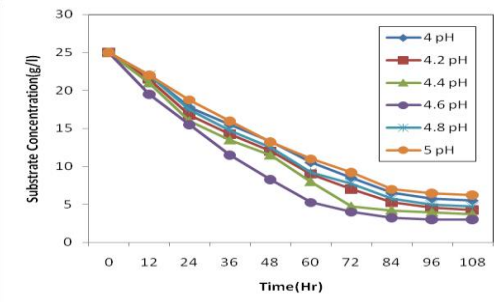


Fig. 7. Effect of pH on substrate concentration in the production of bioethanol

Concentration of the biomass is monitored at different time interval with the varying pH. The pH is varied from 4 to 5 at a constant increase of 0.2. Thus based on the substrate concentration, product formation and biomass production graphs, optimum pH is found to be 4.6. The fermentation process is carried out at this pH.

3.4. Effect of substrate:

The effect of substrate on biomass (Fig. 8), product concentration (Fig. 9) and substrate concentration (Fig. 10) in the production of ethanol was studied.

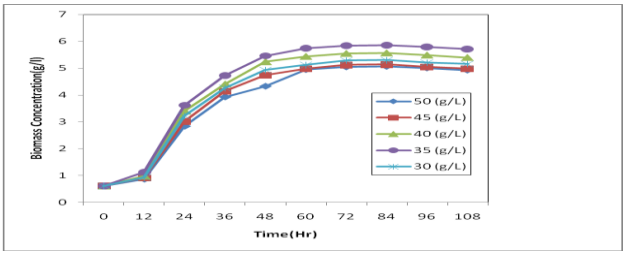


Fig. 8. Effect of substrate on biomass concentration in the production of bioethanol.

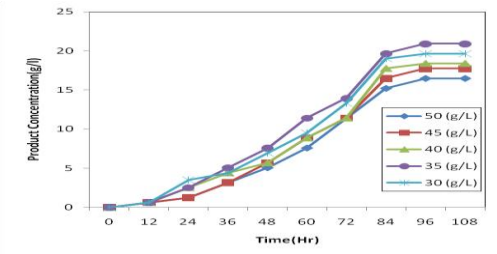


Fig. 9. Effect of substrate on the product concentration in the production of bioethanol

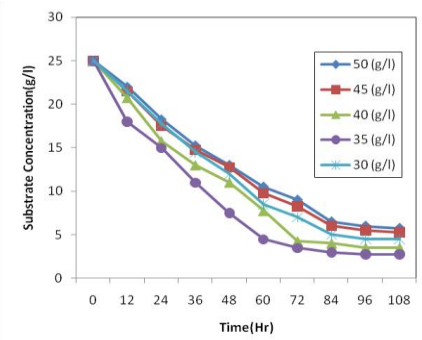


Fig. 10. Effect of substrate concentration in the production of bioethanol.

Substrate concentration too plays a vital role in the bioethanol conversion. Only when there is a maximum utilization of the substrate there can be proper conversion of the substrate to product and biomass. Thus it can be inferred form the above graphical discussions that at a substrate concentration of 25 g/L, there is maximum formation of biomass and product.

3.5. Effect of the concentrations in optimum conditions:

The graph for the biomass, substrate and product concentration at optimum conditions of temperature, pH and substrate concentration are given as a graphical representation below.

Effect of concentration in the production of bioethanol at optimum conditions (Fig.11)

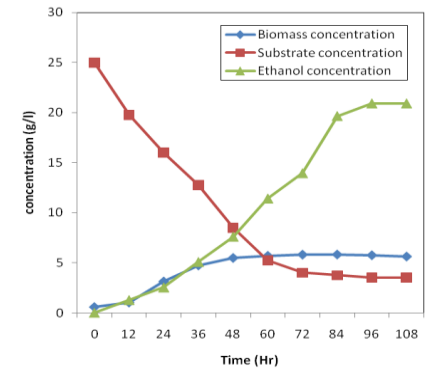


Fig. 11. Effect of concentration in the production of bioethanol at optimum conditions.

Sensitive analysis

Moringa oleifera when tested with *Kluyveromyces marxianus* MTCC 1388 showed a higher yield of ethanol at temperature of 42 ºC with a concentration of around 22.81g/l. The optimized pH with a yield of 19.65g/l was 4.6. Continuous increase in ethanol content occurred while the substrate content remained low, showing good yeast fermentation performance. This effect of substrate accumulation is not evident in many other biomass assays, where the substrate content increase at the end of the experiment is negligible. The presence of substrate in the media in the last stage indicates the cellulose activity continuation whereas the yeast fermentation has finished. Yeast performance may be affected both by very low concentration resulting in metabolic stress conditions and ethanol presence in fermentation media.

4. Conclusion

Based on the experimental results and the above mentioned advantages of this study, it is suggested that the thermo tolerant, ethanol producing yeast strain tested is proven to be a very novel candidate in the conversion of cellulose substrates to ethanol at 42 ºC. Nevertheless, it is assumed that yields obtained are relatively low for industrial ethanol production processes and that the further improvements in terms of increased ethanol yields, are necessary to achieve an economic process in a fed-batch basis, appears to be a promising means to increase final ethanol yields which ultimately when followed in a proper manner will

minimize the risks of global warming and acid rains, leaving the world green and a paradise for the future generation to live.

References:

1. Hui Li, Nag-Jong Kim, Min Jiang, Jong Won Kang, Ho Nam Chang,. Simultaneous saccharification and fermentation of lignocellulosic residues pre-treated with phosphoric acid-acetone for bioethanol production. Bioresource Technology, 2009, 13, 3245-3251.
2. Aloia Romaní, Gil Garrote, Juan Carlos Parajó. Bioethanol production from autohydrolysed *Eucalyptus globulus* by Simultaneous Saccharification and Fermentation operating at high solid loading. Fuel, 2011, d.o.i: 10.1016/j.fuel.2011.12.013.
3. Aloia Romaní, Gil Garrote, José Luis Alonso, Juan Carlos Parajó,. Bioethanol production from hydrothermally pretreated *Eucalyptus globulus* wood. Bio resource Technology, 2010, 101, 8706-8712.
4. Sánchez, Ó.J., Cardona, C.A.,. Trends in biotechnological production of fuel ethanol from different feed stocks. Bio resource Technology, 2008, 99, 5270-5295.
5. Nibedita Sarkar, Sumanta Kumar Ghosh, Satarupa Bannerjee, Kaustav Aikat,. Bioethanol production from agricultural wastes: An overview. Renewable energy, 2012, 37, 19-27.
6. Svetlana Nikolić, Ljiliana Mojović, Marica Rakin, Dušanka Pejin . Bioethanol production from corn meal by simultaneous enzymatic Saccharification and fermentation with immobilized cells of *Saccharomyces cerevisiae* var. *ellipsoideous*. Fuel, 2009, 8, 1602-1607.
7. Keikhosro Karimi, Giti Emtiazi, Mohammad J. Taherzadeh. Ethanol production from dilute acid pretreated rice straw by simultaneous Saccharification and fermentation with *Mucor indicus*, *Rhizopus oryzae* and *Sachharomyces cerevisiae*. Enzyme and Microbial Technology, 2006, 40, 138-144.
8. E.Tomás-Pejó, J.M. Oliva, A. González, I. Ballesteros, M.Ballesteros,. Bioethanol production from wheat straw by thermo tolerant yeast *Kluyveromyces marxianus* CECT 10875 in a simultaneous Saccharification and fermentation fed-batch process. Fuel, 2009, 88, 2142-2147.
9. Sadasivam S & Manickam A. Biochemical methods. New age international publishers, New Delhi 1996.
10. Lezinou V, Christokopoulous P, Li L W, Kekos D & Macris B J. Study of single and mixed culture for the direct bioconversion of sorghum carbohydrates to ethanol. Applied Microbial biotechnology. 1995, 43412-415.

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