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OPTIMIZATION OF FERMENTATION PARAMETERS FOR ENHANCED PRODUCTION OF BIOETHANOL USING *SACCHROMYCES CEREVISIAE*

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ABSTRACT

Sugarcane bagasse is one of the most promising agricultural by-products for conversion to Biofuel. Ethanol fermentation has been achieved using an integrated process combining chemical pre-treatment by acid-alkali with enzymatic hydrolysis and fermentation. The pre-treated cellulosic residue was hydrolyzed by a crude enzyme preparation from *Aspergillus flavus* KUMBF1308 containing complimentary β– glucosidase activity. This study thus demonstrated the potential use of simple integrated process with minimal environmental impact, with the use of promising alternative on-site enzyme and yeast, for the production of ethanol from this lignocellulosic biomass. The assessment of a novel on-site enzyme preparation on bagasse was reported in combination with an environmentally friendly pre-treatment process. The enzymatic hydrolysis process was used in simultaneous saccharification and fermentation (SSF) processes for evaluating the convertibility of the cellulose and hemicellulose contained in sugarcane bagasse to ethanol. In this investigation bioethanol production was optimized by *S. cerevisiae*. After analysis the reducing sugar content of 52gL^{-1} was recorded, after 72h of incubation, the ethanol yield was maximum at pH 5.5, temperature 32°C and 10% inoculum level. The work thus demonstrated the potential of this alternative process for the production of cellulosic ethanol from bagasse.

KEY WORDS: **Bioethanol, Hydrolysate, Saccharification, *Saccharomyces cerevisiae,* Sugarcane bagasse.**

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INTRODUCTION

The high worldwide demand for energy, fast depletion of petroleum resources, drastic increase in greenhouse gas emissions, expansion of human population, industrial prosperity, climatical changes globally, all have spurred a resurgence of interest in alternative fuels, especially liquid transportation fuels[1]. The significance of lignocellulosic ethanol stems from the possibility to use less costly feedstock, avoid direct and indirect competition with human food reserves and animal feed and reduce the environmental risk i.e. pollution, soil degradation linked to the first generation fuels. Lignocellulose is a complex carbohydrate polymer of cellulose (30-50%), hemicellulose (15-35%) and lignin (10-20%) [2,3]. Cellulose is a homopolysaccharide, composed of repeating β-D glucopyranose. It is linear and crystalline in nature. Hemicellulose is a heteropolysaccharide composed of pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose and galactose) and sugar acids. Lignin a complex polymer of phenyl propane, acts as a cementing agent and an impermeable barrier against enzymatic attack. Lignin provides plants with structural support and impermeability[4]. Because the lignocellulosic material have a robust structure, biomass fractionation step is essential to disrupt the macromolecular complexes, remove hemicellulose and/or lignin, and to increase the surface area accessible to the saccharification enzymes[5,6]. The natural structure of the polymer makes it tough for the microorganisms to utilize the material to produce ethanol. Therefore saccharification technologies are crucial steps in order to liberate fermentable sugars. Sequential enzymatic hydrolysis yield maximal sugar productivity and minimizes loss of sugar after efficient pretreatment[7]. Challenges that remain in the bioconversion process include the recalcitrance of the lignocellulosic matrix, the range of enzymes required to breakdown hemicellulosic material of both five and six carbon sugars and inhibiting effect that lignin and its derivatives have upon hydrolysis and fermentation stages of bioconversion[8].Sugarcane bagasse are classified under the agricultural residues among other raw materials i.e. forest and mill residues, disturbance wood, energy crops and marine algae that fall under lignocellulosic sources[9]. Besides its use as fuel for heating and generating power, its abundance and high cellulosic polysaccharide content makes it suitable for bioethanol production. The use of bagasse as feedstock for bio refinery has been limited due to the structure and high pentose fraction that makes it recalcitrant to enzymatic action unless it is pretreated to a more accessible form. The development of an intergrated process combining pre-treatment, enzymatic hydrolysis, optimization and fermentation is thus of great interest for efficient use of bagasse for ethanol production. Dilute acid, hot water, ammonia fiber explosion, steam explosion, wet explosion and use of an alkali are the current leading pretreatment technologies which were earlier investigated[10]. Saccharification is a critical step for bioethanol production for conversion of complex carbohydrate into simple monomers. Enzymatic hydrolysis requires less energy and mild environment. Cellulase enzyme involves endo and exoglucanase and β-glucosidases. Endoglucanase produces nicks in the cellulose polymer exposing reducing and non-reducing ends. Cellobiohydrolase removes the cellobiose units from the free chain ends and β- glucosidase completes the hydrolysis process by acting upon cellobiose to liberate glucose[11, 12]. The on-look for an efficient lignocellulolytic enzyme and optimization of hydrolysis conditions are due to variation in structure and composition of lignocellulosic substrate which have been the key issues in biomass technology research and together form a crucial foundation for successful process development for an alternative fuel and other products from cellulosic waste[13, 14]. The traditional yeast *S. cerevisiae* can easily ferment hexose sugars (glucose and galactose) to ethanol, but not pentose sugars (xylose and arabinose) present in lignocellulosic hydrolysate. It shows high ethanol productivity, can tolerate up to 14-16% (v/v) of ethanol and also can show tolerance to inhibitory compounds present in the hydrolysate[15]. *S. cerevisiae* has advantages such as, short generation time, can be easily cultured, maintenance is of low cost, also can be transformed allowing for either the addition of new genes or deletion. Therefore the objective of this study is to achieve high yields of fermentable sugars and ethanol production from lignocellulosic material through optimization.

MATERIALS AND METHODS

(i) Pretreatment

This is one of the most crucial methods in order to process the substrate to release the fermentable sugars. Sugarcane bagasse was treated with 2% H_2SO_4 at the ratio of 1g solid per 10 mL liquid and autoclaved for 45 min. They were separated by filtration and washed with tap water until baggase is neutralized and then treated with 2% NaOH at a ratio of 1g original raw material to 6 mL liquid. The solids were separated by filtering, washed with tap water until baggase is neutralized then dried at 105°C[16].

(ii) Enzyme production and hydrolysis

For enzymatic hydrolysis studies, *Aspergillus flavus* KUMBF1308 was cultured on Potato Dextrose Agar slants, incubated at 28°C for 7 days for spore development. 10g of rice straw was autoclaved for 30 min. To the substrate, 20 mL of distilled water was added, and then it was seeded with a spore suspension of *A. flavus* KUMBF1308. The flasks were incubated at room temperature for 72 h. At the end of the fermentation, the supernatant was harvested by centrifuging at 10,000 rpm for 10 min (4°C) and was used as crude enzyme. Cellulase enzyme production was studied by FPU assay[17]. The crude enzyme taken in different quantities (3,6 and 9 mL) were added to 250 mL Erlenmeyer flask containing the substrates, 50mM phosphate buffer (pH 7) and kept on a rotary shaker at room temperature for 72 h. The supernatant of the hydrolysate at different time interval *viz*, 2, 4, upto 12 h were taken for the estimation of reducing sugars by DNS method[18].

(iii) Fermentation medium

The hydrolysate obtained after saccharification with the crude enzyme was filtered and centrifuged to remove the solid wastes, as the pH was adjusted to 5.0 with either ammonium hydroxide or sulphuric acid before inoculation. The clear supernatant was then enriched with 1% molasses to add the macro and micro nutrients and fermentation was carried out at 28-30°C using *S. cerevisiae.*

(iv) Optimization of fermentation parameters

Parameters optima are essential in any type of fermentation process especially when using *S. cerevisiae* which is known to vary with respect to pH, temperature, substrate concentration, inoculum size, incubation period etc. It was imperative to optimize the fermentation conditions for the hydrolysate of the substrate that was used for fermentation by *S. cerevisiae* so that the production efficiency increases.

(v) Effect of incubation period

Effect of incubation time on ethanol fermentation was carried out at varying incubation period from 12 to 84 h.

(vi) Effect of pH on ethanol production

The effect of pH on ethanol production was performed according to Udhayaraja and Sriman, (2012) [15] with slight modification[15]. The hydrolysate pH was adjusted to 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 with either sodium hydroxide or sulphuric acid solution. It was inoculated with *S. cerevisiae* and fermentation was carried out upto 7 days at 28°C. The sample was analyzed for ethanol production.

(vii) Effect of temperature on ethanol production

S. cerevisiae (1%) was inoculated to the fermentation medium; pH was adjusted to 5.0 and incubated at different temperatures *viz*, 24, 28, 32, 36, 40°C for 7 days. Analysis of the ethanol yield was done after fermentation.

(viii) Effect of inoculum size on ethanol production

At pH 5.0 the hydrolysate extract was inoculated with 2.0, 4.0, 6.0, 8.0, 10.0% of yeast and kept for fermentation at 28°C for 7 days. Ethanol yield and the unfermented residual sugars were analyzed.

(ix) Ethanol estimation

Determination of ethanol content was done by spectrophotometric method (Caputi *et al.,* 1968) [19] while the fermentation efficiency was calculated as Ethanol produced/Theoretical maximum ethanol yield from sugar x 100

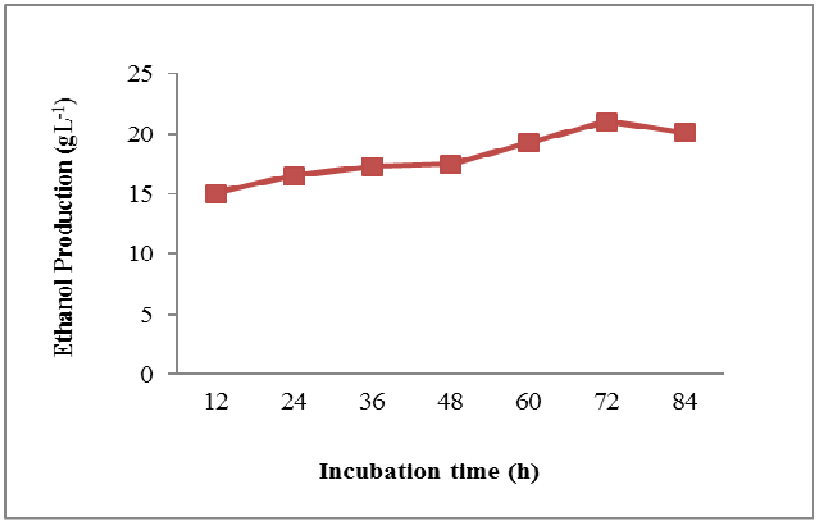
Theoretical maximum ethanol yield= 0.51g ethanol per gram.

RESULTS AND DISCUSSION

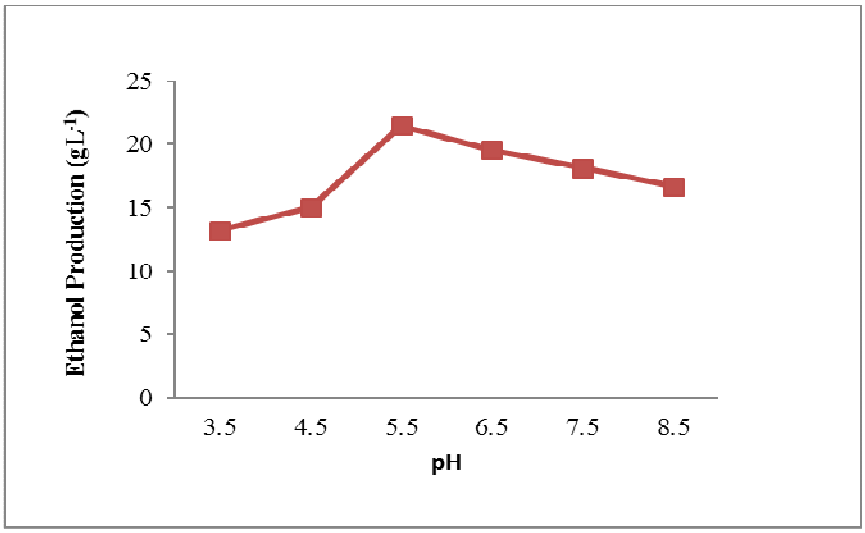
Due to easy adaptability of this fuel to the existing engines, a cleaner fuel with a higher octane rating than gasoline, bioethanol is being considered as an alternative fuel. Global warming, climatical changes, industrial development and prosperity and more factors have generated concern for this kind of fuel as an option over fossil fuels. The purpose of the pre-treatment was to remove lignin and /or hemicellulose, to disrupt the crystalline structure of cellulose and increase porosity of the substrate, making the material more accessible to enzyme attack.

Graph 1

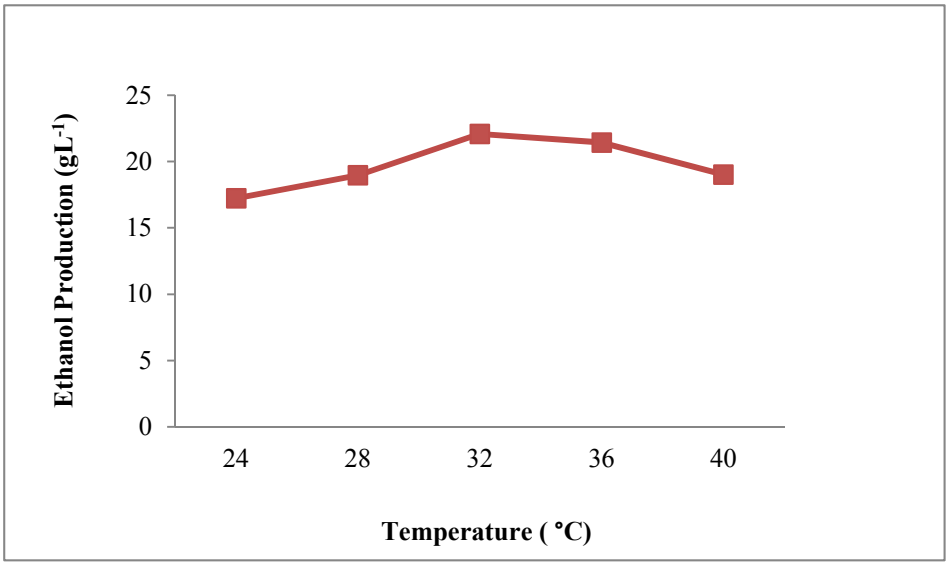
Effect of incubation time on ethanol production



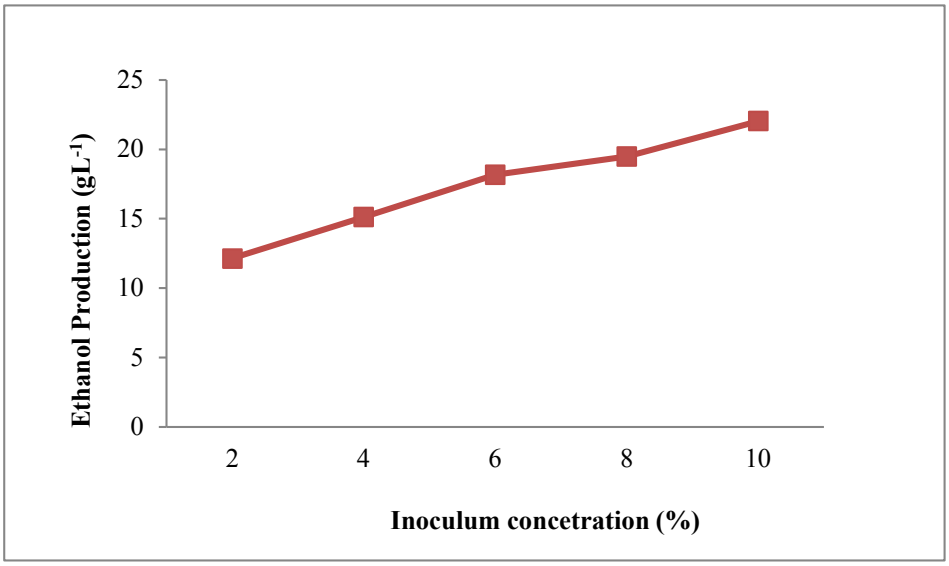
Graph 2
Effect of pH on ethanol production



Graph 3
Effect of temperature on ethanol production



Graph 4
Effect of inoculum size on ethanol production



In the present study, sugarcane bagasse was selected to find out its suitability for alcohol production and obtain a maximum yield of biomass based ethanol. By using an appropriate yeast strain, optimizing fermentation parameters and also developing viable technology, it is important to study ethanol production from sugarcane bagasse. Hurdles associated with the pre-treatment methods, hydrolysis and fermentation projected more improvement in order to lower the operating cost for an ethanol production plant. *S. cerevisiae* along with the crude cellulase enzyme on the acid/alkali pretreated hydrolysate exhibited maximum ethanol yield (0.404gg^{-1}) at 72 h of incubation (Graph-1; Table 1). In a bench scale solid state fermentation process carried out under optimal conditions within 61.5h Srichuwong *et al.,* (2009) [20] achieved 16.61 (v/v) ethanol yield. Arthe *et al*., (2008) [21] reported maximum alcohol content of about 8.9gL^{-1}after 72 h from a hydrolysed sample pretreated with 3% sulphuric acid.

Table 1
Effect of incubation time on ethanol production

Time (h)	Ethanol concentration (gL^{-1})	Ethanol yield (gg^{-1})	Fermentation efficiency (%)
12	15.12	0.291	57.06
24	16.53	0.318	62.35
36	17.31	0.333	65.29
48	17.54	0.337	66.08
60	19.32	0.372	72.94
72	21.03	0.404	79.22
84	20.14	0.387	75.88

The effect of pH on ethanol yield from sugarcane bagasse using *S. cerevisiae* was estimated and the results were furnished in (Graph-2; Table 2). The minimum yield was recorded at pH 3.5 (13.21g/L) while the maximum was at pH 5.5 (21.47g/L). Sreenant and Jeffries (2010)[22] reported at pH 4.5 a maximum of 5.8 (v/v) alcohol concentrations from aqueous carob. At pH 6 Panesar *et al*., (2001)[23] also observed maximum ethanol production of 3.19 per cent (v/v) and sugar utilization of 82.5 per cent with different tested pH *viz*, 4.5, 5.0, 5.5, 6.0 and 6.5.

Table 2
Effect of pH on ethanol production

pH	Ethanol concentration (gL^{-1})	Ethanol yield (gg^{-1})	Fermentation efficiency (%)
3.5	13.21	0.254	49.80
4.5	15.02	0.289	56.67
5.5	21.47	0.413	80.98
6.5	19.56	0.376	73.73
7.5	18.13	0.349	68.43
8.5	16.66	0.320	62.75

The results of the present study (Graph-3; Table 3) showed the highest ethanol yield was observed at 32°C (22.08g/L) while the lowest at 24°C (17.23g/L). At different temperatures *viz*, 25, 30, 35 and 40°C from starch Verma *et al*., (2000)[24] reported a maximum ethanol concentration of 21.8g/L at optimum temperature of 30°C in 48 h of fermentation. Wati *et al.,* (2007)[25] reported that 30°C was the optimum temperature for the production of ethanol (31.6g/L) with different temperatures from alkali treated paddy straw.

Table 3
Effect of temperature on ethanol production

Temperature (ºC)	Ethanol concentration (gL^{-1})	Ethanol yield (gg^{-1})	Fermentation efficiency (%)
24	17.23	0.331	64.90
28	18.96	0.365	71.57
32	22.08	0.425	83.33
36	20.43	0.393	77.05
40	19.01	0.366	71.76

The effect of the inoculum size for maximization of ethanol yield that was studied during the research work (Graph-4; Table 4) shows the level is increasing with inoculum size. Nimbkar *et al.,* (1987)[26] obtained a maximum alcohol concentration of 12.45 and 12.23 (v/v) at 6% and 2% respectively. The finding of their research coincides with the present study by giving a maximum ethanol yield at the maximum inoculum level at 10%.

Table 4
Effect of inoculum size on ethanol production

Inoculum size (%)	Ethanol concentration (gL^{-1})	Ethanol yield (gg^{-1})	Fermentation efficiency (%)
2	12.14	0.233	45.69
4	15.12	0.291	57.06
6	18.16	0.349	68.43
8	19.48	0.375	73.53
10	22.03	0.424	83.14

CONCLUSION

In this study, an alternative process for ethanol fermentation of bagasse was demonstrated. High ethanol yield was obtained from a simple process employing acid-alkali pre-treatment, enzymatic saccharification and fermentation. The use of local microbial strain for the onsite production of crude enzyme and the development of fermentation process by optimizing its parameters have been considered a platform for promising utilization of biodiversity resources for the establishment of sustainable microbiology based industry. Further improvement would include the reduction of the inhibitors during the pre-treatment step by simple detoxification and also increasing the nutrient level for the microorganisms by using molasses as the supplement in place of nitrogen and metal ions i.e casein, albumin, peptone, urea, $MgSO_4$, CaCl, KCl, which would lead to the increase of ethanol production efficiency from this potentially valuable lignocellulosic agricultural residues.

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