Pakistan J. Zool., vol. 40(2), pp. 99-103, 2008.

Ethanologenic Potential of Thermophilic Bacterium and Yeast From Acid Saccharified Sugarcane Bagasse

NAUREEN CHAUDHARY AND JAVED I. QAZI*

Environmental Microbiology Laboratory, Department of Zoology, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan.

Abstract.- Prospective renewable fuel supply has taken the potential of ethanol as one of the reliable alternatives for increasingly depleting fossil fuels. This paper reports the ethanologenic potential of the bacterium, *B. cereus* and yeast (*S. cerevisae*) from aqueous extract and acid hydrolysates of sugarcane bagasse. Aqueous extract of the substrate was found to contain 68, 24.4 and 7.7 mg/ml of total sugars, glucose and xylose, respectively. The corresponding values following mild acid digestion of the aqueous extracted bagasse were found to be 31.4, 22.42 and 3.7 mg/ml. This clearly indicated the presence of valuable soluble as well as acid saccharifiable sugars in the substrate. The bacterium and yeast grew and produced ethanol in the aqueous extract as well as acid hydrolyzates. Above 0.7% ethanol yield in batch culture conditions employing sugarcane bagasse acid hydrolysates is suggestive to develop the process by optimizing various factors that may enhance the yield and growth of the microorganisms as well. In this regard isolating ethanologenic microorganisms capable to resist or detoxify acid hydrolysis allied inhibitory substances appears imperative for obtaining the biofuel from cellulosic materials.

Keywords: Acid hydrolysis of cellulose, Ethanol, Sugarcane bagasse, *B. cereus. S. cerevisae*

INTRODUCTION

Production of ethanol from renewable resource, such as lignocellulosic materials, including wood forest and agriculture residues is being considered an alternative resource to fossil fuels (Bergeron *et al*., 1989; Galbe and Zacchi, 2002). Among the agriculture residues, sugarcane bagasse is a substrate of high potential for biotechnological processes. The polymeric material comprises 44 to 49% cellulose, 24 to 28% hemicellulose and 10 to 14% lignin (D'Arce *et al*., 1985; Palmqvist and Hahn-Hagerdal, 2000; Rodrigues *et al*., 2001). Owing to its very stable nature, hydrolysis of the bagasse is required for any level of its bioconversion. This can be achieved either by chemical treatments, or by employing microorganisms (Pessoa Jr. *et al.*, 1997; Neureiter *et al*., 2002; Nilsson *et al*., 2005). The saccharified substrate can then be fermented to yield ethanol mainly by employing yeast inoculation.

For microbiological saccharification microbes of specific characteristics are required. The bacteria capable of growing on the native substrate with minimum chemical supplement requirements and with ethanol approaching >0.3 % (w/v) are being considered potential candidates for saccharification and fermentation of cellulose rich agriculture wastes (Hari and Chowdary, 2000). However, the commodity can be mobilized by chemical attack. By applying the arkenol process using highly concentrated sulphuric acid, various biomass feedstock, including cedar tree, rice straw, newspaper and bagasse have been successfully processed and converted into glucose and xylose for fermentation (Yamada *et al.*, 2002).

Bioethanol can contribute to a cleaner environment and with the implementation of environment protection laws in many countries, demand for this is increasing (Zaldivar *et al.*, 2001). Brazil is the largest producer of ethanol and sugarcane is the main raw material. Bioethanol is produced by both batch and continuous processes and in some cases, flocculating yeast is used (Zanin *et al*., 2000). Production of ethanol from lignocellulosic biomass is achieved through hydrolysis of cellulose to produce reducing sugars which are subsequently fermented to ethanol (Sun and Cheng, 2002) . Advancements that have taken place to convert xylose efficiently to ethanol by

* Corresponding author: e-mail: qazi@scientist.com

0030-9923/2008/0002-0099 $ 8.00/0

xylose-fermenting microorganisms are promising for economical consideration of the bioconversion process (Chandarkant and Bisaria, 1998; Chaudhary and Qazi, 2006a).

In Pakistan sugarcane is a popular and attractive crop. The commodity is mainly used for sugar production. Huge amounts of sugarcane bagasse are generated and wasted every year. The present study was thus undertaken to initiate steps that may lead to bioconversion of this agricultural residue to value added products. Results of the present study demonstrate the extraction of soluble sugars and then acid hydrolysis of the washed sugarcane bagasse to yield the monomeric sugars. Ethanologenic potential of the fermentative bacterium *B. cereus* (NB-19) and the yeast, *S.cerevisae* (NY-2) employing these sugar syrups has also been worked out.

MATERIALS AND METHODS

Processing of bagasse

Ten gram of dried powdered sugarcane bagasse was suspended in 500ml of distilled water and autoclaved at 15 lb/inch^2 for 20 minutes. The material was then allowed to sediment for 15 minutes at room temperature. The aqueous phase was carefully separated and saved for further analysis. The soaked bagasse was then suspended in 500ml of 0.54% H_2SO_4 and autoclaved as described above. The acid phase was separated and saved. The same procedure was repeated for three times. Each experiment was performed in quadruplicates. The liquid phase, 10ml, of each of the above experiment was processed for determination of glucose, total sugars, xylose, proteins and fat contents as described by Hartel *et al*. (1969), Dubois *et al*. (1956), Raphael (1976), Lowry *et al*. (1951) and Zöllner and Kirsch (1962), respectively. The solid remnants from the last experiment were only processed for the measurement of fiber content according to the method of Ranganna (1986).

Source of microorganisms

The aqueous and acid extracts were fermented employing the bacterial isolate, *B. cereus* NB-19 and the yeast, *S. cerevisiae* NY-2. The microorganisms were obtained from the culture collection of local isolates maintained in this laboratory. They were revived on nutrient agar. The bacterium has earlier been identified, while the yeast was designated in the previous paper as NS-6 (Chaudhary and Qazi, 2006b). The yeast was characterized for its vegetative reproduction, sexual reproduction, ascospore's enumeration and morphology, vegetative structure, nitrate utilization, urea hydrolysis, glucose and starch fermentation, and growth at 25°C, 37°C and 45°C after the Benson (1994) and Merck (1996-1997). The yeast was then identified as described by Barnett *et al*. (2000). Optimum conditions for the growth of yeast were 50°C, 10% inoculum size and pH 7 and it needed aeration. The bacterium on the other hand, had been described to grow optimally at 50°C, pH 6, 1% innoculum size and had requirement of aeration (Chaudhary and Qazi, 2006b).

Ethanol fermentation

For ethanol fermentation, the aqueous and first and second acid extracts were used only. Third and fourth acid extracts contained lesser carbohydrate contents and hence were not used for ethanol fermentation. For inocula preparation a separate colony of a given microorganism from the nutrient agar plates was inoculated into 10 ml of respective aqueous/acid extract and incubated at 50°C for 48 hours. Fifty ml of a given extract pH 6-7 in 100ml culture bottle was inoculated with optimum inocula of microorganism and incubated at 50°C at 100 rpm in an orbital shaker for 48 hours. The culture bottles were then fitted with air tighten rubber hungs fitted with 5ml plastic syringes with their pistons adjusted at zero readings and incubated at 50°C without aeration. The bottles were checked daily for the piston positions and volume filled with fermentative gases was recorded. On fourth day of non aerated incubations, 10ml sample from each bottle was withdrawn with the help of fitted syringes. The culture bottles were again incubated as mentioned above and after another 4 days the samples from the cultures were taken out in the same way. The first and the second samples were processed for determination of growth (O.D.), glucose (Hartel *et al*., 1969) and xylose (Raphael, 1976) contents. Then 1 ml of each sample was proceeded for alcohol estimation. The ethanol was

estimated by oxidation in diffusion vessel employing 0.43% $K_2Cr_2O_7$ and 50% sulphuric acid as described by Snell and Snell (1973).

RESULTS AND DISCUSSION

The exposure of dried bagasse to steam under pressure revealed that this waste contains appreciable amounts of soluble sugars. The total sugar content were upto 68 mg/ml, whereas glucose and xylose contents were 25.4 and 7.7 mg/ml, respectively. The aqueous extract was also found to contain 17.7 and 9.4 mg/ml of protein and fat contents, respectively (Table I). In a comparable study, Saska and Ozer (1995) reported 60% to 89% yield of xylose, mostly in the form of a water soluble xylan from aqueous extraction of sugarcane bagasse at optimum level of severity. These results clearly indicate that the mechanical milling process fails to extract all the contents from sugarcane and the valuable materials go waste along with bagasse, which can easily be extracted by simple aqueous treatment at raised temperature. Because of high ambient temperature for most part of the year in this country, solar heat can be used as economically feasible methods to obtain soluble sugars from bagasse.

When the aqueous extracted bagasse was subjected to mild acid digestion at elevated temperature, the solution was found to contain 31.4, 22.42 and 3.7 mg/ml of total sugars, glucose and xylose, respectively. The acid hydrolysate also contained 1.6mg/ml of protein, while the fat became non detectable. Total and the monomeric sugars turnover decreased, in general, retrogressively in the 2nd, 3rd and 4th attempt (Table I). However, total sugars in the second attempt turned out to be 36.6mg/ml, which was 16.56% higher than the one obtained in the first attempt of acid hydrolysis. This could be because the previous acid exposure led to more access for acid attack during the second attempt. During 3rd attempt of acid hydrolysis the yield of total sugar, glucose and xylose was 57%, 80% and 62% less when compared with the corresponding values obtained at first acid hydrolysis. Similarly, after third attempt these ingredients decreased respectively, 63%, 34% and 52% compared with the second round of acid hydrolysis. The fourth round of hydrolysis could yield only 1.10 and 0.23 mg/ml of glucose and xylose, respectively. Since 1st and 2nd round of the mild acid hydrolysis could yield workable levels of sugars, only these solutions were used in the subsequent ethanol fermentation experiments, besides the aqueous extract. Bagasse remnants from the fourth time acid hydrolysis had only 0.19% fiber content. As already reported from other laboratories, less time required for acid hydrolysis of lingocellu-losic materials (Taherzadeh *et al*., 1997) and lesser cost as compared to enzymatic hydrolysis (Sheehan and Himmel, 2001) are main advantages of the process.

When the sugar solutions obtained from the aqueous extracts and the first two acid hydrolysates were fermented with ethanologenic bacterium and yeast in batch cultures it appeared that the *B. cereus* grew very weakly and produce ethanol upto 0.113% after 4 days of fermentation and consuming about 62% and 93% of glucose and xylose contents, respectively (Tables I, II). However, the yeast produced about 0.23% ethanol at the second sampling period in the aqueous extract.

It is very interesting to note that initial reduction in glucose and xylose contents observed on 4th day of bacterial and yeast fermentations were followed by appreciable increases on 8th day of fermentations. In case of bacterial culture the glucose and xylose contents were measured as 22 and 0.85 mg/ml, respectively as compared with the corresponding values of 9.46 and 0.52 mg/ml at the first sampling period. For yeast culture glucose and xylose contents at the second sampling period turned out to be about 22 and 0.96 mg/ml, respectively. However, these values were 64.58% and 44% higher than the corresponding values of the culture fluids sampled at the first observational period (Table II). The increases found for glucose and xylose contents between the two sampling periods in the case of microbial cultures for the acid hydrolysates also appeared in aqueous extracts and depicted more or less comparable trends. The increase in the monomeric sugars at the second sampling period indicates the sccahrification potential of these microbes from the cellulosic material. The microorganisms had already been reported to grow and ferment in media containing intact bagasse (Martin, 2002; Chaudhary and Qazi, 2006b). When the acid hydrolysates were fermented

the bacterium could produce ethanol upto 0.25% at the first sampling period, whilst the yeast gave a yield of about 0.7% on 8th day of the fermentation of

Table I.- Aqueous extract and acid hydrolysed sugar, protein and fats contents (mg/ml) of sugarcane bagasse at different attempts.

Extract	Attempts	Total sugar	Glucose	Xylose	Protein	Fats	Fiber %
Aqueous extract	1st	68.10±22.5	25.4±1.64	7.7±1.3	17.7±2.7	9.4±1.24	-
Acid digestion	1ST	31.4±9.4	22.42±2.56	3.7±0.25	1.6±0.31	N.D	-
	2ND	36.6±16.2	6.86±0.29	2.89±0.31	N.D	N.D	-
	3RD	13.5±.36	4.49±2.29	1.37±0.174	-	-	-
	4TH	N.D	1.10±0.10	0.23±0.027	-	-	0.19±0.017

Values are means of four replicates ± S.E.M. N.D =not detectable **,** - = test not done

Table II.- Ethanol fermentation (%) of aqueous extract and acid hydrolysed sugar solutions (mg/ml) of sugarcane bagasse by *B. cereus* NB-19 and *S. cerevisiae* NY-2 cultivated at their optimum growth conditions on 4th and 8th day of incubation.

Microorganism	Days	Aqueous extract				Attempts	Acid hydrolysis			
		Growth (O.D)	Ethanol (%)	Glucose	Xylose		Growth (O.D)	Ethanol (%)	Glucose	Xylose
***B. cereus* NB-19**	4	N.D	0.113 ±0.02	9.46 ±1.7	0.52 ±0.01	1st	0.029 ±0.0695	0.25 ±0.046	9.81 ±2.69	0.56 ±0.15
						2nd	0.0165 ±0.006	0.211 ±0.026	3.28 ±3.08	0.22 ±0.012
	8	0.002 ±0.009	0.023 ±0.015	22 ±2.3	0.848 ±0.054	1st	0.038 ±0.0115	0.122 ±0.034	14.6 ±2.68	2.29 ±0.21
						2nd	-0.006 ±0.0085	0.146 ±0.085	4.43 ±0.17	0.88 ±0.13
***S. cerevisiae* NY-2**	4	0.0185 ±0.0065	0.132 ±0.039	0.33 ±0.035	0.528 ±0.005	1st	0.040 ±0.0105	0.193 ±0.049	6.93 ±1.54	0.54 ±0.007
						2nd	0.0167 ±0.0065	0.081 ±0.0176	11.4 ±0.46	0.2 ±0.02
	8	0.027 ±0.011	0.226 ±0.032	21.64 ±3.37	0.964 ±0.076	1st	0.049 ±0.014	0.674 ±0.38	14.01 ±3.11	1.62 ±0.13
						2nd	N.D	0.058 ±0.028	5.07 ±0.58	1.21 ±0.35

Values are means of four replicates ± S.E.M. N.D = not detectable**,** - = test not done

first acid hydrolysate. Chaudhary and Qazi (2006b) have reported that *S. cerevisae* (NS6) could produce upto 0.42% ethanol, while fermenting the bagasse presaccharified with the help of cellulolytic *B. cereus* strain with initial glucose content of 0.94mg/ml. Comparing the differing ethanologenic potentials of *S. cerevisae* NY-2 in acid hydrolyzed bagasse in this study and the yield that has been reported in microbially saccharified bagasse (Chaudhary and Qazi, 2006b). It may be concluded that microbial saccharification processes for cellulosic biomass appear more promising for the product yield as well as regarding environmental issues.

REFERENCES

BARNETT, J.A., PAYNE, R.W. AND YARROW, D., 2000.*Yeast characteristics and identification.* Cambridge University Press. UK. Pp. 23-85, 630-656.

BENSON, H.J., 1994. *Microbiological application, Laboratory manual in general microbiology.* W.M.C. Brown Publishers, Dubuque, U.S.A.

BERGERON, P. W., WRIGHT, J. D. AND WYMAN, C.E., 1989. Dilute acid hydrolysis of biomass for ethanol production. *Ener. Biomass Wast.*, **12**:1277-1296.

CHANDRAKANT, P. AND BISARIA, V.S., 1998. Simultaneous bioconversion of cellulose and hemicellulosic to ethanol. *Crit. Rev. Biotechnol.,* **18:** 295-331.

CHAUDHARY, N. AND QAZI, J.I., 2006a. Potential of locally isolated *Saccharomyces cervisiae* PUZ1Y strain to ferment xylose to ethanol. *Mycopath,* **4**: 5-8.

CHAUDHARY, N. AND QAZI, J.I., 2006b. Microbiological saccharification and ethanol production from sugarcane bagasse. *Biotechnology,* **5**: 517-521.

D'ARCE, R.D., BOIN, C. AND MATTOS, W.R.S., 1985. *Utilizacao de Residuos Agroindustriais da cana-de-Acucar na Alimentacao de Ruminantes*. Fundacao de Estudos Agrarios Luiz de queiroz, Piracicaba, Brazil.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. AND SMITH, F., 1956. Colorimetric methods for determination of sugars and related substances. *Anal. Chem.,* **28:** 350-356**.**

GALBE, M. AND ZACCHI, G., 2002. A review of the production of ethanol from softwood. *Appl. Microbiol. Biotechnol.,* **59**:618-628.

HARI, K.S. AND CHOWDARY, G.V., 2000. Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic biomass. *J. Agric. Food. Chem.* **48**:1971-1976.

HARTEL, A., HELGER, R. AND LANG, H., 1969. A method for determination of glucose. *Z. klin. Chem. klin. Biochem.,* **7:** 183.

KRISHAN, M.S., BLANCO, M., SHATTUCK, C.K., NGHIEM, N.P. AND DAVSION, B.H., 2000. Ethanol production from glucose and xylose by immobilized *Zymomonas mobilis* CP4(PZB5). *Appl. Biochem. Biotechnol.,* **84-86**: 525-541.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.J. AND RANDALL, R.J., 1951. Protein measurements with the folin phenol reagent. *J. biol. Chem.,* **193:** 265-275

NILSSON, A., GORWA-GRAUSLUND, M.F., HAHN-HAGERDAL, B. AND LINDEN, G., 2005. Cofactor dependence in furan reduction by *Saccharomyces cerevisiae* in fermentation of acid-hydrolyzed lignocellulose. *Appl. environ. Microbiol.,* **71**: 7866-7871.

MARTIN, C., GALBE, M., NILVEBRANT, N.I. AND JÖNSSON, L.J., 2002. Comparison of the fermentability of enzymatic hydrolyzates of sugarcane bagasse pretreated by steam explosion using different impregnating agents. *Appl. Biochem. Biotechnol.,* **98-100:** 699-716.

MERCK, 1996-1997. *Microbiology manual*. Merck KGA, Darmstadt, Germany.

NEURIETER, M., DANNER, H., THOMASSER, C., SAIDI, B. AND BRAUN, R., 2002. Dilute acid hydrolysis of sugarcane bagasse at varying conditions. *Appl. Biochem. Biotechnol.,* **98-100**: 49-58.

PALMQVIST, E. AND HAHN-HÄGERDAL**.** B., 2000. Fermentation of lignocellulosic hydrolysates. II. Inhibitors and mechanisms of inhibition. *Bioresour. Technol*., **74:**25-33.

PESSOA, J.R., MANCILHA, I.M. AND SATO, S., 1997. Acid hydrolysis of hemicellulosic from sugarcane bagasse. *Braz. J. chem. Eng.,* **14**: Sao Paulo Sept. www.scielo.br

RANGANA, S., 1986. *Analysis of fruits and vegetables products*. Tata McGraw Hill Pub. Comp. Ltd. New Dehli.

RAPHAEL, S.S., 1976. *Lynch's Medical laboratory technology*. W.B. Saunders Co. Tokyo Japan.

RODRIGUES, R.C.L.B., FELIPE, M.G.A., ALMEDIA, E., SLIVA. J.B., VITOLO, M. AND GOMEZ, P.V., 2001. The influence of pH, temperature and hydrolyzate concentration on the removal of volatile and non volatile compounds from sugarcane bagasse hemicellulosic hydrolysate treated with activated charcoal before or after vacuum evaporation. *Braz. J. chem. Eng.,* **18**: Sao Paulo. www.scielo.br

SASKA, M. AND OZER, E., 1995. Aqueous extraction of sugarcane bagasse hemicellulose and production of xylose syrup. *Biotechnol. Bioeng.,* **45:** 517-523.

SHEEHAN, J.S. AND HIMMEL, M.E., 2001. "Outlook for bioethanol production from lignocellulosic feed stocks: Technology hurtles". *Agro. Food Ind. Hi. Tech.,* **12**: 54-57.

SNELL, F.D. AND SNELL, C.T., 1973. *Colorimetric methods of analysis. Volume III A.* Robert K. Krieger Publishing Co.

SUN, Y. AND CHENG, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.,* **83**: 1-11.

TAHERZADEH, M. J., EKLUND, R., GUSTAFSSON, L., NIKLASSON, C. AND LIDÉN, G., 1997. Characterization and fermentation of dilute-acid hydrolysates from wood. *Ind. Eng. Chem. Res.,* **36:** 4659-4665.

YAMADA, T., FATIGATI, M.A. AND ZHANG, M., 2002. Performance of immobilized *Zymomonas mobilis* 31821 (PZB5) on actual hydrolysate produced by Arkenol technology. *Appl. Biochem. Biotechnol.,* **98-100**: 899-907.

ZALDIVAR, J., NIELSEN, J. AND OLSSON, L., 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic enginnering and process integration. *Appl. Microbiol. Biotechnol.,* **56**: 17-34.

ZANIN, G.M., SANTANA, C.C., BON, E.P., GIORDANO, R.C., DE, MORAES, F.F., ANDRIETTA, S.R., DE CARVALHO, NETO, C.C., MACEDO, I.C., RAMOS, L.P.O. AND FONTANA, J.D., 2000. Brazilian bioethanol program. *Appl. Biochem. Biotechnol.,* **84-86**: 1147-1161.

ZÖLLNER, N. AND KIRSCH, K., 1962. Micro determination of lipids by the sulphophosphocanillin reaction. *Z. Ges. exp. Med.,* **135:** 545-561.

(Received 18 May 2007; revised 9 January 2008)