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Microbiological Saccharification and Ethanol Production from Sugarcane Bagasse

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Abstract: Four strains of *Bacillus cereus* were grown in 5% sugarcane bagasse soaked in water (MI) and 2% bagasse, 0.6% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.075% $MgSO_4$ and 0.35% KH_2PO_4 and 0.2 mL of trace element solution comparing of 1.5% EDTA, 0.45% $ZnSO_4$, 0.1% $MnCl_2$, 0.45% $CaCl_2$, 0.3% $FeSO_4.7H_2O$ and 0.01% KI (M-II). The bacteria showed good growth in both the media. Then these bacteria were optimized for growth conditions in M-III medium comprising of 2% sucrose, 0.6% yeast, 0.1% $(NH_4)_2SO_4$, 0.075% $MgSO_4$, 0.35% KH_2PO_4 and 0.2 mL of trace element solution comprising of 1.5% EDTA, 0.45% $ZnSO_4$, 0.1% $MnCl_2$, 0.45% $CaCl_2$, 0.3% $FeSO_4.7H_2O$ and 0.01% KI. All the four strains grew best at pH 7 and 37°C. Aeration was not required for these bacterial strains. 5% inocula sizes were found optimum for the bacterial isolates except strain No. 23, which yielded best growth with 10% inoculum. After 2nd day of incubation in M-IV medium the bacteria liberated upto 8033 mg/100 mL of total sugar content, which showed reduction in subsequent sampling periods. On the other hand glucose content showed variable response to incubation periods. The strain 11a expressed 1411 mg/100 mL of glucose at 10th day while isolate No. 23 yielded 808 mg/100 mL glucose at 18th day of incubation. Fermentation by ethanologenic yeast of culture fluids saccharification by the isolate 19_4 and 23 expressed about 0.2 and 1.2% ethanol on 3rd and 7th days, respectively. The bacterial strains were identified as *Bacillus cereus* based upon their chemical characterization. These isolates appear potential candidates for saccharifying the sugarcane bagasse and production of ethanol employing ethanologenic yeast.

Key words: *Bacillus cereus*, cellulose degradation, ethanol fermentation

INTRODUCTION

Among the agricultural residues, sugarcane bagasse is a substrate of high potential for biotechnological processes. The polymeric material comprises of 44 to 49% cellulose, 24 to 28% hemicelloluse and 10 to 14% lignin (D'arce *et al*., 1985; Rodrigues *et al*., 2001). Owing to its very stable nature, for any level of bioconversion hydrolysis of sugarcane bagasse is required. This can be achieved either by chemical treatments, or by employing microorganisms (Pessoa *et al*., 1997; Neureiter *et al*., 2002).

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 reasonable to high yield of sugars are being considered potential candidates for saccharification of cellulose rich agriculture wastes. (Hari and Chowdary, 2000). The saccharified substrate can then be fermented to yield ethanol mainly by employing yeast inoculation.

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). 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). Brazil is the largest producer of ethanol and sugarcane is main raw material. Bioethanol is produced by both batch and continuous process and in some cases, flocculating yeast is used (Zanin *et al*., 2000). Production of ethanol from lignocellulosic biomass is achieved through hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars which are subsequently fermented to ethanol (Sun and Cheng, 2002). A lot of attention has been focused on conversion of both glucose and xylose to ethanol. Advancements that have taken place to convert xylose efficiently to ethanol by xylose-fermenting microorganisms are promising for economical consideration of the bioconversion process (Chandarkant and Bisaria, 1998).

In Pakistan sugarcane is a popular and attractive crop. Mainly the commodity is used for sugar production. So huge amounts of sugarcane bagasse are generated and wasted. The present study was thus undertaken to initiate

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steps that would lead to bioconversion of this agricultural residue to value added products. Results of the present study report growth optimization of four bacterial strains capable of saccharifying sugarcane bagasse. The hydrolyzed material was then fermented with the help of yeast into ethanol. Bioethanol is an attractive, sustainable energy resource to fuel transportation

MATERIALS AND METHODS

Four strains of cellulose hydrolyzing bacteria, already isolated in this laboratory, were used in study. Their cultures were revived on nutrient agar. They were characterized and identified by Holt *et al.* (1994). They were then inoculated in medium M-I that contained 2.5 g powdered sugarcane bagasse in 50 mL of water. The bagasse was prepared from extracted sugarcane. It was dried in sunlight and then crushed by electric mill and the powder was kept in oven at 105°C till consistent weight was achieved. The mixture was autoclaved at 15 lb/inch^2 for 20 min. The inoculated bagasse was incubated at (37°C±1).

Growth optima: Bacteria from nutrient agar plates were grown in M-II medium comprising of 2% bagasse, 0.6% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.075% $MgSO_4$, 0.35% KH_2PO_4 and also contained 0.2 mL of trace element solution containing 1.5% EDTA, 0.45% $ZnSO_4$, 0.1% $MnCl_2$, 0.45% $CaCl_2$, 0.3% $FeSO_4.7H_2O$ and 0.01% KI. The cultures were observed under microscope daily upto 4 days post inoculation to assess growth and activity of bacteria. For optimization of growth the bacterial cultures from M-II medium were inoculated in medium M-III. The M-III comprised of same ratio of ingredients as that of M-II except that it contained 2% sucrose instead of bagasse. All the experiments were performed in triplicates and each replicate contained 5 mL of inoculated medium. Bacterial growth was assessed at pH 5, 7 and 9. The bacteria were then grown at room temperature (34°C±1) and 50°C at their corresponding optimum pH. The growth was also checked on orbital shaker at 150 rpm. To optimize inoculum size the bacterial growth was initiated with 1, 5 and 10% inocula. Growth in all these experiments was measured by taking Optical Density (OD) of bacterial cultures at 600 nm after 24 h of incubations. The bacterial strains were then grown in 20 mL of M-IV medium, comprising of 1% bagasse in water at their corresponding growth optima. pH of the two lots of M-IV was adjusted with HCL and H_2SO_4. Fermentation material was sampled in an amount of 2.5 mL at 2, 10 and 18 days. It was centrifuged at 4000 rpm and the cell and bagasse free supernatant was processed for glucose and sugar estimations according to O-toulidine and phenol sulphuric acid by the methods of Hartel *et al.* (1969) and Dubois *et al.* (1956), respectively. To the remaining fermented material water was added in an amount to reachieve 20 mL volume and the containers were autoclaved and inoculated with ethanolegenic yeasts.

Isolation of yeast and estimation of ethanol: Yeast was isolated from soil that was sampled from the vicinity of a sugarcane juice shop on sabouraud agar. Pure cultures of yeast were preserved. Commercial molasses was mixed with 1.5 times water. pH of this solution was adjusted 4.5 with concentrated H_2SO_4 and heated slowly up to 85°C for about 15 min. Then it was allowed to cool in order to settle down the solids. The clear supernatant was diluted by mixing 13.6 mL of it with 86.4 mL of water and used to inoculate yeasts. Twenty four hours old culture of a yeast was transferred from slants and each to 15 mL of sabouraud broth medium. The bottles we incubated at 30°C on orbital shaker at (120 rpm). After 24 h, cells were centrifuged and the supernatant disposed off. Then 10 mL of the diluted processed molasses was poured into each tube having cells, which were subsequently placed on shaker (150 rpm) for 24 h. The growth was checked by simple staining employing methylene blue. The culture was kept at room temperature for 6 to 7 days. Then 1 mL of each sample was processed for alcohol estimation. Ethanol was estimated by oxidation in diffusion vessel employing 0.43% $K_2Cr_2O_7$ and sulphuric acid as described by Snell and Snell (1973). The yeast strain designated NS6 yielded more than 1% ethanol. The yeast inocula, 0.1 mL were obtained from 24 h old culture in dilute molasses. The yeast inoculated fluid of bacterially saccharified M-IV media was sampled for alcohol estimation at 3rd and 7th day. Absorbance of the color developed in the acidic solution of $K_2Cr_2O_7$ was noted at 600 nm and already developed standard curve in this laboratory was used to calculate % ethanol content.

Statistical comparisons were made by employing student's t-test and single factor analysis of variance for two and three values, respectively according to Campbell (1989).

RESULTS AND DISCUSSION

Inoculation of the bacterial strains in medium M-I resulted into good growth that was revealed by microscopic observation of the culture fluids. As in this medium bagasse was the only nutritive source, thus the bacterial isolates possess cellulose degrading potential. It appears that the exocellular enzyme secreated by these bacteria can work well in water milieu and they may not require provision of buffer substances. Zheng *et al.* (2002) compared enzymatic hydrolysis of waste sugarcane bagasse in tap water and in a buffer solution and concluded that similar levels of reducing sugars yields

appeared in both the cases. These authors considered the possibility of formation of a transitional complex of enzyme and the substrate in the hydrolysis process. And that the transitional complex might had provided a buffering capacity. Bacterial isolates showed even more growth in medium M-II. The increased growth might have occurred due to the presence of yeast extract and the minerals contents of this medium.

Optimization of growth conditions in M-III medium: Due to suspended particulate matter of soaked bagasse it was not possible to estimate the bacterial growth by taking optical density. Therefore the growth optimization experiments were conducted in M-III medium. Whose composition was similar to the M-II except that this medium contained sucrose instead of bagasse.

The bacterial strains 3, 19_4 and 23 showed comparable growth at the two pH levels with slight increase at pH 5. The strain 11a, however, showed significantly higher growth at pH 5 (Table 1). All the bacteria grew best at 37°C as compared to 50°C temperature with significant difference only for the strain No. 3. The bacteria showed comparable growth in aerated and non aerated conditions. The bacterial strain, 3 expressed significantly higher growth when the culture was started with 5% inoculum as comparable to 1 and 10% inocula sizes. The 10% inoculum size indicated significantly less growth even than the 1% inoculum size. Bacterial isolate 11a and 19_4 showed best growth with 5% inocula but differences turned out to be non significant as compared to 1 and 10% inocula. The isolate No. 23 yielded significantly higher growth when the medium was inoculated with 10 % culture as compared to 1% inoculum size. The acidophilic, optimum pH 5, of these cellulose hydrolyzing bacterial strains is very valuable. Infact many workers have used dilute acid hydrolysis of sugarcane bagasse as one of successful pretreatment procedures. For example, Neureiter *et al.* (2002) while, studying dilute acid hydrolysis of sugarcane bagasse at varying concentrations have reported that for sugar yield, concentration of the sulphuric acid appeared most important. Similarly, Bustos *et al.* (2003) attempted hydrolysis with sugarcane bagasse with hydrochloric acid and mentioned that at 128°C, 2% HCI, 22.6% xylose, 3.31% arabinose, 3.77% glucose, 3.5% acetic acid and 1.5% were furfural obtained per litre within 51.1 min. Thus in the present report the bagasse acid treatment and the bacteria growth mediated saccharification appeared to occur hand in hand.

Total sugar and glucose contents of culture fluids of M-IV medium: The bacterial strains were grown on medium M-IV at their corresponding optimum pH levels. pH of the media was adjusted with either HCl or H_2SO_4. After 48 h of inoculation the culture fluid indicated comparable amounts of total sugars content in both categories of media. The bacterial strain 19_4 yielded highest sugar contents i.e., 833.0 mg/100 mL. The total sugar contents gradually decreased with the increase in incubation period. The strain No. 3 yielded 1434 mg/100 mL glucose at the first sampling day in the medium containing HCI as compared to the glucose contents in H_2SO_4-medium. The difference was about four folds (Table 2). The glucose yield of 14.34 g L^{-1} is several time higher than 3.77 g L^{-1} obtained by Bustos *et al.* (2003) through acid hydrolysis alone of sugarcane bagasse. Further work will focus isolation and optimization of saccharifying bacterial enzymes. The glucose contents decreased in general drastically in the subsequent sampling days. For the isolate No. 11a significantly higher glucose contents were found at 10th day of incubation in the H_2SO_4- medium as compared to the HCI-medium. The isolate, 23 yielded significantly higher, glucose contents at 10th day of incubation in HCI-medium and highest and at last sampling period in H_2SO_4-medium, respectively (Table 2).

Ethanol production: One week yeast fermentation of sterilized culture fluids of 18th days incubated culture of the bacterial strain No. 3 resulted into production of 0.42% ethanol. Similarly strain 11a yielded 0.23% ethanol after 7 days of fermentation. Highest ethanol contents were found at 3rd and 7th days of fermentation in the culture fluid taken from the growth of bacterial strains 19_4 and 23, respectively in HCI- medium. The corresponding values were 1.94 and 1.197%, respectively. About 2% (Table 3) ethanol content following the fermentation of culture fluid taken from the growth of bacterial strain No. 23 correspond to the highest glucose contents i.e.,

Table 1: Optimization of various growth conditions of the four isolates in MIII medium

Strain	pH		Temperature		Air		Inoculum size		
No.	5	7	37°C	50°C	On shaker	without shaker	1%	5%	10%
3	1.47±0.170	1.27±0.315	0.39±0.046**	0.32±0.138	0.48±0.245	0.61±0.060	0.036±0.004	0.43±0.058***	0.29±0.041•**
11a	1.58±0.008	0.505±0.29*	0.32±0.023	0.28±0.081	0.28±0.047	0.33±0.092	0.09±0.0490	0.698±0.094	0.26±0.087
19_4	1.67±0.017	1.37±0.249	0.38±0.058	0.29±0.063	0.54±0.0282	0.48±0.037	0.019±0.005	0.24±0.110	0.113±0.048
23	1.54±0.064	1.20±0.109	0.47±0.069	0.38±0.052	0.27±0.150	0.29±0.008	0.02±0.0020	0.26±0.190	0.50±0.130*

Values are mean±SEM of three replicates and represent optical density of cultures taken at 600 nm. Those with asterisk(s) are significantly different from the corresponding values in the extreme left column.• = significantly different from the corresponding values in the middle column. Student's t-test and single factor analysis of variance, *p<0.05; ** p<0.01; ***p<0.001

Table 2: Total sugar and glucose contents mg/100mL of supernatants of MIV media following growth of bacteria at different intervals of post inoculation

	Total sugar					
	2nd day		10th day		18th day	
Strain No.	HCL[a]	H_2SO_4	HCL	H_2SO_4	HCL	H_2SO_4
3	561.33±13.184	590.60±68.65	381.0±4.513	312.33±37.53	156.33±12.252	168.73±9.69
11a	7688±181.3	675.30±317.46	407.0±46.14	274.00±47.16	200.40±49.93	243.33±8.462
19_4	741.0±290.87	833.00±216.3	278.6±47.75	325.60±30.37	160.00±19.87	161.30±17.72
23	4970±291.83	575.16±91.15	275.0±54.18	373.00±39.93	142.40±17.076	200.10±48.34*
	Glucose					
	2nd day		10th day		18th day	
Strain No.	HCL[a]	H_2SO_4	HCL	H_2SO_4	HCL	H_2SO_4
3	1434±46.9	363.3±174.6**	441.3±24.74	498.8±31.98	61.59±7.670	93.56±34.57
11a	119.20±103.3	469.4±45.09	399.9±34.50	1411.2±23.34***	146.3±8.2600	87.57±41.82
19_4	149.56±116.10	458.3±41.13	420.4±3.950	473.5±24.0	105.4±417.50	31.29±22.19
23	411.20±66.98	468.0±69.16	411.4±157.7	62.9±20.4**	82.35±12.64	808.3±26.56***

pH of MIV was adjusted either with HCl or H_2SO_4, Values are mean±SEM of three replicates. Those with asterisk(s) are significantly different from the corresponding values in the left column. Student's t-test. *p<0.05; **p<0.01; ***p<0.001

Table 3: Ethanol production by an ethanologenic yeast strain No. NS6 on autoclaved MIV media which were saccharified by 18 days growth of the four strains of *Bacillus cereus*

	3rd day		7th day	
Strain No.	HCl	H_2SO_4	HCl	H_2SO_4
3	0.019±0.011%	0.008±0.004%	0.027±0.015%	0.422±0.243%
11a	0.106±0.06%	0.003±0.0017%	0.053±0.0306%	0.230±0.1329%
19_4	0.194±0.11%	0.029±0.016%	0.066±0.038%	0.043±0.024%
23	0.018±0.010%	0.004±0.002%	1.197±0.691%	0.085±0.049%

Values are mean±SEM of three replicates. Student's t-test; Non significant differences

Table 4: Biochemical characterization of the different strains of *Bacillus cereus**

Strain No.	Endospore staining	Cell morphology	Catalase	Nitrate reduction	Sulphide formation	Fermentation of glucose	Lecithinase test
3	Elliptical, central 1.89×1.155[a]	Streptobacilli 5.25×0.84	+	+	-	+	+
11a	Elliptical, central 1.89×0.945	Bacilli 4.365×0.78	++	+	-	+	+
19_4	Elliptical, central 1.575×1.05	Diplobacilli 3.67×0.42	+	+	-	+	+
23	Oval, central 1.89×1.155	Diplobacilli 2.1×0.42	++	+	-	+	+

The figure indicate dimension of endospores/cells in (m), * All the isolates showed positive reaction for gram's staining, motility, oxidase and anaerobic facultative test

808.3 mg/100 mL at the end of bacterial growth as compared to scanty glucose contents for other bacterial isolates (Table 2). In the present batch culture fermentation limit of about 2% ethanol production may also be an indicative of ethanol tolerance level of the yeast. Thus it can be speculated that for continuous fermentations with simultaneous recovery of the product from the ferments valuable yield of ethanol can be obtained.

Biochemical characterization and identification: All the four bacterial isolates were found gram positive and motile. Similarly all showed positive results for the oxidase and facultative anaerobic tests. All the isolates expressed elliptical endospores except the strain 23 which yielded oval shaped endospores. Sizes of the endospore were comparable in all isolates (Table 4). Cell morphology and other characteristics are also indicated in Table 4. Based upon these characteristics all the strains were found to be identified as *Bacillus cereus*.

In the present model saccharification and ethanol fermentation were carried out separately. While many investigators have reported simultaneous saccharification and ethanol fermentation of sugarcane bagasse and other plant biomass (Hari and Chowdary, 2000; Teixeria *et al*., 2000). Next experiments will attempt co-culturing of the saccharifying bacterial isolates and ethanol fermenting microorganism. In the present study yield of xylose and its fermentation were not taken into account. Being a considerable proportion of plants cellulosic material xylose fermentation can significantly escalate the yield of ethanol from saccharified plant biomass. It has been established that to achieve high ethanol yield, xylose be too converted efficiently to ethanol by xylose fermenting organisms (Chandrakant and Bisaria, 1998; Krishan *et al*., 2000). Determination of xylose content its fermentation to alcohol following the saccharification of sugarcane bagasse have been aimed for further investigation in this laboratory.

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