Research Article

Chemical Pretreatment Method for Enhanced Production of Bioethanol from Sugarcane Bagasse using *Bacillus* sp.

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Abstract | Sugarcane bagasse (SCB) is usually considered agricultural lignocellulosic biowaste attracting worldwide attention as a potential substitute for fossil fuels derived from the sugar and alcohol industry. In this study, ethanol was produced from the acid and alkaline pretreated samples of sugarcane bagasse using a fermentation process. Amylase was partially purified from the bacterial strain N1 and its activity was checked at distinct pH and temperature. The optimum pH was from 6-8 whereas the temperature was 40 °C. *Saccharomyces cerevisiea* was added for enhanced fermentation and ethanol production. The acid pretreated sample produced the highest ethanol (15.2%) than the alkaline. The percent production of ethanol and the amount of sugar in pretreated samples was confirmed using High-Performance Liquid Chromatography (HPLC) and sugar contents were increased in acid pretreated samples.

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Introduction

Lignocellulose is commonly found in agricultural residuals like corn straws, wheat, and other forestry residues used for bioethanol production using second-generation technologies (Gil *et al.*, 2003). This source is abundant in nature and could reduce environmental impacts with low greenhouse gas emissions (Gil *et al.*, 2003). It comprises three significant constituents' lignin, cellulose and hemicellulose. Lignin gives strength and rigidity to plants (Galbe and Zacchi, 2007).

Sugarcane bagasse (SCB) is a lignocellulosic waste used worldwide as a substitute for fossil fuels. Its significance is obtained from the sugar and alcohol industries and used as boilers (Wilkins *et al.*, 2007). SCB is a rich source of xylose and glucose, having five and six-carbon sugars, respectively. Both hemicellulose and cellulose are constituted of SCB, decomposed to hexose and pentose sugars. Microorganisms efficiently hydrolyze hexose for bioethanol production, others have shown to hydrolyze the pentose as a growth substrate (Zhang *et al.*, 2000) and help convert lignocellulose wastes into valuable products like

biofuels. These microbes produce extracellular cellulose and are called cellulolytic microorganisms. Bacilli are the most popular class of cellulase for their commercial production and have very high economic value (Kaneko *et al*., 2006).

Various technologies have been used to improve the conversion of these substrates into bioethanol production (Singh *et al*., 2008). Different pretreatment methods are used to eliminate the lignin and cellulose crystallinity (Wang *et al*., 2014). Various physical (hydrothermal), chemical (alkali, acids, ozone, and solvents), and biological pretreatment methods have been studied over the years by different researchers (Kumar and Kumar, 2017). In acid pretreatment, hemicellulose is degraded and hydrolyzed with the help of cellulase enzyme. Alkaline pretreatment involves the process of delignification to solubilized hemicellulose. It's the saponification of linkage between hemicellulose, xylan and other components. It also removes the compound of acetyl and other uronic acid that reduce accessibility of both hemicellulose and cellulose to enzymes (Rezaei *et al*., 2007).

After pretreatment, the conversion of biomasses into ethanol involves two steps of semi-simultaneous saccharification and fermentation that have been studied as the promising arrangement of enzymatic hydrolysis and fermentation for bioethanol. Enzymes that aid in transforming polymers into fermentable sugars are first made available to cellulose by the breakdown of lignin during this phase (Mosier *et al*., 2005).

Bioethanol is made up of fermenting sugars produced from agricultural products like sugarcane, corn, and potatoes. Due to the depleted demand for fossil fuels, this alternative energy resource from biomass is efficient, sustainable, cost-effective, and safe (Chum and Overend, 2001). Bioethanol has been previously produced through starch and sucrose containing materials like sugarcane and corn (Manzoor *et al*., 2020, 2022; Tsigie *et al*., 2013). Therefore, the present study used acid and alkali pretreatment methods to produce enhanced production of bioethanol from sugarcane bagasse. The enzyme amylase was partially purified for enhanced fermentation process and bioethanol production. Sugar contents and ethanol concentration were determined using HPLC.

Materials and Methods

Sample collection

Sugarcane bagasse and soil were collected from the area of district Kohat and brought to the Microbiology Laboratory, Kohat University of Science and Technology. Sugarcane was thoroughly washed and dried, then cut into smaller pieces. At the same time, the collected soil samples were transferred to sterile plastic bags.

Isolation and identification of amylase producing bacteria

The soil sample (2 g) was weighed and 18 mL of sterile distilled water was added. Spread it into nutritional agar supplemented with 1% starch after serial dilution of 10^5. The plates were incubated at 37 °C overnight. The isolated strain was examined for its color, shape, size, and nature under the microscope (Codex, 2019). The tests for biochemical characterization were performed. The single purified cells were used as a template for PCR analysis with the universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'), and the 16S rRNA gene sequences compared for molecular identification as reported (Manzoor *et al*., 2018).

Partial purification of amylase enzyme

Bacterial culture was transferred from starch nutrient agar slants to the nutrient broth at pH 7 for activation and incubated for 24 hours in a shaker at 40 °C and 120 rpm. Fermentation media consisted of 5 $g{\cdot}L^{-1}$ of peptone, 10 $g{\cdot}L^{-1}$ soluble starch, 2 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$, 1 $g{\cdot}L^{-1}$ KH_2PO_4, 0.01 $g{\cdot}L^{-1}$ $MgCl_2$, which had a pH of 7. The activated culture was added to the fermentation medium and incubated for 24 hours at 37 °C in a shaker. The crude extract, which was used as an enzyme source, was obtained by centrifuging the culture media at 10,000 rpm for 15 minutes (Indrianingsih *et al*., 2018).

Starch iodine test for amylase activity

Starch hydrolysis testing on a starch agar plate was used to check bacterial cultures for amylolytic activity. The pure isolated colonies were streaked on starch agar plates. The separate plates were flooded with Gram's iodine (250 mg iodine crystals were added to 2.5 gm potassium iodide solution with 125 mL of water) after incubating for 24-48 hours at 37 °C. Based on screening and detection of amylase enzyme, no blue color develops in the degradation zone. The

amylase producers showing the largest diameter of the clearing zone were further examined as described (Gupta *et al*., 2003). The starch-nutrient agar slants were kept at 4 ˚C. The pure cultures were subcultured at regular intervals.

Enzyme assay

Enzyme assay for Amylase activity was conducted as described (Indrianingsih *et al*., 2018). Briefly, the solution containing 1% starch (1.5 mL), 2 mL of 0.1M phosphate buffer (pH 6.5), and diluted enzyme (0.5mL) were incubated for 15 min at room temperature. Adding 1 mL of DNS reagent was then diluted with 8 mL of distilled water and maintained in a boiling water bath for 10 minutes. The absorbance was measured at 540 nm against a blank.

Determination of optimum pH

Substrate solution containing 1% Starch was prepared in various test tubes at optimum pH 6, 6.5, 7, 7.5, and 8 with sodium phosphate buffer. Buffer was added in 0.5 mL of each diluted crude enzyme solution. The mixture was then heated for 10 minutes in a water bath before being let to cool at room temperature. After the mixture had been incubated for 15 minutes, the reactions were stopped by adding 1 mL of DNS reagent. The final volume (12 mL) was made with distilled water, and the enzyme activity was measured at 540 nm.

Determination of optimum temperature

Six distinct test tubes were filled with the substrate (1.5 mL each) and phosphate buffer (2 mL) at pH 7. Different temperatures were given to the tubes at 30, 35, 40, 45, 50, and 55 ˚C. In each tube, 0.5 mL of diluted enzyme solution was added. After that, the tubes were incubated for 10 minutes at a particular temperature. The reactions were stopped after adding 1 mL of DNS reagent, and the mixture was then put in a water bath for 10 minutes. The final volume 12 mL was prepared with distilled water, and the enzyme activity was assessed at 540 nm.

Preparation of buffer, NaOH and H_2SO_4

α-amylase dilution was prepared using a buffer. It was kept at room temperature and wrapped in aluminum foil for subsequent use. Sodium hydroxide (0.4 gm) was added to 100 mL of the sterilized bottle and mixed properly with water. It was added to the sample until the pH became 8. Sulfuric acid (0.27 mL) was added to 100 mL of distilled water in a sterilized bottle and mixed properly. It was added to the sample until the pH became 5.

Pretreatment of sugarcane bagasse

The sugarcane bagasse was carefully cleaned and diced. It was then dried in an oven for four to five days at 600 h. The sugarcane bagasse was ground using a machine that grinds solid materials into powder by a sharp spinning blade. The sample was sealed in a polyethylene bag and stored at room temperature. 10 g of sugarcane was weighed. It was put into a conical flask with 200 mL of distilled water inside of it. Add 0.5 mL of NaOH and H_2SO_4 and adjust the pH to 4.5 and 8 separately. Then add 0.2 mL of the α-amylase enzyme to the mixture. The enzyme was diluted with phosphate buffer before being added. Until 50 ˚C, the mixture was heated. 10 mL of *Saccharomyces cerevisia* (yeast) were added after the mixture had been chilled to 32 ˚C.

Fermentation of sugarcane

The simple sugar was fermented with *Saccharomyces cerevisiea* (baker yeast) to produce ethanol and carbon dioxide. Different parameters, such as pH and temperature, were used to check the efficiency of bioethanol. The temperature varied from 20, 25, 30, 35 and 40 ˚C at constant pH of 4.5. The pH ranged from 3, 3.5, 4, 4.5, and 5 at 37 ˚C. The fermentation process went on for 48 hours.

Bioethanol yield determination

After 48 hours, the sample was filtered with Whatman Filter Paper to remove the residue from the ethanol. A rotary evaporator was used to distill the bioethanol at 75 ˚C. Bioethanol was analyzed on a Shimadzu HPLC equipped with a Photodiode array detector, column oven, auto sampler, and an Aminex column (300 × 7.8 mm), for the analysis of carbohydrate and organic acid by using diluted H_2SO_4 as a mobile phase with flow rate 1 mL/min and temperature 60 ˚C. The detection wavelength was set to 210 nm. 20 µL of the sample was injected into the HPLC system for bioethanol determination.

Results and Discussion

Identification of α-amylase producing bacteria

The bacterial strain that could produce α-amylase was isolated from the soil. The starch hydrolysis test noted a zone of clearance with iodine solution. The bacteria isolated from starch-rich substrates produce enzymes

even in harsh conditions. Amylase-producing microorganisms were previously isolated from soil (Fossi *et al*., 2005).

The cultures grown in nutrient broth were used to extract the genomic DNA and were amplified. BioEdit software was used to align the acquired sequence. BLAST analysis was performed, and the Clustal W tool was used to obtain and align the most similar sequences. The phylogenetic tree (Figure 1) was made with the help of Mega 6 software by using the Neighbor-Joining method, and the isolated bacterium was identified as *Bacillus* sp.

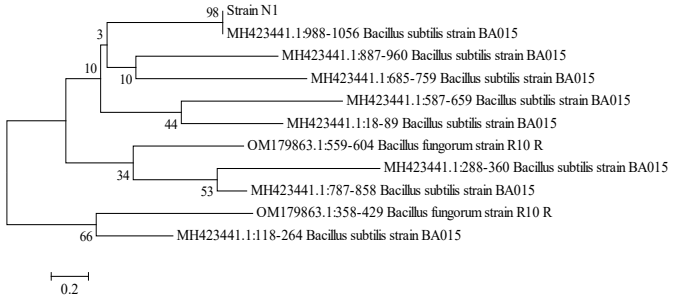

Figure 1: *Phylogenetic analysis of Strain N1 based on 16S rRNA gene.*

Optimum pH and temperature

The pH of the growth medium influences morphological changes in microorganisms and enzyme secretion. A pH range between 6.0 and 7.0 is necessary for most starch-degrading bacterial strains for average growth and enzyme activity (Đurović and Đorđević, 2011). The stability of the medium was affected by the pH change seen during microbial development. The isolate could grow in the pH range of 6 to 8, as shown in Table 1. However, pH 7.0 was ideal for the growth of the cultures. Temperature played an essential role in the stability of enzyme activity. It was found that 40 °C was the perfect temperature for increasing enzyme activity (Table 2).

Effect of pretreatment on ethanol production

Lignin decomposition from plant biomass is accomplished through pretreatment. During the pretreatment process, a proper catalyst concentration and temperature under pressure play a vital role in exposing maximum cellulose from biomass. The proximate analysis of processed sugarcane bagasse is shown in Table 3 on a dry basis weight (w/w) where the moisture content was decreased. Due to the alkaline pretreatment effect, the pretreated substrate's ash contents were also reduced.

Table 1: *Varying pH and its effect on α-amylase activity.*

pH	α- amylase activity (μ/mL)
6	6.74
6.5	8.22
7	8.99
7.5	7.67
8	7.12

Table 2: *Varying temperature and its effect on α-amylase activity.*

Temperature	α- amylase activity(μ/mL)
20	5.43
30	6.73
40	9.12
50	8.93
60	5.92

Table 3: *Chemical composition of sugarcane bagasse (Dry weight basis %).*

1	Cellulose	52.8
2	Hemicelluloses	71.5
3	Lignin	18.5
4	Pectin	1.5
5	Ash	1.76

Table 4: *Production of ethanol (%) from acid and alkali pretreated samples.*

Pretreated samples	Ethanol (%)
Acid Pretreated	15.3
Alkali Pretreated	11.2

Ethanol fermentation was started by adding dry yeast to the pre-hydrolyzed slurries. The high ethanol production rate was seen due to glucose availability in the acid pretreated samples. In contrast, a decline in the production rate is due to the cause of yeast that rapidly consumes glucose. Enzymes helped to hydrolyze the substrate. This was the cause of the rise in bioethanol production following the nearly full use of the initial glucose. The pretreatment technique significantly impacts how the process turns out. As the lignin concentration of the solid wastes dropped, ethanol production rose. The least amount of ethanol was produced during the fermentation of the alkali-treated samples (Table 4).

Due to the interdependence of the three main components of plant cell walls (cellulose,

hemicelluloses, and lignin), lignocellulosic materials frequently need to undergo a first pretreatment stage to make the monomeric sugars fermentable to ethanol. Different techniques have been used, including steam explosion, alkali hydrolysis, and acid hydrolysis. The most popular pretreatment method, known as acid hydrolysis, uses diluted sulfuric acid to separate the components of cell walls to produce hemicellulose hydrolysate and lignin (Dussan *et al.*, 2014).

A calibration curve was drawn to determine the total ethanol concentration from sugarcane bagasse samples (Figure 2). The ethanol standard (95%) was prepared at different concentrations such as 20, 40, 60, 80 and 100%. The concentration of the ethanol standards were plotted against their peak area determined through HPLC.

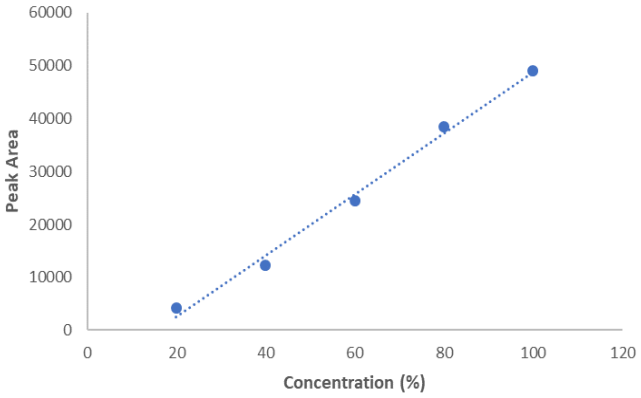

Figure 2: *A standard calibration curve of Ethanol at different concentrations.*

Analysis of sugar contents using HPLC

Monosaccharides were separated from higher oligomers of sugars using HPLC. A separation of glucose from fructose and galactose and a typical profile were obtained with hydrolyzed samples (Figures 3, 4). No detectable quantities of higher oligomers were observed in the hydrolysate. The retention time was used to identify glucose, melibiose, raffinose, stachyose, and galactose. Standard solutions of these sugars were prepared, and their retention time was compared with the peaks eluted in the samples. It was observed that acid hydrolysis gave the highest rate of bagasse degradation to sugars.

Acid-based chemical pretreatments are efficient and cost-effective (Pandey *et al.*, 2000). Acids break down hemicellulose, creating a liquid phase that is rich in xylose and contains lignin derivatives. This method is good in recovering hemicellulose (Lavarack *et al.*, 2002), and sugarcane bagasse has been successfully treated using it (Rocha *et al.*, 2011). Geddes and colleagues in a study for comparing the efficacy of sulfuric and phosphoric acids in removing hemicellulose from sugarcane bagasse, found that both acids produced maximum sugar yields at 1% concentrations (Volpe *et al.*, 2018). Rocha and his colleagues used a blend of sulfuric and acetic acids to remove high yields of hemicellulose from sugarcane bagasse (up to 90%), while only losing 15% of the cellulose (Rocha, 2011).

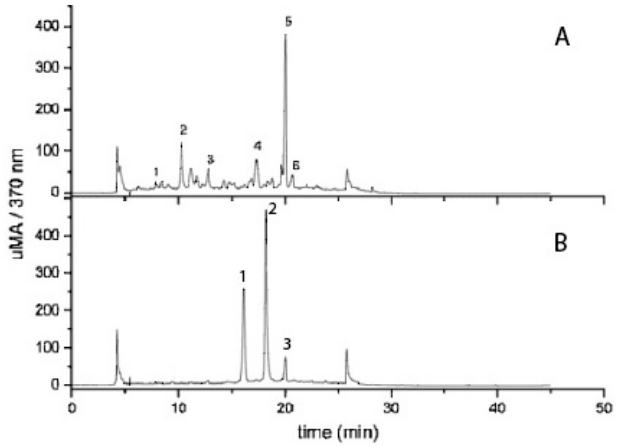

Figure 3: *HPLC Chromatogram of Acid (A) and Alkali (B) pretreated samples of sugarcane bagasse.*

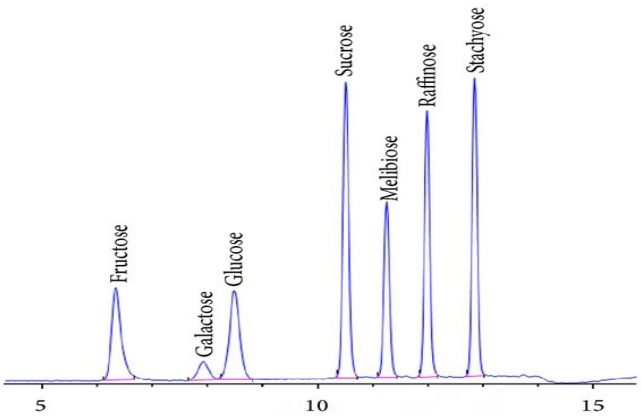

Figure 4: *Chromatographic separation of distinct monosaccharides from the acid hydrolyzed sugarcane bagasse sample.*

Conclusions and Recommendations

This study used acid and alkaline pretreatment followed by enzymatic hydrolysis with amylase for ethanol production from sugarcane bagasse. Ethanol and distinct sugar concentrations were analyzed on HPLC. Ethanol concentration was increased in acid pretreated sample whereas low ethanol concentration was observed in alkali pretreated sample. Sugar contents were increased in acid pretreated sample.

Further study should be conducted on other parameters that affect the ethanol production such as fermentation time, role of pH, enzyme concentration and temperature are major factors. This will give an overall view of how these parameters would affect ethanol concentration.

Novelty Statement

This research demonstrates the feasibility of extracted amylase enzyme from the bacterial strain N1 used as biocatalyst for enzymatic hydrolysis using sugarcane bagasse for bioethanol production.

Author's Contribution

Nazish Manzoor: Designed and supervised the study.
Alim Khan, Muhammad Yaseen and Abid Ullah: Experimented and collected the results.
Zulqarnain: Analyzed and interpreted the results of HPLC.
Iftikhar Jan: Phylogenetic analysis.

Conflict of interest
The authors have declared no conflict of interest.

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