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Molecular Characterization, Production and Optimization of Cellulase Producer and its Industrial Applications

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Plant cell walls are composed primarily of cellulose, making it the most common organic polymer on Earth. The utilization of microorganisms to convert cellulose into valuable products has gained significant attention worldwide. This study aims to isolate novel cellulase-producing microorganisms and investigate the influence of dietary and physicochemical factors on cellulase production, as well as evaluate the potential applications of a selected isolated strain. The Bacillus amyloliquefaciens strain was identified as the most efficient producer of cellulase among various cellulase-producing bacterial strains isolated during the research. The morphological and biochemical characteristics of this strain were characterized. To determine the optimal conditions for cellulase production, various parameters such as raw substrates, carbon sources, nitrogen sources, temperature, inoculum age, inoculum size, agitation rate, incubation duration, and pH were investigated. The study found that sugarcane bagasse, molasses, and paper pulp exhibited the highest cellulase production capacity, with respective enzyme activities of 0.97, 0.98, and 0.88 IU/mL. Maximum cellulase production occurred at a temperature of 37°C, an inoculum size of 2.5%, an inoculum age of 24 hours, an agitation rate of 120 rpm, an incubation time of 48 hours, and a pH of 7 with 1% peptone. The Bacillus amyloliquefaciens isolate was successfully applied to convert lignocellulosic waste into ethanol and fermentable sugars through saccharification and fermentation of cellulose, demonstrating its significant effectiveness. Bacillus amyloliquefaciens strain isolated in this study proved to be a highly efficient producer of cellulase. The optimized production media led to improved enzyme production, and the isolate showed promising results in the industrial application of converting cellulosic waste into ethanol. The 16s rRNA sequencing confirmed the identity of Bacillus amyloliquefaciens , which was deposited under the accession number MN081796 in the NCBI database. In this study we discussed the possible low-cost, enzymatic pretreatment methods of lignocellulosic material in order to use it as an efficient raw material for biofuel production. These findings contribute to the understanding and potential utilization of cellulose producing microorganisms in various biotechnological applications.

Keywords: Agricultural waste; Bacillus.sp.; Cellulase activity; Fermentation; Industrial applications.

Global attention has been drawn to using renewable resources, particularly agricultural and forest leftovers, due to rising energy demands. Cellulose, starch, lignin, xylan, and pectin make up the majority. Because of their widespread availability as alternate feedstock and energy sources, these materials have garnered much attention. The Indian government has invited

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research-driven proposals to advance the development of agricultural waste management without burning. In the past ten years, the Indian government has devised a program known as the "National Bioenergy Mission," under which attempts are made to meet most of the country's regular energy needs using bioenergy and other renewable energy sources rather than fossil fuels[1]. By developing various enzymes in various environmental niches, many microbes can use these materials as carbon and energy sources. The most prevalent polysaccharide on earth is cellulose. Most urban solid waste is composed of cellulosic material, the most prevalent biomass and carbon source on earth. The industry needs Cellulase, and there is a big market for cheap Cellulase. An enzyme called Cellulase helps to hydrolyze cellulose[2]. Cellulases degrade cellulose into monosaccharides like beta-glucose or shorter polysaccharides. A few fungi, aerobic bacteria, and anaerobic bacteria, including *Bacillus subtilis* and *B .amyloliquefaciens,* are examples of microorganisms with cellulolytic skill. Cellulolytic bacteria were isolated and identified in urban waste in the current analysis. Additionally, cellulolytic enzymes are produced by microbes such bacteria, fungi, and actinobacteria have a variety of uses in agriculture, textiles, pulp and paper, food and beverage, brewing and winemaking, detergent production, and bioconversion for value-added industrial products[3,4,5]. Samples of urban waste are taken and plated on Carboxymethylcellulose agar[6]. Much effort has gone into finding new microorganisms that can produce cellulase enzymes with higher particular activities and performance.

Experimental section

Sample Collection and Screening for Cellulase Producers

Bacteria for cellulase production were isolated from soil samples collected from 6 different regions of the Ahmedabad district, including the garden area, fruit market, and vegetable market. The soil sample was enriched by placing 100 g. of soil sample and 1g. of cellulose powder in a sterile beaker for a few days at room temperature. A small amount of water was applied to the soil sample to maintain moisture. After that, 1 gram of enriched soil sample was inoculated into enrichment broth containing (g/l): cellulose-10, yeast extract-1, peptone-5, $CaCO_3$-2, and Nacl-2, which was then shaken for 10 days at 200 rpm on a rotatory shaker. The enriched soil sample was then serially diluted and inoculated on Carboxymethyl Cellulose (CMC) agar medium containing (g/l): $NaNO_3$ -2, K_2HPO_4-0.5, $MgSO_4.7H_2O$ 0.2, $MnSO_4.H_2O$-0.02, $FeSO_4.7H_2O$-0.02, $CaCl_2.2H_2O$-0.02, CMC-1 and agar-2.0. After 24 h incubation at 37°C, Cellulose degradation was demonstrated by creating a strong hydrolysis region after pouring off the Congo red solution and 1M NaCI for 15 minutes on plates[7]. For additional research, the colony with the largest zone width was preceded.

Identification of the Bacterial Strain

Different staining methods, as well as biochemical and molecular techniques, were used to classify the bacteria that had been isolated.

Sequencing of the Amplified 16s RNA Gene to Identify the Isolated Bacteria

The amplified PCR product was purified using a Helini DNA purification package, and the PCR product was then commercially sequenced. The collected sequence was blasted into the NCBI database, and a phylogenetic analysis was performed. The FASTA sequence was built using the neighbor-joining (N-J) process.

Genomic DNA Isolation of the Bacterial Isolate

The above sequence was BLASTed against established sequences in NCBI's public libraries, and the findings were shown as a phylogenetic tree. Based on nucleotide homology and phylogenetic analysis, the sample displayed a high degree of resemblance to *Bacillus amyloliquefaciens*. CLUSTAL W. performed a molecular phylogenetic study using the Maximum Likelihood approach for pectinase producers. The final dataset included 1434 locations and MEGA7 was used to run evolutionary studies.

Sequences for Scanning Electron Microscopy of Bacterial Strain

Bacterial isolates were fixed in 6% buffered glutaraldehyde for 24 hours. Scanning Electron Microscope photographs were obtained from MediWave labs, Mumbai**.** The absolute ethyl alcohol was acquired from Hayman Ltd., England. The microbial identification by 16srRNA was conducted by Eurofins Genomics India Pvt Ltd. Bangalore 560048, Karnataka, India.

Optimization of Physico-Chemical Parameters for Cellulase Production by *Bacillus amyloliquefaciens*

Any bacterial species' ability to produce pectinase can vary and be influenced by various physicochemical factors. Different ratios of these variables may impact the enzyme's secretion. Hence, in the current study, pectinase enzyme production was optimized at various temperatures (28 to 60^{O}C), pH (6 to 12), different substrates(sugarcane, a paper pulp, tea leftover, cassava rubbish, orange peels, molasses, wheat cake, and agriculture residues), carbon source(lactose, maltose, mannitol, sucrose, glucose, fructose, cellulose, and starch), various concentrations of nitrogen sources(peptone, beef and yeast extract, casein, urea, potassium nitrate, ammonium chloride, and ammonium sulfate) different inoculum age(18 to 55 hrs.) and size(0.5 to 5%), incubation period(12 to 96 hours) and agitation rate(50 to 300rpm). The experiment was conducted in 250 ml Erlenmeyer flasks for cellulase enzyme production with Berg's mineral salts broth medium as the production medium. After being autoclaved for sterilization, flasks were cooled down and infused with pure cultures of *Bacillus amyloliquefaciens* obtained using selective media.

Production of Cellulase by Submerged Fermentation (SMF)

Industrially important enzymes have traditionally been obtained from submerged fermentation (SMF) because of the case of handling and greater control of environmental factors such as temperature and pH[8, 9, 10]. The best initial pH, temperature, time, nitrogen supply, age, inoculum size, incubation period, agitation rate, and substrate concentration for cellulase development were determined. Free-flowing liquid substrates, such as molasses and broth, are used in submerged fermentation[22]. cellulase fermentation medium comprising of peptone1.0g/L, yeast extract1.0g/L, $NaNO_3$2.0g/L, $KH_2PO_4$1.0g/L, $MgSO_4.7H_20$ 0.5g/L and substrate 5.0 g/L was used for submerged fermentation process. Following the sterilizing procedure, flasks were cooled, sputter-coated with pure cultures of Bacillus amyloliquefaciens, and incubated for three days at 37 °C. The crude enzyme source was the supernatant from the centrifuged manufacturing medium.

Cellulase assay by DNS method (3,5-dinitrosalicyIic acid)[11]

Using CMC as a substrate, the DNS approach was employed to assess the enzyme activity of Cellulase. In a 0.1M acetate buffer with a pH of 6.0, 0.5 ml of the crude enzyme and 0.5 ml of CMC are combined to create the reaction mixture. It was then incubated for 10 minutes at 40 °C. After adding 1 ml of DNS reagent, the mixture was cooked for 5 minutes at 90 °C. Rochelle's salt, one milliliter, was added to halt the reaction. At 595 nm, the absorbance was measured. The creation of a standard graph was done using a standard glucose solution. The quantity of enzyme that releases 1 mmol of glucose per minute was used to define one unit of cellulase activity.

Partial cellulase enzyme purification

Ammonium Sulfate Precipitation

The crude enzyme solution was saturated with solid ammonium sulfate in about 20 ml, and the combination was then left overnight at 40°C to precipitate. Through centrifugation, the precipitates were recovered, and they were then dissolved in 10 ml of a 50 mM sodium acetate buffer with a pH of 5.5.

Dialysis method

After ammonium sulfate precipitation, the enzyme recovered was dialyzed against 30mM sodium acetate buffer (pH 5.5) at 4oC with three different buffer modifications to achieve partial purification. Enzyme activity and protein concentration were assessed in the sample that had only partially been purified.

Statistical Analysis

SPSS 16.0 was used to analyze the collected data quantitatively. A mean and standard variation are used to represent the data. (SD). There was a p-value of 0.05 in the one-way ANOVA.

Application of *B. amyloliquefaciens* in Conversion of Lignocellulossics into Ethanol and Fermentable Sugars

The samples used in this analysis came from ordinary garden soil, namely the interior part of the rock, the rock surface, and grassland. At 4°C, these research samples were held aseptically in sterilized plastic bags. These research samples were serially diluted (10^{-1} to 10^{-5}) before being inoculated on sterile Petri dishes containing Luria

Bertani stable medium (LB). The Petri dishes were kept at 37°C for 3 to 5 days, depending on the speed with which the bacteria grew. Purified bacteria were grown in liquid media after development and stored at -80°C in a 20% glycerol solution. The bacterial research isolate was streaked on cellulose Congo red agar medium to validate its ability to degrade cellulose. Congo red dye is used as a cellulose utilization predictor in the nutrient medium. The appearance of Congo red discoloration identified positive cellulose-degrading bacterial colonies.

The novel *B. amyloliquefaciens* isolate produced a substantial amount of Cellulase, as evidenced by the discoloration of Congo red dye. Similar results have been obtained by Gupta et al**.** and Hendricks *et al*. 1995[12, 13].

Bioethanol Production from the Cellulose Saccharification and Fermentation using Cellulase obtained from B. amyloliquefaciens

Reducing sugar was determined by fermentation medium and fermentation medium was analyzed by GC-MS to determine ethanol concentration after the saccharification process.

Fermentable Sugars (Glucose) Production from the Saccharification and Fermentation of the Cellulose using new Cellulase obtained from B. amyloliquefaciens

After ammonium sulfate precipitation and dialysis, the culture supernatant of *B. amyloliquefaciens* with cellulase activity was used to saccharify brewer spent grains (BSG) [26]. Brewers' spent grains usually contain 16.8–25.4 percent cellulose, 21.8–28.4 percent hemicellulose, and 11.9–27.8% lignin. The raw material used in this analysis has a very high cellulose content and a very low lignin content.

Then pretreatment was given to BSG with aqueous ammonia soaking in the current analysis. In this experiment pre-treated biomass of BSG was treated with cellulase extract (1.5 U g -1 of pretreated biomass) obtained from *B. amyloliquefaciens* and incubated at specific temperature and time intervals. For a comparative study, 5.4 U g"1 of pretreated biomass of commercial *Trichoderma reesei* cellulase was used. After incubation cellulose, glucose and ethanol yield was determined.

RESULTS AND DISCUSSION

Screening and Identification of Bacterial Isolate

A cellulase-producing bacteria were recovered from the soil sample after the primary and secondary screening. The isolate was identified as *B. amyloliquefaciens* based on its appearance, biochemistry, and molecular characterization. The sequence is available under the accession number MN081796 after being submitted to NCBI. A phylogenetic tree is represented in Figure 1. *Bacillus amyloliquefaciens* was studied

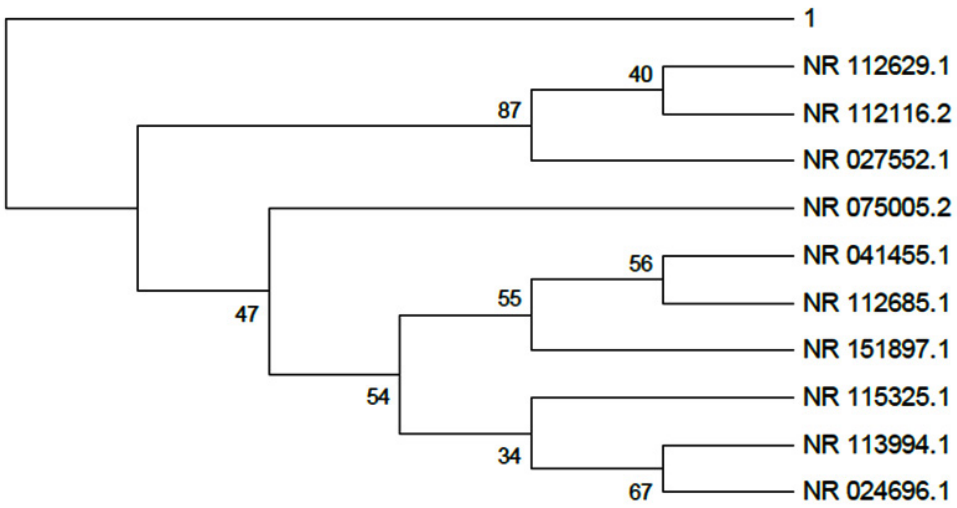

Fig. 1. Maximum Likelihood Molecular Phylogenetic study of Isolate 1 reveals *Bacillus amyloliquefaciens*

in detail for the cellulase production concerning the development of low-cost and easily available medium ingredients by submerged fermentation.

Production and Optimization of Bacterial Cellulase

The optimization of cellulase using bacterial species strain is used for the production of cellulase at the optimum condition of different parameters for industrial application [14, 15]. The production medium was optimized for different physicochemical parameters. Among raw substrates molasses, sugarcane bagasse, and paper pulp showed the highest cellulase activity with 0.98±0.12, 0.97±0.12, and 0.88±0.1U/ml, respectively [Table 1]. One of the most cost-effective sources for the manufacturing of enzymes was discovered to be molasses[16,17]. For commercial cellulase fermentations, various biomass, such as lignocellulosic material, paper waste, pulses, cereals, straw, and bagasse, have been employed as carbon sources[18, 19-26]. Cellulase production was higher at pH 7[Figure 2]. The pH range of 6.0-7.5 was previously found to yield the maximum degree of Cellulase by cellulolytic bacteria[19, 20]. Although Cellulase from bacteria can be stable in neutral to alkaline environments, Cellulase from fungi is unstable in alkaline environments[21]. Cellulase activity was greater at a temperature of 37^{O}C [Figure 3]. However, the optimum temperature for the synthesis of Cellulase may differ from one bacterial species to another [17, 18]. In our study, the cellulase activity of *Bacillus amyloliquefaciens* NP1607 was higher after 48 hours of the incubation period. During 48 hours of incubation, Persian Gulf cellulolytic bacterial isolates produced Cellulase at their highest levels[23]. However, depending on

Table 1. Effect of raw substrates on enzyme production

Raw substrates used	Enzyme activity(U/ml) cellulose	P value
Sugarcane baggase	0.97±0.12	0.12
Paper waste	0.88±0.11	0.13
Cassava waste	0.55±0.06	0.05
Tea waste	0.47±0.05	0.12
Orange peel	0.58±0.07	0.19
Molassses	0.98±0.12	0.01
Wheat bran	0.56±0.12	0.17
Agricultural waste	0.32±0.08	0.15

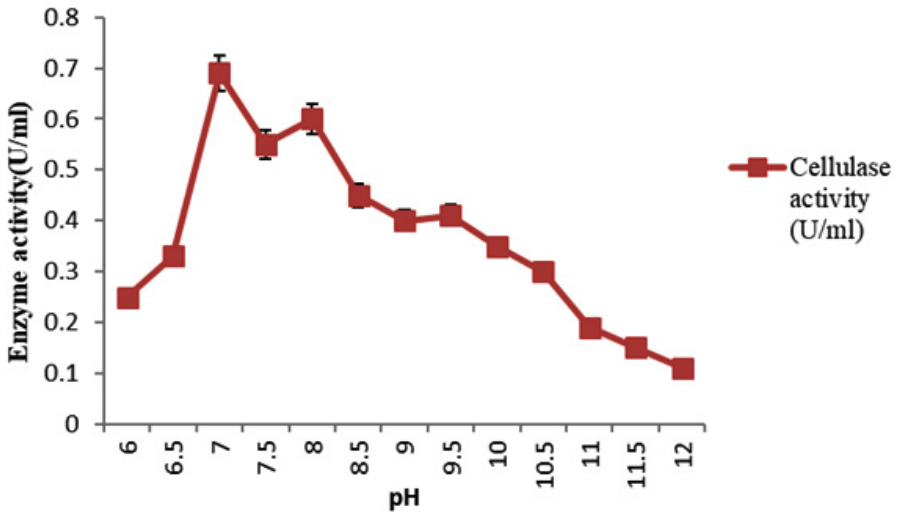

Fig. 2. Effect of pH on Enzyme activity. Error bar in the graph represents standard deviation of the mean value

each strain's qualities and environmental factors, optimal cultivation duration is different [24]. It was observed that the cellulase activity of *Bacillus amyloliquefaciens* NP1607 was showing high value when The only sources of nitrogen utilized were inorganic ones. In my study, ammonium sulfate gives optimum enzyme activity among other inorganic nitrogen sources. Similar findings were also made by Kumar et al. and Kalogeris et al. in their research [27,28]. *Bacillus amyloliquefaciens* NP1607 gives good cellulase activity when using glucose as a carbon source and peptone and yeast extract as an organic nitrogen source. Agitation is required in the submerged fermentation process. I got best enzyme results at 120 rpm agitation rate.

Applications of *B. amyloliquefaciens* and its Enzyme Extract

Due to the high expense of their utilization methods, the majority of agricultural, industrial, and municipal cellulosic wastes have been accumulating

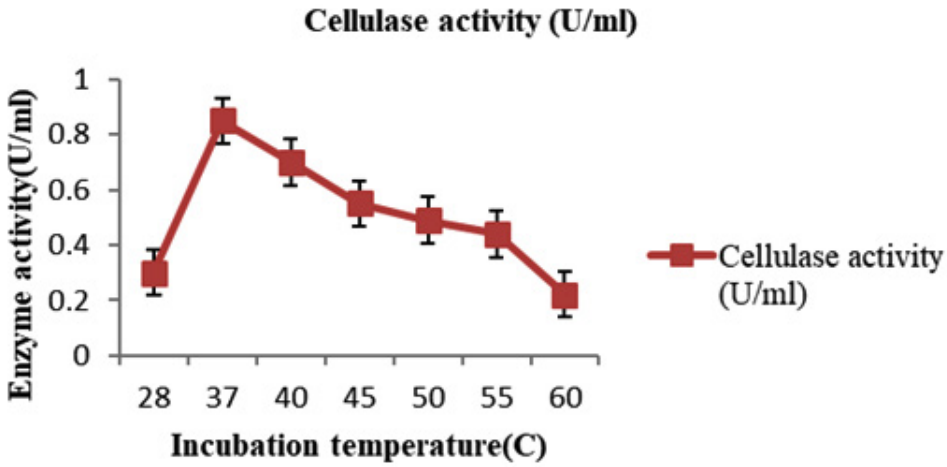

Fig. 3. Effect of temperature on Enzyme activity. Error bar in the graph represents standard deviation of the mean value

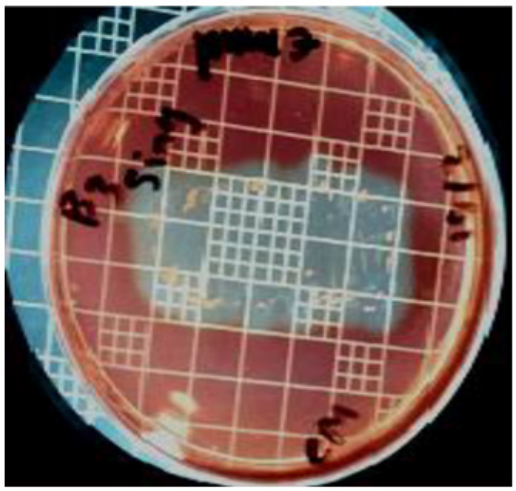

Fig. 4. Cellulase activity of the research variant of *B. amyloliquefaciens*(study strain) grown on a base medium containing Congo red. The transparent zone is a sign that *B. amyloliquefaciens* is degrading the cellulose

or used inefficiently. The most prevalent polymer in the planet is cellulose [29]. Therefore, it has become economically advantageous to create methods for efficiently treating and using cellulosic residues as low-cost carbon sources. A crucial chance for maximizing the benefits of biomass utilization is provided by cellulases. Many scientists have closely studied the cellulases made by fungus like *Aspergillus, Rhizopus*, and *Trichoderma species* [30-35].

Due to their potential use in the textile industry for bio stoning denim clothing to produce softness in fabric and a faded appearance, Cellulases have grown significantly in industrial significance. Due to the use of Cellulase, we can replace the customary industry practice of using pumice stone[36]. Cellulase and pectinase are used in the food business to clarify fruit and vegetable juices, make fruit purees and nectars, and extract olive oil.

The main enzymes responsible for converting lignocellulose into ethanol and fermentable carbohydrates are called cellulases. Enzymatic hydrolysis of polysaccharides produced by biomass pretreatment with cellulases is preferred for the transformation of lignocelluloses into ethanol and fermentable sugars for fuel generation. Cellulases have traditionally been primarily obtained from filamentous fungus. However, bacterial cellulases are most frequently favored because of the benefits that bacteria offer as a source of enzymes, including a faster growth rate and the potential for easy engineering[37].

The results of our study standardization revealed that the new *B. amyloliquefaciens* (study strain) cellulase activity has an apparent half-life of 9 h at 50 °C; at 55 °C and 60 °C, half-lives of 6 h and 4.72 h were found, respectively. When temperatures rise above 60 °C, the enzyme starts to lose its function. With a half-life of five days at pH4, pH5, pH6 pH7, and pH8, the cellulase activity of the research strain of *B. amyloliquefaciens* exhibits extremely high stability over a wide pH range.

The novel *B. amyloliquefaciens* (study strain) isolate produced substantial amount of Cellulase as evidenced by discoloration of Congo red dye (Figure 4). Gupta et al. (2012) and Hendricks et al. (2013) both achieved the same findings[12, 13].

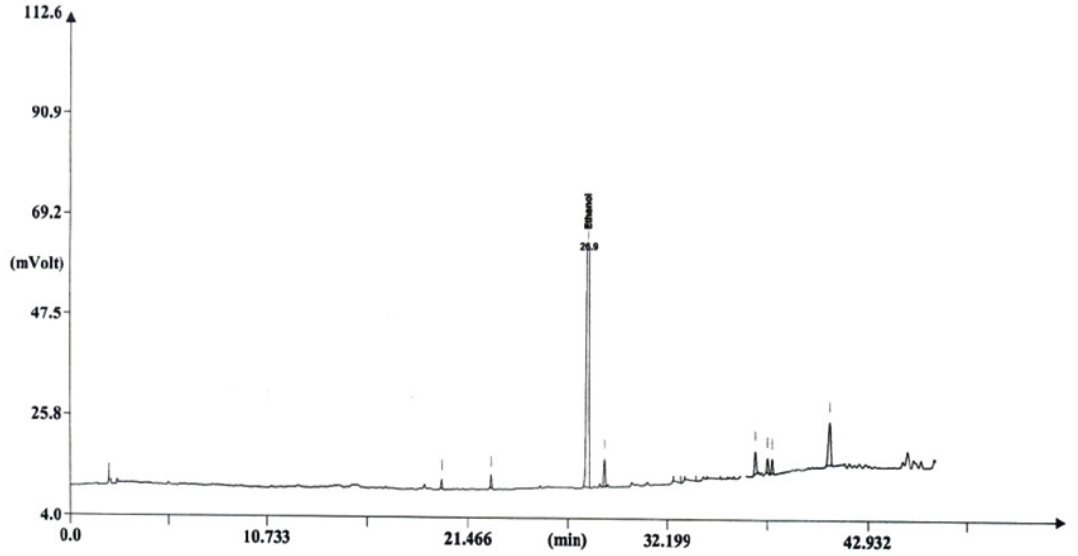

Fig. 5. Chromatogram of the ethanol generated by *B. amyloliquefaciens* culture supernatant

Table 2. Results of saccharification with sigma enzymes – cellulase from *Trichoderma reesei* ATCC 26921 (5.4 U g-1 of pretreated biomass) and cellulase from *B. amyloliquefaciens* (study strain) (1.5 U g -1 of pretreated biomass)

Composition	Cellulose yield (%)			Glucose yield (%)			Ethanol yield (%)		
	24h.	48h.	72h.	24h.	48h.	72h.	24h.	48h.	72h.
cellulase from Trichoderma reesei ATCC 26921 (5.4 U g-1 of pretreated biomass)	35.5	48.5	52.3	32.9	41.2	51.5	35.5	48.5	52.3
cellulase from *B. amyloliquefaciens* (study strain) (1.5 U g-1 of pretreated biomass)	36.7	79.2	91.2	44.6	63.6	79.8	36.7	79.2	91.2

Synthesis of Bioethanol via Saccharification and Fermentation of the Cellulose using new Cellulase obtained from B. amyloliquefaciens (Study Strain)

Before the saccharification procedure, the fermentation medium's reducing sugar content was 0.32 g/L. The GC-MS measurement of the fermentation medium revealed a 16.2 g/L ethanol concentration [Figure 5].

The findings above demonstrated that when *B. amyloliquefaciens* (the study strain) was used to hydrolyze cellulose, there was a higher concentration of ethanol. Cellulase enzymes' enzymatic degradation is very substrate-specific. According to a 2013 study, Caldicellulosiruptor DIB 004C, a wild-type bacteria, can produce 3.5 g/L of ethanol at most [38]. According to a recent research by Sato (1993), *Clostridium thermocellum* produced 4 g/L of bioethanol [39].

Fermentable Sugars (Glucose) Production from the Cellulose Saccharification and Fermentation using Cellulase Obtained from B. amyloliquefaciens (Study Strain)

The findings of saccharifying the pretreated BSG using a protein preparation of *B. amyloliquefaciens* (study strain) endowed with commercial cellulase activity (1.5 U g1 pre-treated biomass) are shown in the following table. The commercial Cellulase from *Trichoderma reesei* (Sigma) was used at the same dosage along with the novel enzymatic activity discovered in this study. It's important to note that using the enzyme preparation from the study strain of *B. amyloliquefaciens* produced outcomes that were comparable to those obtained with commercial Cellulase from *Trichoderma reesei* (Sigma). After a 48-hour hydrolysis reaction, the maximum cellulose recovery for the study enzyme and the commercial enzyme, respectively, was around 52.3% and 91%. Long incubation times had no effect on the yield figures. It's interesting to note that using the cellulase preparation from *B. amyloliquefaciens* (study strain) improved the degradation of the glucose fraction, which increased from 51.5 to 79.8% after 72 hours of incubation. However, the majority of the cellulose conversion in this instance was also finished in 48 hours [Table 2].

In our study we used crude enzyme and partially purified enzyme to perform industrial applications. Considering how expensive commercial cellulases are, it is currently uneconomical to convert biomass to ethanol or fermentation products using pure enzymes. A microbial enzyme isolated from a natural ecosystem possesses special qualities that may make them suitable candidates for enhancing the efficiency of biomass conversion into high-value products, chemicals, and fuels. The study of the components of cellulosic biomass and the natural sources of the microbial enzymes that govern the efficiency of biomass conversion, however, is still in its infancy [40,41].There are still issues that need to be addressed, so active study must be undertaken. Cellulosic biomass is an important resource that can serve as a substrate for the production of cellulases and other metabolites with additional value. To increase the mass production of bioethanol from cellulose, it is crucial to further improve microorganism abilities by introducing or altering traits like tolerance to ethanol and inhibitors, hydrolysis of cellulose/hemicellulose, thermotolerance, reduced need for nutrient supplementation, and improved sugars transport. The use of ethanol as a fuel for transportation has the ability to help protect the environment. The effective use of lignocellulose is anticipated to help the bioethanol industry.

CONCLUSION

Cellulase producer *Bacillus amyloliquificiens* was identified by 16s rRNA sequencing as having been isolated from a soil material. Production media was optimized for better enzyme production and isolate was applied for industrial application where we got prominent results. The use of isolate *Bacillus amyloliquificiens* in industry could have several advantages, including a rapid rate of growth and the capacity to secrete proteins extracellularly, which are all traits of the *Bacillus species*. This study has demonstrated the production of significant quantity of ethanol from pre-treated BSG by using cellulase from *Bacillus amyloliquificiens*. More knowledge from these analyses will help determine whether to use the entire organism for industrial purposes, harvest the enzymes for use in subsequent processes, or purify the gene to see if it should be added to the genetic pool for protein

engineering and directed evolutionary research to create super enzymes.

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Conflict of interests

Authors declare no conflict of interest

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