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Original Article

Isolation of Cellulolytic Bacteria from Soil, Identification by 16S rRNA Gene Sequencing and Characterization of Cellulase

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Microbial Cellulases have an escalating demand in many industries and constitute a major group of the industrial enzymes. It has attracted the attention of many researchers because of its tremendous industrial applications including textile industry, pulp, and paper industry, laundry and detergent industry, food and animal feed industry. The present study pursues to unfold a novel cellulase that can overcome existing challenges in biorefineries as well as to reduce biofuel production cost. Therefore, soil from a dairy farm was screened for potent cellulase producers on carboxymethylcellulose agar. Out of 68 isolates, 31 expressed cellulase activity. The best isolate so far had an extracellular crude enzyme activity of 0.167 U/ml and specific activity of 0.333 U/mg. The cell morphology, cultural characteristics, and biochemical tests presumptively identified it to belong to the genus *Bacillus*. Molecular analysis using 16S rRNA gene of the isolate indicated it to be *Bacillus subtilis*. The optimum pH and temperature for the activity of the crude enzyme were determined to be 5 and 65°C respectively.

Keywords: *Bacillus subtilis*, Cellulase, 16S rRNA

Introduction

Cellulose is a major component of plant biomass. Every year production of photosynthetic biomass is estimated to be approximately 40 billion tons, making cellulose the most abundant polysaccharide produced in the biosphere and also a renewable as well as a potential resource for bioconversion[1,2]. This biological conversion of the cellulosic biomass to fuels and chemicals offers a high yield of products vital to economic success and the potentiality for very low cost produce[3]. Cellulase is responsible for the bioconversion of cellulosic and lignocellulosic residues. It hydrolyses â-1, 4-glycosidic bonds in cellulose. The extensive intermolecular bonding pattern of cellulose creates a phenomenal crystalline substrate, particularly resistant to microbial degradation[4]. Hence, cellulolytic activity is a multi-complex enzyme system and complete enzymatic hydrolysis requires a synergistic action of 3 enzymes: endo-glucanase, exo-glucanase and glucosidase[5]. These enzymes act sequentially in the synergistic system and efficiently degrade cellulose converting it into an utilizable energy source. Thus, cellulases play a vital role in biomass utilization.

Although most efficient cellulase activities are observed in fungi, there is increasing interest in cellulase production by bacteria since they have a higher growth rate as compared to fungi and has good potential to be used in cellulase production[6]. Most common cellulolytic microorganisms include fungi like *Trichoderma, Humicola*, *Penicillium*, *Aspergillus* and among bacteria, *Bacillus*, *Pseudomonas*, *Cellulomonas* and within Actinomycetes, *Streptomyces* and *Actinomucor*[7].

Few pieces of evidence from previous studies performed in Bangladesh reveals that at present due to the urbanization and rapid growth of population, municipal solid waste is raising in the amount causing a threat to the environment. It mostly consists of cellulosic organic content which could be easily biologically converted to bioresources using potent organisms from the studies, in order to prevent environmental pollution and public health hazards in urban areas. This makes cellulase research imperative in Bangladesh[8]. Also, celluloses have a high demand in the textile industry for sustainable washing as a finishing treatment in the denim garments in order to make them comfortable to wear[9]. Thus, cellulases are used in wide range of industries such as agriculture, detergents, fermentation, food, pulp and paper, textile and others. The need to import cellulases by means of expensive transportation or the usage of harmful chemicals instead of cellulases in the industries can be prevented if the enzyme is produced in Bangladesh in a substantial amount and expanding the economy thereby. New enzyme sources can be developed using emerging biotechnological tools to provide desirable enzyme features, which include increasing specific activities with more balanced synergism, better thermal stability, better resistance to environmental inhibitors and improved combination of various enzyme activities that maximize sugar yields at low cost[3]. Lignocellulosic biomolecules are also used as an alternative

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source of energy to prevent global warming and air pollution[10]. Although, cellulases have a wide range of industrial applications in Bangladesh, the obstacles to developing cost-effective processes for converting biomass to fuels and chemicals are yet to be fully realized. The aim of this research work included isolation, identification, and screening of bacteria with high cellulase activity from soil samples and optimization of different parameters such as optimum pH and temperature for better enzyme activity. The present study further aimed to obtain a cellulase producer that would allow cellulase production at low cost; that can be utilized in the industries that would passively reduce environmental pollution through biodegradation and cellulose decomposition.

Materials and Methods

Sample collection, isolation and primary screening of cellulolytic bacteria

Soil sample was collected from 'Bagan Bari Dairy Farm', Keranigonj. The samples were collected in a sterile container and stored at 4°C until use. Ten-fold serial dilutions of the soil sample were prepared in autoclaved saline water. One hundred microlitre sample from 10^{-4}, 10^{-5} and 10^{-6} dilutions were spread plated on Nutrient Agar (NA) plates. The NA plates were then incubated at 37°C for 24 hours. Pure cultures of bacterial isolates were transferred onto carboxymethylcellulose agar plates by needle inoculation and incubated for 48 hours. The carboxymethlycellulose agar medium used for isolation of cellulolytic bacteria contained 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin[11]. After incubation at 37°C for 48 hours, the CMC agar plates were flooded with gram's iodine and allowed to stand at room temperature for around 10 minutes. The ratio of the clear zone diameter to colony diameter of CMC hydrolysis was measured[6]. The bacterial colonies having the lergest ratio were analyzed for cellulase activity.

Estimation of cellulase activity and specific activity

Cellulase activity was assayed using dinitrosalicylic acid (DNS) reagent by estimation of reducing sugars released from CMC solubilized in 0.05 M phosphate buffer at pH 8. Crude enzyme was added to 1.0 ml of 1 % CMC (used as substrate) in 0.05 M phosphate buffer (pH 4.8) and incubated in a water bath at 50°C for 30 min. After incubation, the reaction was stopped by the addition of 1.5 ml of DNS reagent, boiled at 100°C in a water bath for 10 min and were cooled under running tap water. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using a glucose calibration curve. Using equal volumes of distilled water and 0.05 M phosphate buffer as blank, the absorbance was taken and a graph was plotted for the values of glucose concentration against the corresponding absorbance. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1 ìmol of glucose per minute under standard assay conditions[12].

Protein concentrations in the crude enzymes were determined by using the Folin Lowry method with bovine serum albumin (BSA) as a standard[13]. This was used to calculate specific activity.

Presumptive identification of cellulase producing bacteria

The best isolate was tentatively identified by means of morphological, cultural and biochemical characterization. The biochemical tests performed were: Carbohydrate fermentation (Sucrose, fructose, glycerol, maltose and D-xylose), Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), Urease test, Nitrate reduction test, Catalase test, Oxidase test, Casein hydrolysis test, Motility test, Gelatin hydrolysis test, Mannitol Salt Agar, Starch hydrolysis, Blood agar, growth at 45°C, 65°C, in 7% NaCl media and ability to grow in anaerobic condition.

Identification by 16S rRNA gene sequencing

Genomic DNA was extracted according to the protocol provided with the wizard® Genomic DNA Purification Kit.

The 16S rRNA gene from the isolated DNA was amplified by Polymerase Chain Reaction (PCR) using bacterial universal primers 27F (5'–AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). A reaction mixture was prepared adding 5 µl of 10X reaction buffer having 1.5 mM $MgCl_2$, 1 µl of 10 mM dNTPs and 39.75 µl of nuclease free water. Then each of the DNA primers was added in an amount so that the final concentration of DNA primers become 0.2 mM in the final reaction mixture. Afterward, 2 µl of DNA template was added along with 0.25 µl of Taq polymerase. The final reaction volume was 50 µl. All the steps were performed on ice. The PCR reactions were performed in a thermal cycler (Applied Biosystems, USA). The PCR program initiated with an initial denaturation of template DNA at 94°C for 2 minutes, followed by 30 cycles: denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 2 minutes. A single final extension was done at 72 °C for 5 minutes[14].

After PCR reaction, amplification was checked by horizontal electrophoresis in 1.0 % agarose slab gel in Tris –borate EDTA (TBE) buffer. 1 Kb plus DNA marker (Invitrogen, USA) was used to identify the amplicon size. The ethidium bromide-stained DNA bands were observed on a UV transilluminator at 365 nm (UV Transilluminator, Waltec). Amplified PCR products were purified by using the purification kit Wizard® SV Gel and PCR Clean-Up System. These purified PCR products were sequenced by the Sanger sequencing method using the ABI Genetic Analyzer (Model: 3700) in 1st BASE Laboratories, Malaysia. The sequence obtained was subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and was used for constructing a phylogenetic tree using MEGA6 software.

Effect of pH on activity of crude cellulase

The optimum pH for the crude enzyme was determined by incubating crude enzyme with substrate (1% CMC) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0 to 6.0), 0.05 M sodium phosphate buffer (pH 6.0 to 8.0), 0.05 M Tris-HCl (pH 8.0 to 9.0) and 0.05 M glycine-NaOH (pH 9.0 to 10.0). The crude enzyme mixture was added to those buffers and incubated for 30 min at 50°C. Cellulase activity was assayed by the DNS method as previously described. The optical density (OD) was taken at 540 nm in a spectrophotometer.

Effect of temperature on activity of crude cellulases

The effect of temperature on the activity of cellulase was determined by incubating crude enzyme with 1% CMC in 0.05 M phosphate buffer (pH 6.5) at temperatures, including 25°C, 35°C, 45°C, 55°C, 65°C, 75°C and 85°C. Cellulase activity was assayed by the DNS method as described above.

Result

Isolation and primary screening for cellulase producing bacteria

A total 68 isolates were obtained on nutrient agar plates, out of which only 31 were capable of degrading cellulose. Their clear zone ratio is presented in figure 1.

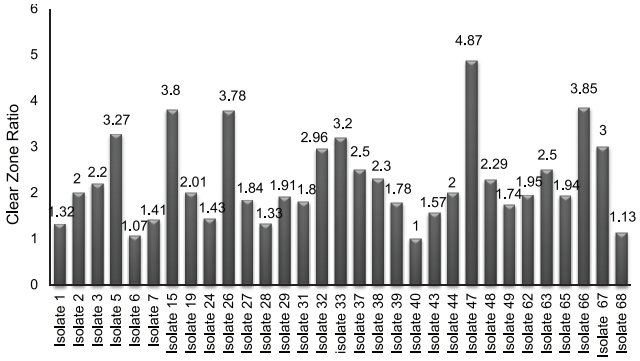

Figure 1*: Ratio of clear zone diameter to colony diameter of total 31 cellulose degrading isolates.*

Estimation of cellulase activity and specific activity

Out of the 31 cellulose degrading isolates, the two best isolates 47 and 66 were evaluated for their enzyme productivity in submerged fermentation process. The cellulase activities of the isolate 47 and 66 were found to be 0.101 U/ml and 0.165 U/ml respectively as shown in figure 2. The specific activity of the isolate 66 was 0.333 U/mg while isolate 47 showed a specific activity of 0.271 U/mg. This is also represented in figure 2.

Since the isolate 66 showed higher enzyme activity along with higher specific activity, it was identified and its enzyme was characterized.

Presumptive identification of the best cellulase producing isolate

The morphological and cultural characteristics were observed and biochemical characteristics are represented in Table 1. The organism was found to be gram positive (Figure 3), facultative anaerobe and spore former. According to the biochemical tests,

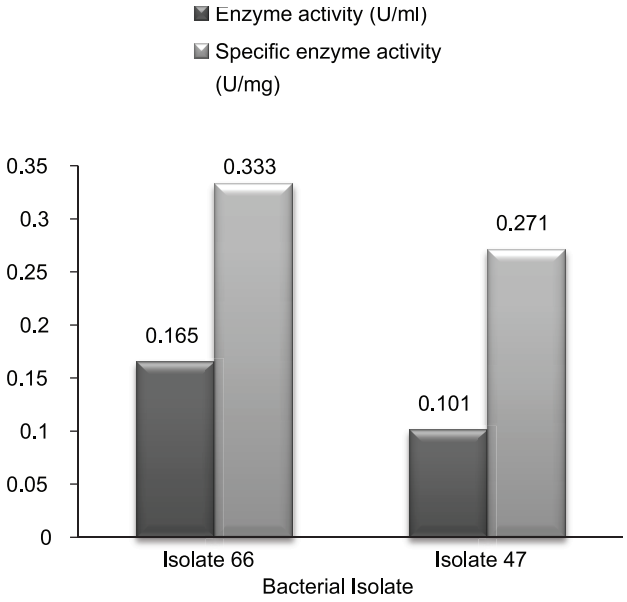

Figure 2: *Cellulase activities and specific enzyme activities of isolated bacteria 47 and 66 in submerged fermentation at 37°C.*

Table 1: *Biochemical reaction and characteristics of the cellulolytic bacterial isolate 66*

Biochemical tests	Results	Biochemical tests	Results
Gram's reaction	+	Anaerobic growth	+
Spores formation	+	Mannitol salt agar test	+
Indole production	+	Starch hydrolysis test	+
Citrate utilization	+	Casein hydrolysis test	+
Voges-proskauer	+	45æ%C	+
Triple Sugar	Alkaline slant (red)	65æ%C	-
Iron agar test	and acid butt (yellow)	7% NaCl	+
Urease	-	Glucose	+
Catalase	+ (weak)	Lactose	-
Oxidase	-	D-xylose	-
Nitrate reduction	+	Fructose	+
Motility	-	Maltose	-
Methyl red	-	H_2S	-
Gelatin hydrolysis	+	Gas	-

+ : indicates positive result; - : indicates negative result

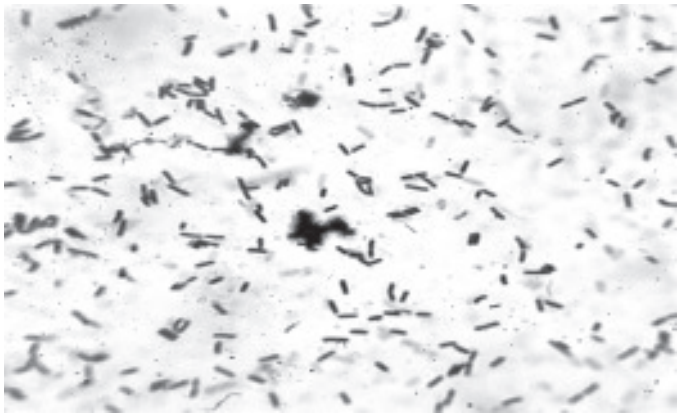

Figure 3: Gram staining of isolate 66 (positive)

the organism belongs to the genus *Bacillus* as compared with Bergey's Manual of Systematic Bacteriology[15].

Identification by 16S rRNA gene sequencing

PCR product size was found to be just below 1650 bp by agarose gel electrophoresis (Figure 4).

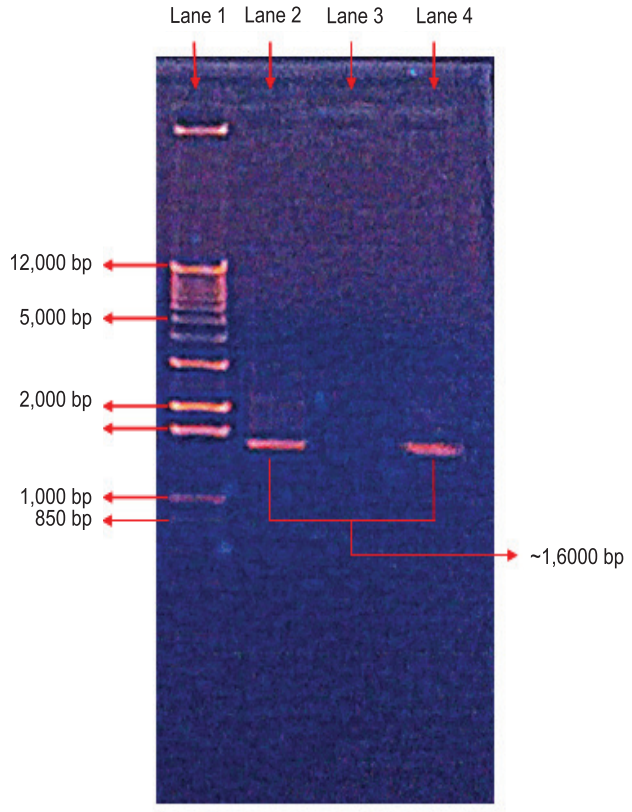

Figure 4: *Agarose gel electrophoresis result of the amplification of 16S rRNA gene of the isolate 66. The DNA band size was estimated to be just below1650 bp according to the 1 Kb plus ladder and a positive control on the other side of the sample. Lane 1: Kb plus DNA Ladder, Lane 2: 16S rRNA PCR product, Lane 3: Negative control, Lane 4: Positive control.*

The sequence obtained was trimmed and cleaned. Most of the homologous sequences received from NCBI nucleotide databank based on higher percentage identity (99%) and 0.0 E-value ($<10^{-5}$) after subjecting the corrected Fasta format of the query sequence to BLASTn program, belonged to *Bacillus* spp. Phylogenetic tree indicated it to be *Bacillus subtilis*. The higher bootstrap value (71) of the branches of *Bacillus subtilis* subsp. *spizizenii* with the query sequence (EMBOSS 001) clearly signified the stability of the branching pattern (Figure 5). Also, the distant branches of the two sequences belonging to the two different genera clearly signify having a distant relationship with the query sequence[16].

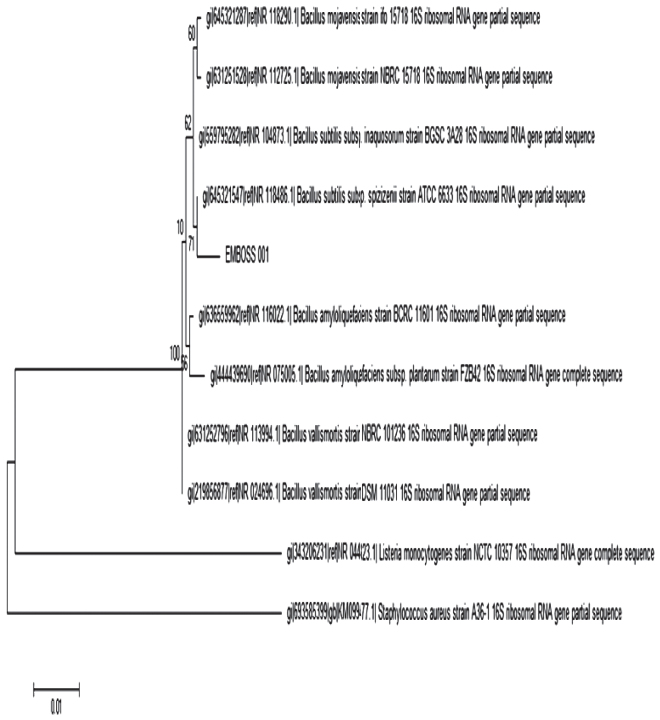

Figure 5: *Molecular Phylogenetic analysis by Maximum Likelihood method illustrating the query sequence (EMBOSS 001) to be identified as Bacillus subtilis with a bootstrap value of 71.*

Effect of pH on cellulase activity

Data illustrated in figure 6 clearly indicated that the highest enzyme activity of the isolate 66 was found to be 0.494 U/ml at pH 5.0 suggesting that the enzyme is an acidic cellulase.

Effect of temperature on cellulase activity

Effects of temperature from 25°C to 85°C were studied. Enzyme activity was found in the range of 0.063 U/ml to 0.133 U/ml. Data represented in figure 7 showed that the highest enzyme activity of isolate 66 was found to be 0.133 U/ml at 65°C.

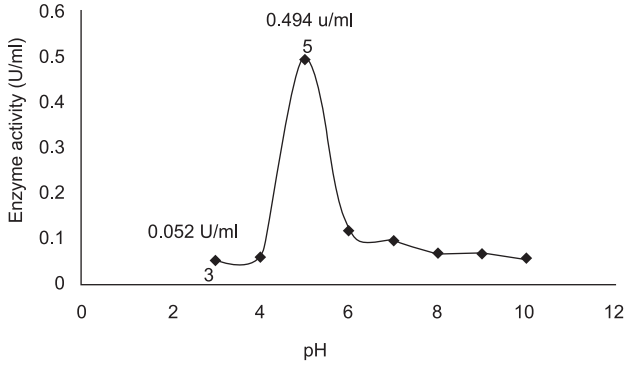

Figure 6: *Effect of pH on activity of cellulase by isolate 66*

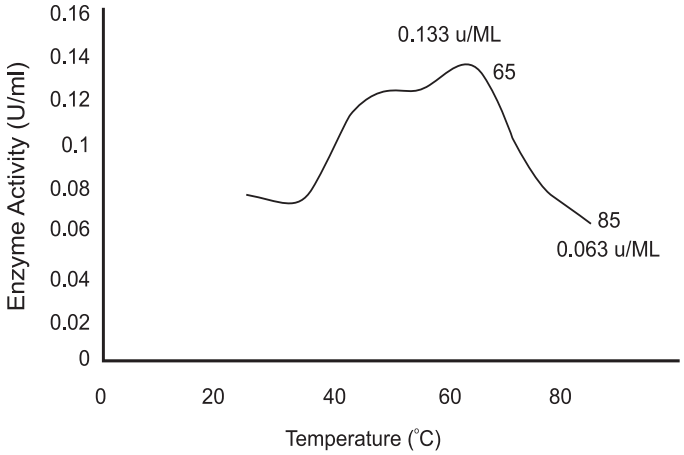

Figure 7: *Effect of temperature on activity of cellulase by isolate 66*

Discussion

Soil from a dairy farm was selected as the sample for the study since cow manure is considered as a desirable source of cellulase producers because of its high content of cellulosic material[17]. Around 50 % of the bacteria showed cellulolytic activity indicating this to be a good source of cellulose degraders. Previous studies conducted with cow dung displayed similar results[17,18]. Sources such as farming soil, forest soil[1], retting ponds and estuary[2], hot springs[19], wood furnishing region, sugar cane farm and water samples collected from paper industry waste and municipal waste[6] are also potential sources of finding cellulolytic organisms.

Compared with the data provided by Bergey's Manual of Systematic Bacteriology, the strain turned out to be facultative anaerobe since it could grow under anaerobic condition due to its ability to ferment in the absence of O_2 as an electron acceptor[15]. Out of the few previous studies using cow manure, one identified *Bacillus subtilis* with the highest homology of 100% and in another study, *Bacillus circulans* and *Bacillus nealsonii*[17] were identified with a sequence identity of 99.04%. Similar experiments with other samples identified *Anoxybacillus flavithermus* and *Geobacillus thermodenitrificans*[19] as well as *Bacillus caldoxylolyticus* and *Bacillus* sp. strain AK1[20] as the potential cellulase producers. The optimum pH was close to that of most bacterial cellulases[21] and a pH of 5.0 to 6.5 was found for *Bacillus subtilis*[22] in some studies. Also, cellulases from *Bacillus subtilis* subsp *subtilis* A-53, *B. subtilis* YJ1 and *Bacillus* strains RH68 have optimum temperatures of 50°C, 60°C and 70°C respectively[11,23] which are similar to the outcome of the present study.

The isolated *Bacillus subtilis* was able to produce a moderate level of cellulases under thermophilic conditions from the submerged fermentation process. The typical high growth rate in *Bacillus subtilis* along with the ability to secrete extracellular proteins is a feature of the bacteria to be implemented in several industrial applications, for example, in biofuel, laundry, and detergent, pulp, and paper industries. Site-directed mutations and implementation of metabolic engineering can be carried out in order to develop the strain. The identification gives us the ability to control the fermentation according to the specific requirements for better industrial performance. A further analysis of the physiology of the microorganism, pre-treatment of cellulosic biomass for enhanced microbial attack, processes for cost-effective production of cellulases, production of various necessary metabolites and finally utilization of the knowledge of protein engineering principle in order to increase the enzyme specific activities, process tolerance and stability can be carried out in the near future.

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