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Isolation, Identification and Cellulase Production by Bacillus Brevis from the Acacia Forest Soil

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ABSTRACT

A *Bacillus* isolate AK5 which was provisionally identified as *Bacillus brevis* isolated from the *Acacia* forest soil at University of Chittagong was found to be capable of producing cellulolytic enzymes during growth on different cellulosic substrates. The isolate AK5 grown under different conditions showed that CMC was the best cellulosic substrates for inducing the synthesis of extracellular cellulolytic enzymes. The isolate also showed heavy growth at different pH, temperature, incubation period, substrate concentration, carbon and nitrogen sources during growth in liquid Winstead's media having 1.2% CMC as cellulose substrate. Maximum level of CMCase is 143.22 U/ml at pH 6.5, 183.89U/ml at 40°C, 248.25U/ml in 1% substrate concentration, 259.49 U/ml at 2 hours incubation period, 154.67U/ml when asparagine as a nitrogen source and 105.21U/ml when CMC as a carbon source. Maximum 228.81 U/ml CMCase activities were recorded in liquid media when asparagine was used as a nitrogen source along with CMC as carbon source. The crude enzymes of the isolates were found to show highest enzyme activities i. e. CMCase 179.66 U/ml, FPase 162.71 U/ml, Avicelase 92.37 U/ml and β-glucosidase 81.53 U/ml.

Keywords: *Cellulose, Cellulase, CMCase, Bacillus brevis*

INTRODUCTION

Cellulose along with hemicellulose makes up the fibrous material of plants. Cellulose is an important structural component of the primary cell wall of green plants. Cellulose $(C_6H_{10}O_5)n$ is a long chain polymeric polysaccharide where glucose units are joined together by 1, 4 β-linkage [7, 37]. The cellulose content of cotton fiber is 90%, that of wood is 40–50% and that of dried hemp is approximately 45%. Some animals, particularly ruminants and termites, can digest cellulose with the help of symbiotic microorganisms that live in their guts.

Bacterial cellulolysis has recently gained importance as a potential source for development of commercial process because of high growth rate, wide genetic variability and adaptability and high amenability to genetic manipulation [18, 38]. The commercial possibility of using cellulase preparations to produce glucose, alcohol and protein from cellulose is under intensive study [21]. Cellulose of plants can be converted to constituent glucose by cellulase system and this glucose could be fermented to ethanol [12, 39]. Cellulose is the most abundant organic source of food, fuel and chemicals. However, its usefulness is dependent upon its hydrolysis of glucose [35]. The biological degradation of cellulose has a great importance in the activities of the living systems. The degradation of cellulosic biomass represents an important part of the carbon cycle within the biosphere. For the same reason, treatment of cellulose by cellulolytic enzymes for practical purposes has attracted the continuing interest of biotechnologists [1]. A large part of cellulosic substance is added to the soil by the green vegetation. In nature cellulose is usually associated with other polysaccharides such as xylan or lignin. Microbial cellulase converts cellulose into glucose or a disaccharide. Cellulase is a group of enzymes that degrade the plant polysaccharide called cellulose. The bioconversion of various complex cellulosic waste materials such as sugarcane baggase [19], corncob, saw dust [34] have been reported. The animal source of cellulase enzyme is produced from the bacteria found in the digestive system of ruminating herbivores such as deer, sheep and cows. It is produced by microorganisms, such as plant pathogens, to breach these cell walls, and by plants to help fruits soften as they become ripe.

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In the present study, cellulolytic bacterium *Bacillus brevis* was isolated and characterized based on morphological, cultural and biochemical analysis and identified by comparing with the standard descriptions given in Bergey's Manual of Systematic Bacteriology [5, 14]. It has been studied for its cellulase activity, extracellular reducing sugar, protein, saccharification and biomass production.

MATERIALS AND METHODS

Isolation, Purification and Screening of the Cellulase Producing Microorganism

Acacia forests soil was collected to isolate cellulolytic bacteria using Czapek's agar medium having 2% CMC as carbon source. Again the isolates were tested for cellulolysis in Winstead's medium having 1.2% CMC as carbon source. Among the numerous isolates a *Bacillus* sp. (AK5) was found promising cellulose degrader. After isolation the organism was purified through repeated plating in Nutrient Agar Media. For screening the isolate firstly, 8 ml Winstead's agar without carbon source was poured in a sterile Petri plate (9 cm) and allowed to solidify. After solidification, 8 ml Winstead's agar with 1.2% CMC as a cellulose source was poured aseptically over the first one. After solidification, the plates were inoculated and then incubated at 37 ± 1^0C for 3 to 5 days. Then the plates were flooded with 10 ml Congo red solution (0.1%), after 20 minutes replacing with 10 ml of 5M NaCl solution [36]. After 20 minutes the salt solution was removed. An orange red colour around the colonies revealed CMC-ase activity. Besides, the clear zone in CMC containing Winstead's broth media would indicate the capability of cellulose production by the isolates. For the identification of selected isolates different morphological, cultural characteristics (size, shape, arrangement, colour, growth on agar plate, agar slants, in liquid or in deep agar media etc.) and biochemical characteristics (indole test, methyl red test, Voges proskauer test, catalase test, oxidase test, urease test, nitrate reduction test, gelatin hydrolysis test, Starch hydrolysis test, H_2S production and carbohydrate fermentation test) were observed. Finally the characteristics were compared with the standard description of Bergey's Manual of Systematic Bacteriology [5, 14] and provisionally identified as *Bacillus brevis.*

Saccharification

For saccharification the enzyme preparation (crude) was adjusted to pH 4.0 and sodium azide (0.2%) was added to inhibit the microbial growth. Hydrolysis was carried out under stationary condition in 25 ml screw cap test tubes at 50°C with substrate (cellulose) concentration of 7.5% (w/v) for 24 hour and 48 hour of intervals [2]. The sugar content in the hydrolysate was measured by Nelson's modification of Somogyi method [29].

Saccharification percentage was calculated by applying the following equation:

$$\text{Saccharification \%} = \frac{\text{mg of reducing sugar per ml}}{\text{mg of substrate per ml}} \times 100$$

Biomass Yield

Bacterial biomass was determined by measuring absorbance at 600nm [13].

Optimization of Culture Condition

An attempt was also made to determine the optimum culture conditions such pH, temperature, incubation period, carbon and nitrogen sources requirement for maximum growth and production of extracellular protein, reducing sugar level, cellulase, saccharification (%) and biomass yield.

Incubation Period and Substrate Concentration

To determine the optimum incubation period of the isolate for maximum enzyme production, the supernatant were collected after 2, 3, 4, 5, 6, & 7 days of incubation and substrate concentration was 0.5%, 1.0%, 1.5% and 2.0%.

Medium pH and Temperature

To determine the optimum pH for maximum production of enzyme, selected medium of different pH (such as 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5) was inoculated with the isolate. To determine the optimum temperature for maximum enzyme production the culture medium was incubated at 27°C, 35°C, 40°C, 45°C and 50°C temperature was inoculated with the isolate.

Carbon and Nitrogen Sources

The production of extracellular under different carbon and nitrogen sources was studied in the liquid Winstead's culture medium four carbon sources (CMC, rice bran, rice straw and saw dust of 1.2%) and five nitrogen sources (asparagine, beef extract, ammonium sulphate, peptone and urea of 0.2%) were used and the effects of these carbon and nitrogen sources on the production of cellulase, protein, reducing sugar, saccharification (%) and biomass yield were recorded.

Production of Sugar

When microorganisms are allowed to grow on cellulosic materials, they degrade the cellulose into sugar. So estimation of reducing sugar in the culture filtrate by Nelson's modification of Somogyi method [29] indicates the rate of degradation of cellulosic substances.

Production of Protein

When microorganisms are allowed to grow on cellulosic waste material they convert cellulose into protein, popularly known as single cell protein. Soluble protein contents of each enzyme extract were determined by the method of Lowry [23].

Enzyme Assay

For CMCase activity 2 ml of the filtrate was added to 2 ml of 1% CMC prepared in phosphate buffer at pH 6.5, then added 1 ml of phosphate buffer and incubated at 45°C for 120 min. For FPase activity 2 ml of filtrate was added to 1 ml of phosphate buffer at pH 6.5 along with 50 mg Whatman No. 1 filter paper strip (1×6 cm) in a test tube and incubated at 45°C for 120 min. For avicelase and β-glucosidase all procedures were same as CMCase activity. The amount of reducing sugar's released in CMCase, FPase, Avicelase and β-glucosidase assay after incubation was measured by Nelson's modification of Somogyi method. Enzyme activity was expressed by the amount of glucose released in µg/ml of crude enzyme/hour (U/ml) enzyme substrate reaction at given conditions. Soluble protein in culture filtrate was estimated following the method described by [23] measuring the absorbance at 650nm.

RESULTS AND DISCUSSION

Isolation and Screening of the Cellulase Producing Microorganism

Cellulose degrading bacteria were enriched and isolated by inoculating in Winstead's media; the bacterial culture showed growth as the medium turned cloudy. Bacteria isolate found to be positive on screening media producing clear zone. Physiological and biochemical characteristics are shown in Table 1.

Effects of Medium pH, Temperature, Substrate Concentration and Incubation Period

The Effects of medium pH, temperature, substrate concentration and incubation period on the production of CMCase, extracellular protein, reducing sugar, saccharification (%) and biomass yield are shown in following Tables. Maximum production of CMCase, extracellular protein, reducing sugar, saccharification (%) and biomass yield was recorded in culture media with pH at 6.5 but 7.5 for CMCase activity as shown in Table 2, temperature at 40°C but 35°C for saccharification (%) shown in Table 3 and incubation period 5 days shown in Table 4 and substrate concentration 1% shown in Table 5.Optimum nitrogen and carbon sources for maximum production of cellulases were asparagine and CMC but rice bran for extracellular protein. Heavy growth at pH 6.5 and 7.5 with different microorganisms was reported by many workers [9, 24, and 31] which are in accordance with our observation.

Liquefaction of Winstead's medium (with 1.2% CMC) due to enzyme activity at 35°C and 45°C which was recorded here in found similar to the finding's of [3, 8, 9, 15, 24, 31, 33]. Production of CMCase by fungi at 25-28°C was reported by [2, 22], 40°C was reported by [22]. Our result with the isolate *Bacillus brevis* is in concurrence with the above reports. Production of maximum CMCase at 42± 2°C was reported by [25].

Effects of Carbon and Nitrogen Sources

The influence of various carbon and nitrogen sources on the production of CMCase, extracellular protein, reducing sugar, saccharification (%) and biomass yield by the isolate AK5 in the Winstead's

broth media as shown in Table 6 & 7. Among the cellulosic substrates CMC supported good growth and maximum CMCase by the isolate AK5 in the Winstead's broth media. The microbial cellulase enzymes production due to addition of different carbon and nitrogen sources in the medium reported by some workers [4, 26, 32].

In the present investigation maximum enzyme activity were found when CMC was used as a carbon source (1.2 %) and asparagine as a nitrogen source with Winstead's medium. Similar observation has also been made by other workers [15, 16, 22, and 35].

Table1. *Physiological and biochemical properties of strain AK5*

Characteristics	Reaction
Catalase	+
H_2S production	–
Nitrate reduction	+
Voges-Proskaur test (V.P.test)	–
Methyl red	–
Casein hydrolysis	+
Indole test	–
Motility	–
Hydrolyzing ability	
Starch	+
Gelatin	+
Acid fermentation	
Arabinose	+
Xylose	+
Starch	+
Mannitol	–
Galactose	–
Sucrose–	–

"+": positive reaction; "–": negative reaction.

Table2. *Effects of pH on extracellular protein, reducing sugar and production of cellulase by Bacillus brevis (AK5)*

pH	Extracellular protein µg/ml	Reducing sugar µg/ml	CMCase activity U/ml	Saccharification (%)	Biomass yield (absorbance at 600 nm)
3.5	60.50*	72.88	45.21*	0.63	0.129*
4.5	71.19	77.12	158.47	0.64	0.149
5.5	103.26	85.59	174.56	0.79	0.167
6.5	172.10**	163.56**	247.46	1.57**	0.288**
7.5	143.12	61.86	266.10**	0.54	0.159
8.5	63.41	43.54*	51.27	0.34*	0.149

** indicates minimum, ** indicates maximum*

Table3. *Effects of temperature on extracellular protein, reducing sugar and production of cellulase by Bacillus brevis (AK5)*

Temperature (°C)	Extracellular protein µg/ml	Reducing sugar µg/ml	CMCase activity U/ml	Saccharification (%)	Biomass yield (absorbance at 600 nm)
27°C	97.83	54.24	121.19	0.52*	0.105*
35°C	102.36	193.22	176.27	2.24**	0.235
40°C	202.90**	233.89**	248.31*	1.15	0.457**
45°C	135.87	56.78	168.64	0.79	0.141
50°C	72.46*	42.37*	88.12*	0.74	0.113

** indicates minimum, ** indicates maximum*

Table4. *Effects of incubation period on extracellular protein, reducing sugar and production of cellulase by Bacillus brevis (AK5)*

Incubation period	Extracellular protein µg/ml	Reducing sugar µg/ml	CMCase activity U/ml	Saccharification (%)	Biomass yield (absorbance at 600 nm)
2	53.44	48.31	91.53*	0.39	0.176
3	170.29	71.19	133.05	0.62	0.144
4	182.97	155.93	141.53	1.34	0.257
5	305.25**	231.36**	246.61**	2.14**	0.325**
6	155.79	41.53*	116.95	0.37*	0.129
7	48.91*	54.83	94.07	0.47	0.107*

** indicates minimum, ** indicates maximum*

Table5. *Effects of substrate concentration on extracellular protein, reducing sugar and production of cellulase by Bacillus brevis (AK5)*

Substrate concentration	Extracellular protein µg/ml	Reducing Sugar µg/ml	CMCase activity U/ml	Saccharification (%)	Biomass yield (absorbance at 600 nm)
0.5%	125.91	113.56	121.19	1.16	0.222
1.0%	128.62**	170.34**	243.22**	2.41**	0.447**
1.5%	109.60	85.59	95.76	1.17	0.221
2.0%	61.59*	69.49*	94.07*	0.81*	0.216*

** indicates minimum, ** indicates maximum*

Table6. *Effects of nitrogen sources on extracellular protein, reducing sugar and production of cellulase by Bacillus brevis (AK5)*

Nitrogen sources	Extracellular protein µg/ml	Reducing sugar µg/ml	CMCase activity U/ml	Saccharification (%)	Biomass yield (absorbance at 600 nm)
Asparagine	153.08**	140.68**	228.81**	2.71**	0.572**
Beef extract	87.86	71.19	172.88	1.26	0.254
Peptone	121.38	81.76	122.03	1.24	0.240
Ammonium sulphate	124.09	50.85*	164.41	1.19*	0.238**
Urea	77.89*	68.64	117.79*	1.30	0.252

** indicates minimum, ** indicates maximum*

Table7. *Effects of carbon sources on extracellular protein, reducing sugar and production of cellulase by Bacillus brevis (AK5)*

Carbon sources	Extracellular protein µg/ml	Reducing sugar µg/ml	CMCase activity U/ml	Saccharification (%)	Biomass yield (absorbance at 600 nm)
CMC	179.35	157.63**	182.20**	2.46	0.447**
Rice bran	231.62**	80.51	154.24	1.47*	0.312*
Saw dust	86.96*	65.18*	164.41	1.61	0.253
Rice Straw	103.26	133.90	150.00*	2.66**	0.322

** indicates minimum, ** indicates maximum*

Effects of Enzyme-Substrate Reaction Time on CMCase Activity

The quantitative CMCase activity of crude enzyme produced by the selected isolate AK5 while grown in liquid Winstead's medium having with their suitable carbon and nitrogen source different incubation periods (0.5, 1.0, 1.5, 2 hour) in a water bath shown in Table 8. The highest CMC-ase activity was recorded for 2 hours. Optimum conditions (incubation period, temperature, pH and substrate concentration) are important factors for maximum enzyme activity. Our results at optimum conditions during enzyme substrate reaction are in concurrence with another worker [11].

Effects of Enzyme-Substrate Reaction pH and Temperature on Enzyme Activity

The quantitative CMCase activity of crude enzyme produced by the selected isolate AK5 while grown in liquid Winstead's medium having with their suitable carbon and nitrogen source different pH (4.5, 5.5, 6.5, 7.5 and 8.5) in a water bath is shown in Table 9. The highest CMCase activity was recorded at pH 6.5. The optimum temperature during enzyme substrate reaction of crude enzyme of the selected isolates was recorded to be the best at 25°C, 30°C, 35°C, 40ºC, 45°C and 50°C. The highest CMCase activity was recorded at Temperature 40ºC is shown in Table 9. Similar observation with enzyme-substrate reaction temperature and pH was reported by [22, 31].

Effects of Different Carbon and Nitrogen Source on Enzyme Activity

The quantitative CMCase activity of crude enzyme produced by the selected isolate AK5 while grown in liquid Winstead's medium having CMC as a carbon source and different nitrogen source were determined. The highest CMCase activity was recorded when CMC as a carbon source is shown in Table 10. Besides the highest CMCase activity was recorded when asparagine as a nitrogen source is as shown in Table 10. Due to addition of different carbon and nitrogen sources in the medium was reported by some workers [17, 20, 26, 27, 28, 30, and 32]. Our observation shows similarities with their reports.

Table8. *Relative cellulolytic activities (Reducing sugar released) of crude enzymes at different incubation period that produced by Bacillus brevis (AK5)*

Incubation period (hour)	CMCase activity U/ml
0.5	95.76
1.0	100.00
1.5	53.39*
2.0	269.49**

** indicates minimum, ** indicates maximum*

Table9. *Effects of enzyme substrate reaction pH and temperature on relative CMCase activity of crude enzyme produced by Bacillus brevis (AK5)*

pH	CMCase activity U/ml	Temperature	CMCase activity U/ml
4.5	37.68*	25°C	78.81
5.5	55.45	30°C	69.49
6.5	143.22**	35°C	105.08
7.5	105.18	40°C	183.89**
8.5	83.10	45°C	67.79
		50°C	54.24*

** indicates minimum, ** indicates maximum*

Table10. *Effects of enzyme substrate reaction of carbon and nitrogen sources temperature on relative CMCase activity of crude enzyme produced by Bacillus brevis (AK5)*

Carbon sources	CMCase activity U/ml	Nitrogen sources	CMCase activity U/ml
CMC	105.21**	Asparagine	154.67**
Rice bran	82.05	Beef extract	106.13
Saw dust	43.32	Peptone	63.07
Rice Straw	39.62*	Ammonium sulphate	53.58
		Urea	53.03*

** indicates minimum, ** indicates maximum*

Comparative Activities of Different Cellulase

Other than CMCase activity, the isolate AK5 produced appreciable levels of FPase (92.37 U/ml), Avicelase (162.71 U/ml), and β-glucosidase (81.53 U/ml) when CMC as a carbon and asparagine as a nitrogen source were used. Comparative study of enzyme activity indicated that CMCase (179.66 U/ml) activity was higher compared to FPase, Avicelase and β-glucosidase as shown in Table 11.

Comparative study of enzyme production by the isolate indicated that in maximum cases, CMC-ase activity is higher compared to that of FP-ase activity, Avicelase and β-glucosidase which is in accordance with the findings of many workers [6, 10, 22, and 25].

Table11. *Effects of different optimum condition on relative cellulase activity of crude enzyme produced by Bacillus brevis (AK5)*

CMCase activity U/ml	FPase activity U/ml	Avicelase activity U/ml	β-glucosidase activity U/ml
179.66**	92.37	162.71	81.53*

** indicates minimum, ** indicates maximum*

CONCLUSION

Our investigation suggest *Bacillus brevis* that has been isolated from the forest soil of Chittagong University campus has the potentiality for using as organic matters decomposer, biofuel production as well as essential organism for cellulases. As cellulase is important microbial enzyme that is used widely in different purposes further work with this microbe can make it important organism for biotechnology.

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Environmental Pollution and Bioremediation: Microbial Remediation of Environment.

Microbial degradation of Organic matter: Microbial Degradation of Cellulose, Hemicellulose and Lignin.

Isolation and characterization of Antibiotic Producing Microbes and their improvement

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Microbial degradation of Organic matter: Microbial Degradation of Cellulose, Hemicellulose and Lignin.

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