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Isolation, Purification, Characterization of Cellulolytic Enzymes Produced by the Isolate *Streptomyces omiyaensis*

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Abstract: A *Streptomyces* strain A_2 which was provisionally identified as *Streptomyces omiyaensis,* isolated from goat's rumen was found capable of producing cellulolytic enzymes during growth on different cellulosic substrates. The isolate A_2 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 and liquefaction at pH 6.5, temperature 35 to 40°C and 5 days of incubation period during growth in liquid Winsted's media having 1.2% CMC as a cellulose substrate. Maximum level of CMC-ase (230.56 U mL^{-1}) in liquid media was found to produce when beef extract used as a nitrogen source along with CMC as cellulose source. Maximum level of reducing sugar (473.33 µg mL^{-1}) and protein (324.27 µg mL^{-1}) was obtained when CMC used as a growth substrate. The crude enzyme of the isolate was found to show highest enzyme activity (CMC-ase of 269.44 U mL^{-1}, FP-ase of 64.81 U mL^{-1}, Avicelase of 200 U mL^{-1} and β-Glucosidase of 138.89 U mL^{-1}) at pH 6.5 and temperature 45°C during enzyme substrate reaction. The molecular weight of enzyme of the isolate *Streptomyces omiyaensis* was determined by SDS-PAGE technique and found 85 kDa.

Key words: CMC-ase, FP-ase, Avicelase, β-Glucosidase, SDS-PAGE, *Streptomyces omiyaensis*

INTRODUCTION

Cellulose is the most abundant of all naturally occurring organic compounds which accumulates every year in large quantities in the form of agricultural, industrial, forest and residential wastes[1]. Due to the abundance and renewability, there has been a great deal of interest in utilizing cellulose as an energy resource and as a feedstock[2]. Many fibrous by-products have a substantial potential value as animal feedstuffs. Ruminants, especially, have the unique capacity to utilize cellulose, because of their microbes[3]. Bacterial cellulolysis has recently gained importance as potential source for development of commercial process because of high growth rate, wide genetic variability and adaptability and high amenability to genetic manipulation[4-6]. Effective bioconversion processes of cellulosic materials depend mainly on the good sources of cellulolytic enzymes, the nature of cellulosics and the optimal conditions for production and catalytic activity of the enzymes. Many attempts had been taken to detect potential sources and characterize their enzyme systems in the last century. Various microbial enzyme systems have been recognized for degradation of these materials under natural habitats[7]. Bioconversion of such materials to desirable products are complex processes requiring a number of different enzymes and from industrial viewpoints, production of extracellular cellulolytic enzymes especially carboxymethyl cellulase and Avicelase from aerobic bacteria like *Cellulomonas, Bacillus, Cytophaga, Streptomyces* etc. would be advantageous[8-11]. In natural habitats, most of the cellulose (90-96%) is degraded aerobically and the rest is degraded anaerobically[12]. Cellulosic materials, as agricultural and forest residues, are available in huge quantity, scattered over wide areas in many agro based countries including Bangladesh. Microbiological utilization of these residues may help in the production of food, feed and other valuable organic products. It is necessary to search for a potential bacterium and to find optimum environmental condition for the growth and enzyme activities and to improve their potentiality. Recently, biotechnologists have given importance on the production of extracellular cellulolytic enzymes from aerobic microbial strains for utilization of cellulosic materials.

The present study was carried to find out optimum conditions for growth and enzyme activities of a streptomyces isolate under aerobic condition.

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MATERIALS AND METHODS

Substrate preparation: Two natural cellulosic substrates such as rice bran and saw dust and two commercial semisynthetic cellulosic substrates such as CMC and avicel were used for growth and enzyme production by the selected isolate. The natural cellulosic substrates were pretreated by boiling in 0.5% NaOH (0.1g NaOH/g substrate) for 1 h following the method of Gray *et al*.[13].

Microorganisms: A *Streptomyces* species (A_2) was isolated from goat's rumen. After isolation the organisms were purified through repeated plating in Nutrient Agar media. For the identification of selected isolates different morphological (Fig. 1) and cultural (Fig. 2) characteristics (size, shape, arrangement, colour, growth on agar plate, agar slants, in liquid or in deep agar media etc) were observed. Finally the characteristics were compared with Buchanan and Gibbons[14] and provisionally identified as *Streptomyces omiyaensis.*

Biomass yield: Biomass was measured by dry weight method. After collection of the supernatant, the biomass residue was dried at 80°C and the yield was expressed as mg g^{-1} of substrates.

Optimization of culture conditions: An attempt was also made to determine the optimum culture conditions such as pH, temperature, incubation period and carbon and nitrogen source requirements for their maximum growth and activities. The biomass yield, extracellular protein, reducing sugar level and cellulase production of the selected isolate was recorded.

Incubation periods: To determine the optimum incubation period of the isolate for maximum enzyme production, the supernatant were collected after 2, 3, 5 and 7 days of incubation.

Medium pH: To determine the optimum medium pH, for maximum enzyme production, selected medium of different pH (such as 4.5, 6.5 and 8.5) was inoculated with the isolate. The effects of medium pH on growth and liquefaction were recorded.

Temperature: To determine the optimum temperature for enzyme production the culture medium was incubated at 25, 35, 40 and 45°C temperature at optimum pH and incubation period. The effects of temperature on growth and liquefaction were recorded.

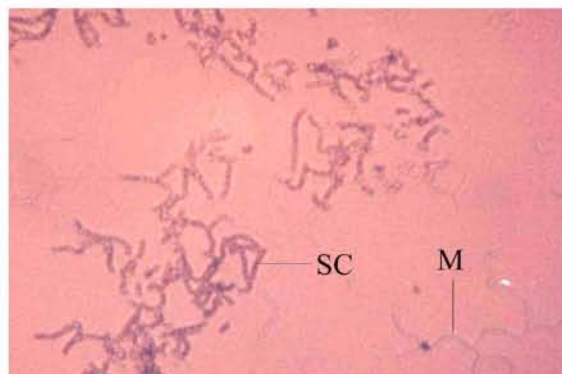

Fig. 1: Spore chain (SC) and mycelium (M) of the isolate A_2

Fig. 2: Growth (colony) of isolate A_2 on ISP-V medium

Carbon and nitrogen sources: The production of extracellular cellulase under different carbon and nitrogen availability was studied in the liquid Winstead's culture medium. Four carbon sources (CMC, avicel, ricebran, sawdust at the rate of 1.2%) and five nitrogen sources (asparagine, beef extract, yeast extract, urea, $(NH_4)_2SO_4$ at the rate of 0.2%) were used and the effects of these carbon and nitrogen sources on the production of cellulase, change of pH, biomass yield, protein and reducing sugar were recorded.

Culture conditions and enzyme preparations: For inoculum preparation, a loop of the isolate were inoculated in 150 mL conical flask containing 50 mL of Winsted's broth medium with different carbon and nitrogen sources and then incubated at 37±2°C for 5 days. After incubation the culture filtrate was collected by centrifugation at 8000 rpm for 15 min at 4°C and then filtered to remove the hydrophobic spore.

Crude enzyme activity: To determine the optimum conditions during enzyme substrate reaction phase for maximum activity of crude cellulase, different temperature and pH were studied.

Effects of pH: The effects of pH on the cellulase (crude) activity were studied in three different phosphate buffer solutions range from 4.5 to 8.5.

Effects of temperature: The effects of temperature on the cellulase (crude) activity were studied at different temperature such as 30, 35, 40, 45 and 50°C.

Enzyme assay: For CMC-ase activity 2 mL of the filtrate was added to 2 mL of 1% CMC prepared in phosphate buffer (pH 6.5), then added 1 mL of phosphate buffer and incubated at 45°C for 120 min. For FP-ase activity 2 mL of the filtrate was added to 1 mL of phosphate buffer (pH 6.5) along with 50 mg Whatman No.1 filter paper strip (1X6 cm) in a test tube and incubated at 45°C for 120 min. For β-glucosidase activity, 1% salicin and for avicelase activity, 1% avicel were used which were prepared in phosphate buffer (pH 6.5) and other procedures were same as CMC-ase activity. The amount of reducing sugars released in CMC-ase, FP-ase, Avicelase and β-glucosidase assay after incubation was measured by Nelson's modification of Somogyi method[15]. Enzyme activity was expressed by the amount of glucose released in μg mL^{-1} of crude enzyme/hour (U mL^{-1}) enzyme substrate reaction at given conditions[16]. Soluble protein in culture filtrate was estimated following the method described by Lowry *et al.*[17], measuring the absorbance at 650 nm.

Saccharification: Saccharification (%) was calculated by applying the following equation:

$$\text{Saccharification }\% = \frac{\text{mg of reducing/mL}}{\text{mg of substrate/mL}} \times 100$$

Enzyme precipitation by ammonium sulphate: Sixty percent ammonium sulphate was added to the filtrate slowly with continuous stirring condition at low temperature (in an ice bath/ beaker) for 5 to 10 min and leave for over night in the refrigerator. After that, the mixture (salt+filtrate) was centrifuged at 12000 rpm for 20 min at 4-6°C. Then the pellet was collected and dissolved in 0.01 M Sodium phosphate buffer (pH 7) using 2 volumes of buffer. During the collection of enzymes, the tube and the funnel were washed with the same buffer and collected; the process was repeated thrice. Care was taken so that the volume should not be the 20% of total volume of the filtrate (10% is better). The enzyme salt solutions were dialyzed with the same buffer for 24 h with continuous stirring and the buffer was changed time to time (3 to 5 times) for removing the ammonium salt. The enzyme was concentrated with polyethylene glycol. Then the enzyme was transferred to another test tube and determined the enzyme activity and molecular weight by SDS-PAGE technique.

Determination of molecular weight: The molecular weight of the enzyme of the isolate A_2 was determined by SDS-PAGE[18,19] with the standard protein BSA (molecular weight 66 kDa), egg albumin (molecular weight 45 kDa) and casein (molecular weight 23.5 kDa).

RESULTS AND DISCUSSION

Effects of medium pH and temperature: The selected isolate A_2 was allowed to grow in different pH and temperature to determine their optimum growth pH and temperature (Table 1). At pH 6.5 the isolate showed heavy growth and high cellulase activity (liquefaction Fig. 3) but at pH 4.5 the isolate showed no growth and liquefaction and at pH 8.5 A_2 showed moderate growth and liquefaction. At 35 and 40°C the isolate A_2 showed heavy growth and high cellulase activity (liquefaction).

The growth and liquefaction of A_2 was found to decrease with the increase as well as decrease of temperature (Table 1).

Heavy growth at pH 6.5 to 7.5 with different microorganisms was reported by many workers[20-23], The maximum growth of mesophilic organisms at 35°C was reported by Shibli[24]; 37°C by Malek *et al.*[20] and Farhana *et al.*[23] and 42±2°C by Manchur and Anwar[25]. Present observations are in concurrence with their reports. Both pH and temperature have an effect on cellulose liquefaction. The higher liquefaction of cellulose due to enzyme activity at pH 6.5 to 7.5 was reported by many workers[20-22,26-28]. Present observations also showed similarities with their reports. Liquefaction of winstead's medium (with 1.2% CMC) due to enzyme activity at 35 and 40°C which was recorded herein found similar to the finding's of Malek *et al.*[26], Brakee *et al.*[29], Donnelly[30], Shailendra *et al.*[21], Farhana *et al.*[23], Hossain *et al.* [28], Shibli *et al.*[31], Huq *et al.*[11] and Shibli *et al.*[32].

Effects of incubation period: The isolate was found to show heavy growth and liquefaction and maximum enzyme activity after 5 days of incubation period (Table 1).

Fig. 3: Growth vigour and clearance of viscosity of the selected isolate A_2 in Winstead's medium having 1.2% CMC

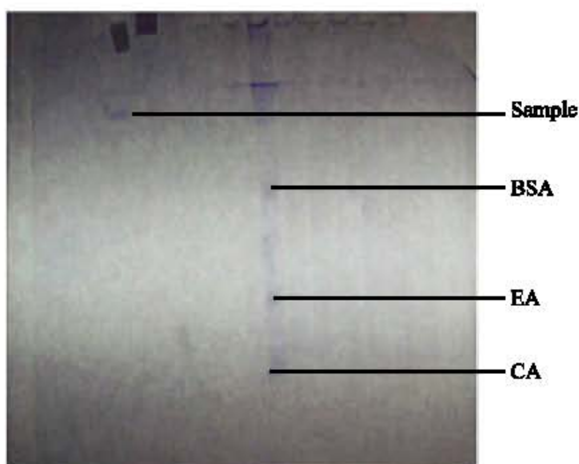

Fig. 4: The protein bands in the gel after destining

Effect of carbon and nitrogen sources: The highly cellulolytic isolate A_2 allowed to grow in Winstead's medium having 1.2% CMC as a carbon source and different nitrogen sources (Asparagine, Beef extract, yeast extract, $(NH_4)_2SO_4$ and urea) for the determination of optimum nitrogen sources for maximum production of cellulase, reducing sugar level, extracellular protein, saccharification (%) biomass.

The change of pH of the culture supernatant ranged from 6.9 to 8.2, the maximum protein and reducing sugar level of culture supernatant 430.19 and 497.78 $\mu g\ mL^{-1}$, respectively when yeast extract used as a nitrogen source. The highest saccharification percentage and biomass yield was found 4.17% and 316.67 $mg\ g^{-1}$ cellulose, respectively when yeast extract used as a nitrogen source.

The highest CMC-ase activity (230.56 $U\ mL^{-1}$) was recorded with the crude enzyme of the isolate A_2 when

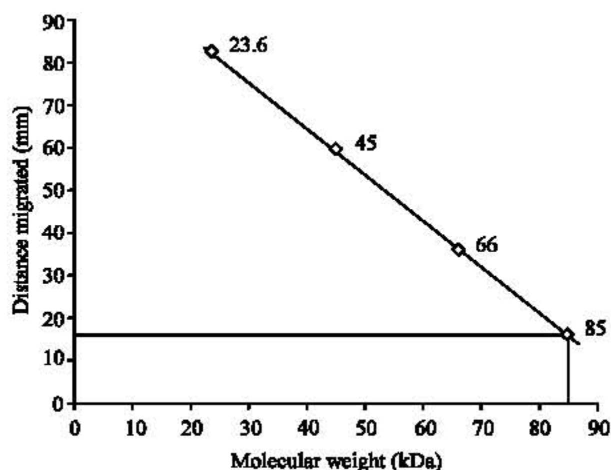

Fig. 5: Standard curve with known protein markers [BSA-bovine serum albumin (66 kDa), EA-egg albumin (45 kDa), CA-casein (23.6 kDa)]

Beef extract used as a nitrogen source and the lowest CMC-ase activity (111.11 $U\ mL^{-1}$) when asparagine used as a nitrogen source (Table 2).

The Induction or repression of microbial cellulase enzymes production due to addition of different nitrogen sources in the medium was reported by some workers[11,22,33,34]. Present observation showed similarities with their reports.

The isolate A_2 was also allowed to grow in Winstead's medium having Beef extract as a nitrogen source and 1.2% CMC/ Avicel/ Saw dust/ Rice bran as a carbon source for the determination of optimum carbon sources for maximum production of cellulase, reducing sugar level, extracellular protein, saccharification (%) and biomass. The change of pH of the culture supernatant ranged from 7.6 to 7.9. The maximum protein and reducing sugar level of culture supernatant 324.27 $\mu g\ mL^{-1}$ (CMC used as a carbon source) and 473.33 $\mu g\ mL^{-1}$ (CMC used as a carbon source), respectively. The highest saccharification percentage and biomass yield were found 3.92% (CMC used as a carbon source) and 332.33 $mg\ g^{-1}$ cellulose (CMC used as a carbon source), respectively. The highest CMC-ase activity (233.56 $U\ mL^{-1}$) was recorded with the crude enzyme when CMC used as a carbon source and the lowest CMC-ase activity (11.11 $U\ mL^{-1}$) when saw dust and rice bran used as a carbon source (Table 3).

Induction or repression of microbial cellulase enzyme production due to addition of different carbon sources to the cellulose medium was reported by many workers[34-41]. Present observations are in concurrence with many of the above reports.

Table 1: Effects of pH, temperature and incubation period on the growth and liquefaction of the isolate A_2 (*Streptomyces omiyaensis*)

pH	Growth	Liquefaction	Temp. (°C)	Growth	Liquefaction	Days	Growth	Liquefaction
4.5	-	-	25	-	-	2	-	-
6.5	++++	++++	35	++++	++++	3	++	+++
8.5	+++	+++	40	++++	++++	5	++++	++++
			45	+++	+++	7	+++	++++

-= no growth, ++, = low growth, +++, = moderate growth ++++, = heavy growth, initial medium pH 6.5

Table 2: Effects of nitrogen sources on the pH, production of extracellular protein, reducing sugar level, saccharification(%), biomass yield and CMC-ase activity of the isolate A_2 (*Streptomyces omiyaensis*)

Sources of nitrogen	Final pH	Extracellular protein (μg mL^{-1})	Reducing sugar (μg mL^{-1})	Biomass yield (mg g^{-1}) cellulose	Saccharification (%)	CMC-ase activity (U mL^{-1})
Aspara-gine	7.7	150.53*	373.33	233.33*	3.08	111.11*
Beef extract	7.7	329.25	473.33	327.33	3.92	230.56**
Yeast extract	7.6	430.19**	497.78**	316.67**	4.17**	180.56
$(NH_4)_2SO_4$	6.9*	162.77	494.44	315.67	4.08	194.44
Urea	8.2**	183.51	330.00*	330.33	2.75*	119.44

* indicates minimum,** indicates maximum, initial medium pH 6.5, Enzyme substrate reaction temperature 35°C and pH 6.5

Table 3: Effects of carbon sources on the pH, production of extracellular protein, reducing sugar level, saccharification (%), biomass yield and CMC-ase activity of the isolate A_2 (*Streptomyces omiyaensis*)

Sources of carbon	Final pH	Extracellular protein (μg ml^{-1})	Reducing sugar (μg ml^{-1})	Biomass yield (mg g^{-1}) cellulose	Saccharification(%)	CMC-ase activity (U mL^{-1})
CMC	7.7	324.27**	473.33**	332.33**	3.92**	233.56**
Avicel	7.8	219.00*	21.11*	266.67*	0.18*	33.33
Sawdust	7.9**	251.00	34.44	316.67	0.25	11.11*
Ricebran	7.6*	260.10	37.78	316.67	0.33	11.11*

* indicates minimum,** indicates maximum, initial medium pH 7.5, Enzyme substrate reaction temperature 35°C and pH 6.5

Table 4: Effects of pH on the CMC-ase, FP-ase, Avicelase and β-Glucosidase activity of the isolate A_2 (*Streptomyces omiyaensis*) during enzyme substrate reaction

	Activity (U mL^{-1})			
pH	CMC-ase	FP-ase	Avicelase	β-Glucosidase
4.5	47.22	18.25	67.36	33.68
6.5	228.00**	44.44**	177.77**	116.67**
8.5	183.33	33.12	140.37	88.75

** indicates maximum, Enzyme substrate reaction temperature 35°C

Table 5: Effects of temperature on the CMC-ase, FP-ase, Avicelase and β-Glucosias activity of the isolate A_2 (*Streptomyces omiyaensis*) during enzyme substrate reaction

	Activity (U mL^{-1})			
Temperature (°C)	CMC-ase	FP-ase	Avicelase	β-Glucosidase
30	127.78*	37.04*	116.67*	63.89*
35	231.48	43.43	179.70	114.62
40	250.00	53.70	191.67	125.00
45	269.44**	64.81**	200.00**	138.89**
50	227.78	55.56	186.11	122.22

* indicated minimum, ** indicated maximum, Enzyme substrate reaction pH 6.5

Determination of optimum conditions for the maximum enzyme activity: To study the optimum pH and temperature for maximum enzyme activity, the crude enzyme was collected and enzyme activity was assayed with CMC, avicel, salicin and filter paper as substrate at various temperature ranged from 30 to 50°C and pH ranged from 4.5 to 8.5. At pH 6.5 the isolate showed maximum CMC-ase (228.00 U mL^{-1}), FP-ase (44.44 U mL^{-1}), Avicelase (177.77 U mL^{-1}) and β-Glucosidase (116.67 U mL^{-1}) activity (Table 4). The isolate showed maximum CMC-ase (269.44 U mL^{-1}), FP-ase (64.81 U mL^{-1}), avicelase (200.00 U mL^{-1}) and β-Glucosidase (138.89 U mL^{-1}) at 45°C in pH 6.5 (Table 5).

Optimum pH 6.5 to 7.5 was reported by Hachiro and Kazuhiko[27], Shailendra *et al.*[21] and optimum temperature 50°C was reported by Hachiro and Kazuhiko[27] and 40°C by Shailendra *et al.* [21]for maximum cellulase activity.

Comparative study of enzyme production by the isolate indicated that CMC-ase activity was higher compared to that of FP-ase activity, which is in accordance with the findings of many workers[25,31,32,42-45].

Determination of molecular weight: The molecular weight of the cellulolytic enzymes of the isolate (*Streptomyces omiyaensis*) was found to be 85 kDa (Fig. 4 and 5).

Results of this study suggested that *Streptomyces omiyaensis* can produce maximum cellulase with CMC as a carbon source and beef extract as a

nitrogen source in liquid medium at incubation temperature 37±2°C and pH 6.5. The crude cellulase from *Streptomyces omiyaensis* can show the highest activity at 45°C and pH 6.5 during enzyme substrate reaction.

So, the present study concluded that optimum pH, temperature, incubation period, carbon and nitrogen sources are the important limiting factors for the maximum cellulase production as well as enzyme activity.

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