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Research Article

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Production, Partial Purification and Characterization of Extracellular, Alkalophilic, Carboxy Methyl Cellulase from *Baccillus Megaterium*

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ABSTRACT

A total of five bacterial strains were isolated from soil samples of Selakui area of Dehradun, India, and were subjected to screening for their carboxy methyl cellulose (CMC) hydrolysis activity. Results revealed that, MJSH 1205 have maximum exocellulase activity and after fermentation at temperature 27°c, pH 7 and 1.5 % CMC concentration and 0.5% of urea as nitrogen source, bacterial strain MJSH-1205 showed the highest CMC hydrolysis activity of 0.01038 ±0.000504 U/ml/min. The enzyme produced is stable at varied range of temperature and pH, but the optimum temperature and pH was found to be 30°C temperature pH 9 respectively. This alkalophilic property of enzyme makes it industrially important. Presence of Mg^{+2} ions enhance the activity of enzyme while reduction in the activity of enzyme was found in the presence of SDS.

Keywords: Corboxy Methyl Cellulose (CMC), Cellulase, Bacteria, Fermentation

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INTRODUCTION

Cellulose is the major component of plants cell wall and one of the most abundantly present forms of carbohydrates on earth. It is linear polymer of D-glucose units linked together by β 1-4 glycosidic linkage and crystalline in nature [1]. The annual biosynthesis of cellulose by land plants and marine algae occurs at the rate of 0.85×10^{11} tonnes per annum [2]. Degradation and consequent use of cellulose is important for global carbon sources. Enormous amounts of agricultural, forestry, industrial and municipal cellulosic wastes have been accumulated in fields/soils if it was not degraded or suitably used. The cellulosic biomass can be utilized by microorganisms for the production of commercially important products such as ethanol, methane, glucose syrup and single cell protein. This approach also solves the waste disposal problem and diminishes the dependence over conventional fuels by the production of ethanol [3-4]. Due to rising cost of fossil fuel and environmental pollution global effort was shifted towards the utilization of renewable resources and production of green energy [5].

Cellulase is a group of enzymes which degrade cellulose in to glucose and cellooligosaccharides. Cellulase is a complex enzyme and composed of endo-β-glucanase (EC 3.2.1.4), exo-β- glucanase (EC 3.2.1.9.19) and β-glucosidases (EC 3.2.1.21) which are acts synergistically [6]. Cellulase are industrially important enzymes and used in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry [7-10]. The high production cost and low yield is the major problem associated with their industrial application [11]. Cellulase production was found to be most expensive step about 40% of total cost in the production of bioethanol from cellulosic biomass. To reduce the

relative cost of this process cellulose production by microorganisms were studied. Cellulase can be synthesized by microorganisms during their growth on cellulosic materials [12].

A large number of Bacteria, Actinomycetes and filamentous fungi have ability to produce cellulase by secreting the enzyme freely in the solution or in the form of cell bund enzyme cellulosmes. The main difference between cell free cellulase and cellulosomes is in the component of cellulosome-cohesine containing scaffolding and dockerine containing enzyme. The free cellulase contains cellulose binding domains (CBMs) which are replaced by dockerin in cellulosomes, and single scaffolding born CBM directs the entire cellulosome complex to cellulosic biomass [13-14]. Most commonly used bacterial sp. for the commercial production of cellulase is *Acidothermus sp., Bacillus sp., Clostridium sp., Pseudomonas sp. and Rhodothermus sp.*

The present study was aimed to isolate and screen microorganisms which produce cellulolytic enzyme. Further effort was made to optimize the culture condition for enhance the yield of enzyme. After purification and characterization of enzyme was done to investigate the stability of enzyme at the varied range of pH, temperature and under the influence of heavy metals.

MATERIALS AND METHODS

2.1. Isolation and screening of microorganisms

Soil samples were collected from Selakui, Dehradun India, at a depth of about 15-20 cm from the top. Samples were collected from the area where decomposing leaves were present because the probabilities of occurrence of cellulolytic microbes were higher at these places. The soil was serially diluted and spread on sterilized nutrient agar (NA) plates and incubated at 37°C. Five bacterial (MJSH-1201, MJSH-1202, MJSH-1203 MJSH-1204 and MJSH-1205) were purified by streak plate method at suggested by Khan et al. [15]

All purified cultures were subjected to screening for CMC hydrolysis. Pure cultures were transferred on Minimal Agar Media (NaH_2PO_4, 6.0g L^{-1}; KH_2PO_4, 3.0gL^{-1}; NaCl, 5.0gL^{-1}; NH_4Cl 2.0g L^{-1}; $MgSO_4$ 0.1g L^{-1}; Glucose 8.0g L^{-1}; Agar 17g L^{-1};) supplemented with 1% CMC and incubated for 48 hrs [16]. Secondary screening was done by flooding the plates with 0.1% Congo red solution for 15 minutes, after that the plates were washed with 1N NaCl [17].

2.2. Identification of isolated microorganisms

Microorganisms which were showing cellulose hydrolysis were subjected to staining (Gram's staining and endospore staining) and biochemical tests like catalase, manitol fermentation and, Voges- proskauers for their identification [18].

2.3. Optimization of physico-chemical conditions for the production of enzyme on laboratory level by shake flask fermentation

Among all the isolates, microorganism which shows maximum zone of hydrolysis during screening was grown in cellulase production media having composition of peptone 5.0g L^{-1}; yeast extract 5.0g L^{-1}; K_2HPO_4 1.0 g L^{-1}; NaCl 5.0g L^{-1}; $MgSO_4$ 0.2g L^{-1}; Agar 17g L^{-1}; pH 7 and 1% CMC [15]. Production of cellulase enzyme was optimized at different temperature (10°C, 20°C, 30°C, and 40°C), pH (5, 7, 9, and 11), incubation time (1 to 7th day) and nitrogen sources (peptone, yeast extract, beef extract, ammonium chloride, urea, ammonium sulphate, and soyameal) in different concentrations of 0.01%, 0.05, 0.1, 0.15 and 0.2% [19-21].

2.4. Estimation of protein concentration and enzyme activity in crude

For extraction culture was centrifuge at 5000 rpm for 5 min. After centrifugation 86 ml of crude was obtained. The crude extract was transferred to a 250 ml conical flask and stored at cold temperature. 40% of the crude volume ammonium sulphate salt was added pinch by pinch to the crude extracts with continuously stirring. When the total salt gets dissolved then the mixture was kept overnight at 4°C [15]**.**

Concentration of protein in crude extracellular extract was determined by Lowry's method using BSA as standard [22]. CMCase activity is assayed using a standard method given by Mendales, [23]. The activity was estimated using 1% solution of CMC in 0.1M Tris buffer (pH 7.0) as substrate. 0.5 ml substrate and 0.5 ml of enzyme solution were allowed to react on 37^{o}C for 15 minutes after that the 30 min of incubation at 50°C was given to the reaction mixture. 2 ml of DNS solution was then added and samples were boiled for 15 min and then allowed to cool in cold water for color stabilization and optical density was taken at 540 nm [19].

2.5. Partial purification of crude enzyme

Dialysis was performed for the partial purification of salt precipitated protein. Pretreatment of dialysis bag was done by boiling in distilled water for one min. and then washed in 0.1% SDS for one minute and finally cooled in distilled water. The enzyme was filled in dialysis bag and kept in 200 ml of 100 mM Tris buffer (pH 7.0) on magnetic stirrer for one and half hrs at 4°C. After that dialysis bag was replaced in new 200 ml of 100 m M Tris buffer and kept overnight at 4°C. After salt precipitation and dialysis concentration of protein was estimated by Lowry's method. While for the estimation of enzyme activity 0.5 ml of substrate and 0.5 ml of enzyme was reacted at 37°C for 15 minute and the absorbance was compared with the standard graph [24].

2.6. Characterization of enzyme

Enzymes are catalytically active only in a narrow pH range, temperature, substrate specificity and under the influence of metal ions and inhibitor. For the characterization of enzyme, a reaction between CMC as substrate and enzyme was carried out at different pH (5, 7, 9 and11), temperature (0°C, 10, 20, 30 and 40°C) under the influence of different activators like Ca^{+2}, Mg^{+2}, Fe^{+2}, and Zn^{+2} and inhibitors such as SDS and EDTA for 15 min. The absorbance was read by using spectrophotometer at 540 nm wavelengths and the activity was calculated by the formula given by Irfan et al. [25]

Unit/ml/min= [(mg/ml glucose released X 0.180)/ ml enzyme used]

2.7. Statistics

During present study, all the measurements were done in triplicates. The mean ± standard deviation (SD) was calculated and graphs were plotted using GraphPad Prism 5 statistical software.

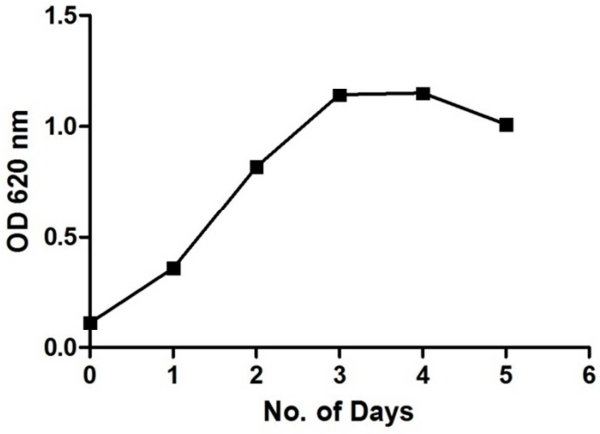
RESULTS AND DISCUSSION

3.1. Isolation and screening

On spreading the serially diluted soil sample over nutrient agar plates mixed colonies were obtained and differentiated on the basis of morphological characteristics. Five different bacterial colonies were obtained were numbered as MJSH-1201, MJSH-1202, MJSH-1203, MJSH-1204 and MJSH-1205 and purified by streak plate method. On screening with congo red dye, among all the isolated bacterial strains MJSH-1205 showed the highest zone of CMC hydrolysis. This indicated the production of extracellular cellulase that hydrolyze the cellulose present in their surroundings and use them as carbon source. The zone was appeared due to congo red dye stained that area, and after washing with NaCl solution elutes the dye in the zone where the cellulose has been degraded into simple sugars by the enzymatic activity. While the remaining part of the plate retained the congo red dye even after washing with NaCl and a clear zone of cellulose hydrolysis was observed around the organisms colony [26].

3.2. Study of growth parameters

Study of growth curve revealed that day 4 and 5 is the stationary phase for the bacterial strain MJSH 1205. The optimum temperature for the growth of MJSH 1205 is found to be 40°C while optimum pH for growth was found to be pH 7.



Growth curve study of MJSH-1205

3.3. Biochemical tests

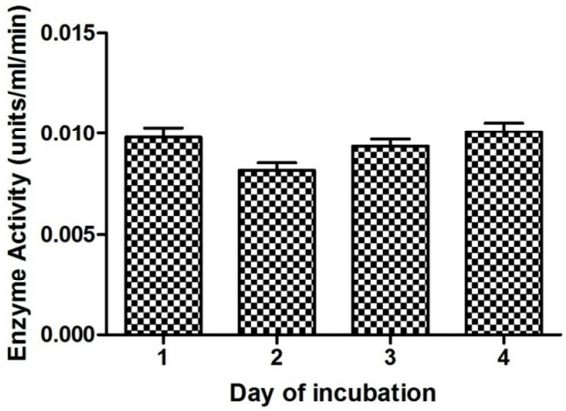
Staining and biochemical characteristics of MJSH-1205 revealed it Gram positive rod shaped bacteria, which was positive in endospore staining, catalase test and mannitol test while negative in Voges-proskauer test. On comparing

the results of biochemical tests with Bergey's manual it was confirmed that the MJSH-1205 was *Bacillus megaterium.*

3.4. Optimization of culture condition to enhance enzyme yield

In order to enhance the yield of enzyme culture conditions was optimized, Effect of incubation time on enzyme activity was studied and the results revealed that MJSH-1205 had maximum enzyme activity on 4th day of inoculation (0.01008 ±0.000457 U/ml/min). Similar results were observed by Krairitthchai and Thongwai, [19], while maximum enzyme activity after 8th day of inoculation was reported by Lalitha et al, [27].

S. No.	Nitrogen Sources	Enzyme Activity (U/ml/min)
1.	Peptone	0.01002 ±0.000516
2.	Yeast extract	0.01008 ±0.000504
3.	Beef extract	0.00932 ±0.000466
4.	Ammonium chloride	0.00912 ±0.000456
5.	Urea	0.00864 ±0.000432
6.	Ammonium Sulphate	0.01038 ±0.000504
7.	Soyameal	0.00840 ±0.000420
8.	Peptone + Yeast extract	0.00624 ±0.000312
9.	Peptone + Urea	0.00624 ±0.000312
10.	Yeast extract + Urea	0.00780 ±0.000390

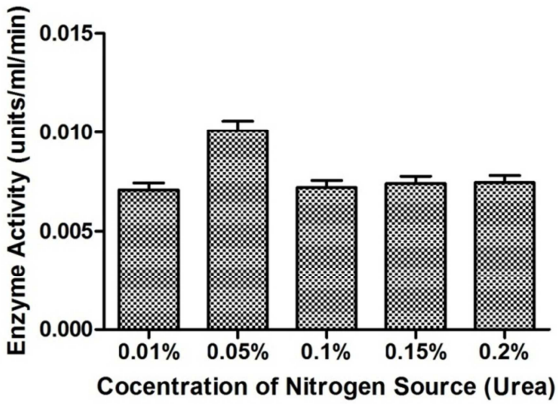


Effect of incubation time on enzyme activity

Nitrogen is one of the most important component of protein and main constituents of protoplasm. Altering the media composition in respect to different nitrogen sources, concluded by DNS assay *Bacillus megaterium* showed maximum enzyme activity with urea as nitrogen source in 0.05% concentration (0.01038 ±0.000504 U/ml/min).

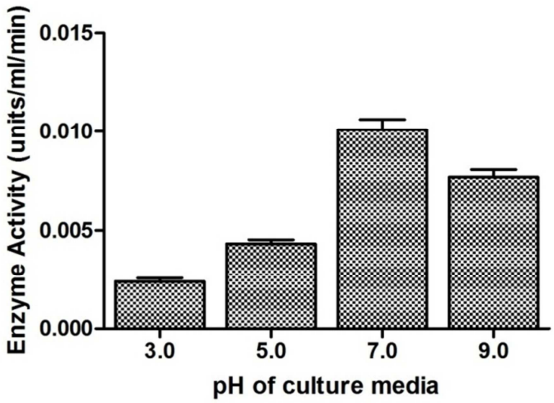
Effect of different nitrogen sources over enzyme activity

This result is supported by previous researchers Das et al. [28] 2010 and Doi, [29] 2008, whereas maximum cellulase production by using ammonium chloride as a nitrogen source was reported by few workers Jardat et al, [30].

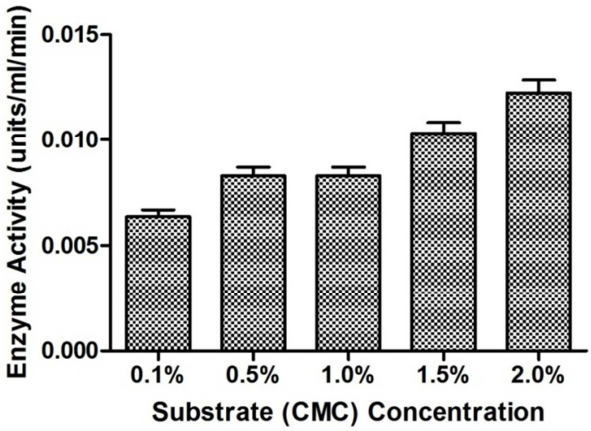


Effect of different concentrations of nitrogen source over enzyme activity

Optimum pH for maximize the yield of enzyme was found to be pH 7 (0.01008 ± 0.000534 U/ml/min) and 2.0% substrate concentration was found to be suitable for the maximum yield of enzyme (0.01224 ± 0.000612 U/ml/min).



Effect of culture media pH over the activity of produced enzyme



Effect of different substrate (CMC) concentration over enzyme activity

3.5. Extraction and partial purification of crude enzyme

Bacillus megaterium was cultured in optimized culture media and crude enzyme was extracted from the fermented media through centrifugation. To precipitate out the dissolve protein, salt precipitation was done by saturating the crude extract with ammonium sulphate. The solubility of protein depends upon the concentration of salt in the solution, as the salt concentration increases; a point of maximum protein solubility is reached. Further increase in the salt concentration leads that there is less or no water available to solubilize the protein. Finally protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This method of precipitation of protein in the presence of excess salt is known as salting out. Precipitation of protein by salt gives a mixture of protein and salt, to remove the salt from the protein, dialysis was performed.

Dialysis operates on the principle of diffusion and osmosis to desalt the protein [31]. Small molecule passes through the pores of dialysis bag and move into or out of the bag, in the direction of decreasing concentration. Larger molecules that have size greater than the pore diameter could not move across the pore of the bag and retained inside the dialysis bag [32]. After purification concentration of protein was estimated and found to be 0.006 ± 0.0004 mg/ml in extracellular crude extract, while 0.05 ± 0.0045 mg/ml after dialysis.

In order to estimate the activity of enzyme in different form viz. crude form, in presence of salt and in pure form, DNS assay was performed. Results revealed that in *Bacillus megaterium* the enzyme activity remains constant in crude enzyme and after salt precipitation i.e; 0.01008 ± 0.000474 U/ml/min, while after dialysis little increase in enzyme activity was observed (0.01032 ± 0.000506 U/ml/min).

3.6. Characterization of enzyme

Different reaction conditions were given to the enzyme during DNS assay incubation to check the activity of enzyme at varied range of conditions. This allow us determine the stability of enzyme at different conditions, like temperature, pH, activators and inhibitors. A reaction of substrate and enzyme was performed at different

temperature, 0°C, 10, 20, 30, and 40°C and results revealed that optimum enzyme activity (0.01032 ±0.000504 U/ml/min) found at 30°C. The pH of substrate in DNS assay was altered from 5 to 11 in the interval of 2, and the enzyme activity was calculated for each pH condition. The result revealed that the enzyme was stable at varied range of pH but maximum activity of enzyme (0.01028 ±0.000496 U/ml/min) was found to be at pH 9.

Characterization of enzyme

S. No.	Parameter	Enzyme Activity (U/ml/min)
1.	10°C	0.00946 ±0.000438
2.	20°C	0.00967 ±0.000451
3.	30°C	0.01032 ±0.000483
4.	40°C	0.00780 ±0.000360
5.	pH 3	0.00584 ±0.000264
6.	pH 5	0.00632 ±0.000302
7.	pH 7	0.00874 ±0.000416
8.	pH 9	0.01028 ±0.000486
9.	Calcium	0.01024 ±0.000472
10.	Magnesium	0.01104 ±0.000538
11.	Iron	0.00964 ±0.000426
12.	Zinc	0.00938 ±0.000406
13.	SDS	0.00924 ±0.000416
14.	EDTA	0.00396 ±0.000194

To access the effect of metal ions over the activity of enzyme DNS assay was done in the presence of different metal ions (Ca^{+2}, Mg^{+2}, Fe^{+2} and Zn^{+2}) the results indicated in the presence of Mg^{+2} ion enzyme activity was found to be maximum (0.01104 ±0.000492 U/ml/min). Effect of inhibitors over the activity of enzyme was also studied by incubation the enzyme with 1% SDS and 1% EDTA during DNS assay; results revealed SDS inhibit the activity of enzyme (0.00396 ±0.000194 U/ml/min). Other workers observed activation in enzyme activity in the presence of Co^{+2} and Mn^{+2} ions [25]. These metals acted as cofactor and enhance the enzyme activity, while SDS and EDTA are the inhibitors and cause the degradation of peptide chain of enzyme which leads to inhibition of enzyme activity.

CONCLUSION

On the basis of present research it can be concluded that soil from forest area are a very good source of cellulolytic microorganisms. The enzyme produced is extracellular which make the downstream processing more easily. The activity of cellulase enzyme varied with the variation in culture media and culture conditions. The enzyme produced is stable at varied range of pH and temperature. The produced enzyme found suitable to use in industries because of its maximum activity even at highly alkaline condition.

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