

ISOLATION AND CHARACTERIZATION OF CELLULASE PRODUCING BACTERIA FROM SALIVA SAMPLE AND OPTIMIZATION OF EXTRACTED CELLULASE

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Abstract: Microorganisms have a great potential in degradation of cellulose. Among which, bacteria have a major contribution. This study was conducted to isolate and characterize cellulose degrading bacteria which was collected from 'Jaffrabadi' breed of buffalo by morphological and biochemical analysis. The cellulose degrading bacteria were isolated, screened and purified on CMC agar plates. Screening resulted in selection of bacterial isolate producing clear zone when plates were flooded with Congo red and washed with NaCl, which confirmed the cellulose degradation. The morphological characterization showed the bacteria to be a Gram positive, motile, nonsporulating rod-shaped bacterium. The biochemical tests indicated that the bacteria belonged to the *Cellulomonas* spp. (according to Bergey's Manual of Determinative Bacteriology). The bacterial isolate was then subjected to cellulase production, which was further optimized with various parameters like incubation time, temperature, pH and also with different carbon and nitrogen sources.

Keywords: Jaffrabadi, degrading, characterize, CMC agar, *Cellulomonas*

INTRODUCTION

Enzymes are the proteins that help speed up the metabolism, or the chemical reactions in the bodies. They are the type of proteins found within a cell. They function to build some substances and break some down. Also, they speed up the chemical reactions of metabolism in the body and support life. To date, scientists have discovered over 10,000 different enzymes. One of which is cellulase. The enzyme cellulase degrades cellulose [22], which comprises of a linear polysaccharide of glucose having β -1,4 glycosidic linkages [10].

Cellulase enzyme is an extracellular, soluble enzyme including the classes- 1,4 β -endoglucanases, 1,4- β -exoglucanases and β glucosidase [19]. The mechanism of action of cellulase consists of endoglucanase that cleaves β -1,4 glycosidic bond whereas exoglucanase cleaves non reducing end of the cellulose chain and β -1,4 glucosidase hydrolyses cellobiose and cellodextrin to glucose [10]. Many of the microorganisms comprises of all three classes of this enzyme [18]. Synergy of all the above three enzymes is necessary for complete degradation of cellulosic material [10].

Ruminants include plants in their diet; but they do not have the machinery of their digestion. Instead, they have microorganisms in symbiotic relationship that produce enzymes to degrade these polysaccharides [30]. They have the ability of converting cellulose into oligosaccharide with the help of enzymes [18]. Cellulolytic bacteria within the ruminants degrade the plant microfibrils and the substrate thus formed are then acted upon by another group of bacteria [30]. They can be found in ruminant secretions of the gut, pancreas, small intestine, abomasum etc. One of the secretions is mouth saliva, which aids in chewing and swallowing of variety of cellulosic plant materials. A rumen of a buffalo secretes 40-150 litres of saliva in a day, which is rich in microflora. [23].

MATERIALS AND METHODS

1. Isolation-

Saliva sample was collected from Jaffrabadi breed of buffalo at Gavalipura, Ahmednagar, Maharashtra. Enrichment was done using 1 ml of saliva sample inoculated into sterile CMC broth medium of composition : Carboxymethylcellulose (CMC) 10 g/L, tryptone 2 g/L, KH_2PO_4 4 g/L, Na_2HPO_4 4 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g/L, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.001 g/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004 g/L. The pH was adjusted to 7 and incubated for 72h at 37°C in shaking incubator.

2. Screening of cellulase activity-

After enrichment, loopful culture was streaked onto sterile CMC agar plates. These plates were incubated at 37°C for 24h. Further, the plates were flooded with Congo red to visualise the zone of hydrolysis that indicate cellulase activity of organisms. The culture producing largest clear zone was selected for further studies.

3. Extraction of cellulase from production medium-

Sterile CMC broth was selected as the production media for cellulase, which was then inoculated and incubated at 37°C for 48h. After incubation, the production media was harvested by centrifugation at 5,000 rpm for 20 min at 4°C. The supernatant was used for enzyme assay of cellulase.

4. Characterization and identification of isolate-

The isolate was subjected to morphological identification by Gram staining and microscopic observation. The colony characters on the CMC agar plates were also recorded. Motility test (hanging drop method) and endospore staining (Schiffor-Fulton method) was performed according to the standard protocol. The isolates were further identified biochemically by enzymatic profiling (catalase and oxidase test) and biochemical test using Sugar fermentation test, IMViC test, Nitrate reduction test, Gelatinase test, Casein hydrolysis test, Urease test, Starch hydrolysis test and H₂S production test. With the help of the test results, the isolate was identified using Bergey's Manual of Determinative Bacteriology 9th edition.

5. Effect of physio-chemical parameters on cellulase produced by the isolate-

5.1 Effect of incubation time on cellulase production

Loopful culture was inoculated into sterile CMC broth and incubated at 37°C for different time intervals. The enzyme assay was performed by DNSA method and readings were recorded at 540 nm after 24h, 48h and 72h.

5.2 Effect of temperature on cellulase production

Loopful culture was inoculated into sterile CMC broth and incubated at different temperatures of 4°C, 37°C, 28°C and 60°C. After 24h of incubation the enzyme assay was performed by DNSA method and readings were recorded at 540 nm.

5.3 Effect of pH for cellulase production

Loopful culture was inoculated into sterile CMC broth having different pH of 2,4,6,7,8,9 and 10. They were incubated at 37°C for 24h. After that enzyme assay was performed by DNSA method and readings were recorded at 540nm.

5.4 Effect of carbon sources for cellulase production

Loopful culture was inoculated into sterile CMC broth. To study the efficiency of various carbon sources, the CMC medium tubes were supplemented independently with 1% glucose, lactose, paper chits and starch respectively. The broth was incubated at 37°C for 24h. After 24h of incubation, the enzyme assay was performed by DNSA method and readings were recorded at 540 nm.

5.5 Effect of nitrogen sources for cellulase production

Loopful culture was inoculated into of sterile CMC broth. To study the efficiency of various nitrogen source, the CMC medium tubes was supplemented independently with 1% ammonium sulphate, ammonium chloride, yeast extract, meat extract and tryptone respectively. The broth was incubated at 37°C for 48h. After 24h of incubation, the enzyme assay was performed and readings were recorded at 540 nm.

RESULTS AND DISCUSSION

3.1. Isolation and screening of cellulase producing bacteria

The screening was carried out by using CMC agar. After incubation at 37°C for 48h, the zone of cellulolytic activity or hydrolysis was seen on CMC agar plate after flooding with 1% Congo red solution. The isolate with highest zone of hydrolysis was streaked onto CMC agar plate and again checked for cellulolytic activity. This isolate was selected for further studies.



Figure 1: Screening of the selected isolate

3.2 CHARACTERIZATION AND IDENTIFICATION-

After incubation at 37°C for 48h, on CMC agar plate, the morphological and biochemical results were recorded as shown in table no. 1 and 2.

Table no 1: Morphological characterization of Isolate

Sr. No	Morphological characters	Observation
1	Size	2 mm
2	Shape	Irregular
3	Colour	Cream
4	Margin	Undulate
5	Consistency	Butyrous
6	Elevation	Convex
7	Opacity	Opaque
8	Motility	Motile
9	Gram character	Gram positive short rods
10	Endospore staining	Non sporulating

Table No. 2- Biochemical characterization of isolate

Sr. No	Biochemical tests	Result
1	Catalase	+ ve
2	Oxidase	+ ve
3	Nitrate reduction	+ ve
4	Urease	+ ve
5	Gelatinase	-ve
6	Casein hydrolysis	+ ve
7	Starch hydrolysis	+ ve
8	H ₂ S production	-ve

Sr. No	IMViC test	Results
1	Indole	-ve
2	Methyl red	-ve
3	Voges proskeur	+ve
4	Citrate	+ve

Sr. No	Sugar fermentation	Results
1.	Glucose	Acid + gas
2.	Lactose	Acid + gas
3.	Fructose	Acid + gas
4.	Sucrose	-ve

After reference from Bergey's manual of determinative bacteriology, the isolate was tentatively identified as *Cellulomonas spp.*

3.3. Effect of physio-chemical parameters on the cellulase produced by the isolate

3.3.1 Effect of incubation time on cellulase production

The effect of incubation time on cellulase production by the isolate was studied. It was found that the amount of cellulase production increases with increase in incubation time until 48h. Maximum cellulase production was obtained after 48h, which was 0.08 U/ml. When incubation time was further increased, the concentration of cellulase was reduced.

3.3.2 Effect of temperature on cellulase production

The effect of temperature on cellulase production by the isolate was studied. It was found that concentration of cellulase increases with increase in temperature. The maximum cellulase production was found at 37°C which was 0.06 U/ml, but when the temperature was further increase, the concentration of cellulase was reduced. This indicate that the optimum temperature for cellulase production by isolate was 37°C.

3.3.3 Effect of pH on cellulase production

The effect of pH (from 2 to 10.5) on the cellulase production by the isolate was studied. It was found that the concentration of cellulase production increases with increase in pH. Maximum cellulase production was observed at pH 8 after 48h, which was 0.075 U/ml. But, when the pH was further increased, concentration of cellulase was reduced. This indicates that the optimum pH for cellulase production by the isolate was 8. The influence of pH on enzyme production was found to be an important parameter.

3.3.4 Effect of carbon sources on cellulase production

On cellulase production, different carbon sources were studied. The maximum cellulase production was obtained when glucose as a carbon source which was 0.3 U/ml followed by Paper chits was 0.2 U/ml. The lowest cellulase production by isolate was obtained by lactose and starch used as a carbon source.

3.3.5 Effect of nitrogen source on cellulase production:

The effect of different nitrogen sourced on production was studied. The maximum cellulase production was obtained when tryptone used as a nitrogen source which was 0.2 U/ml followed by yeast extract was 0.12 U/ml. The lowest cellulase production by isolate was obtained when gelatin, ammonium chloride and ammonium sulphate was used as nitrogen source.

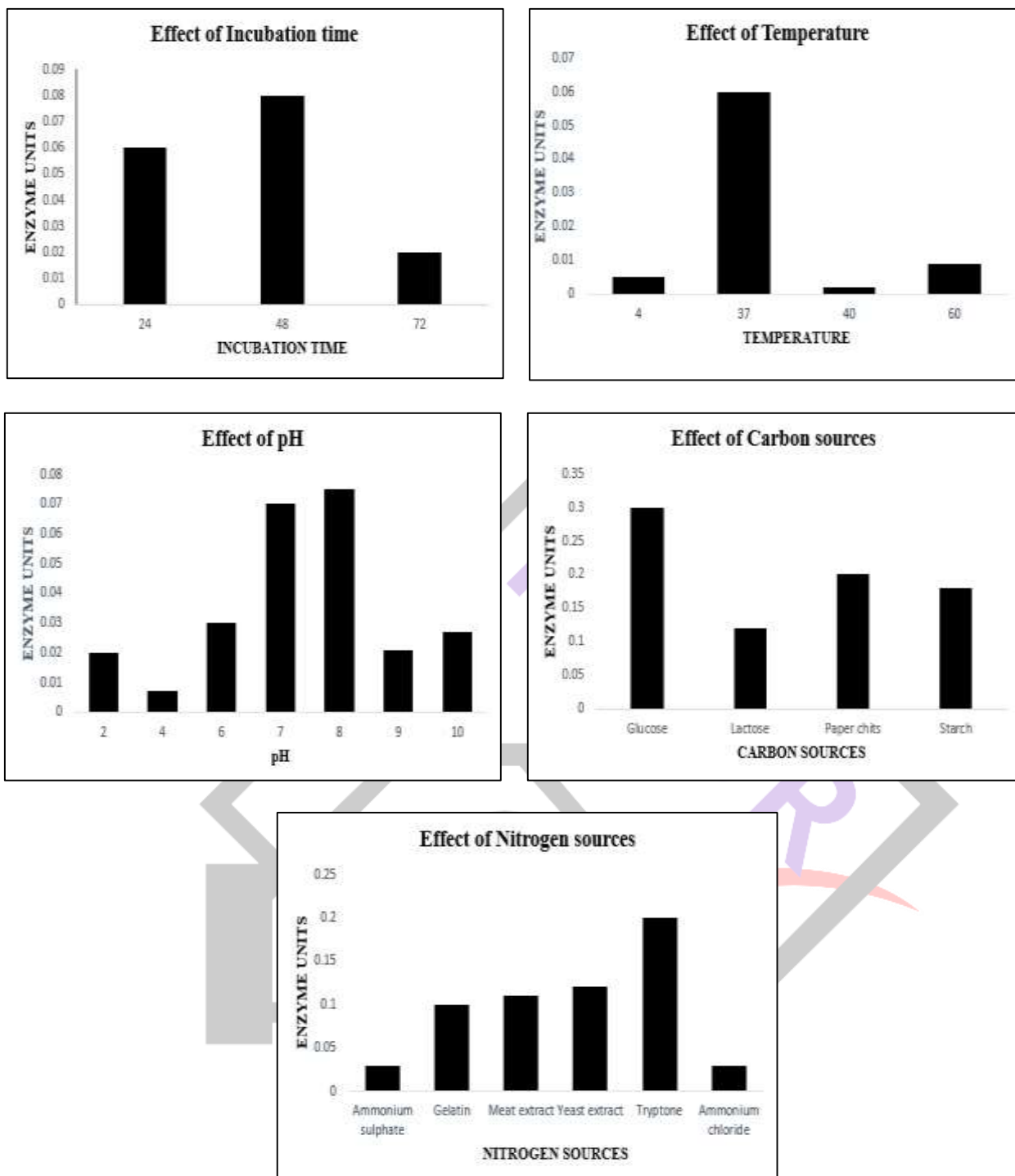


Fig 1: Effect of physiochemical parameters on cellulase production

DISCUSSION

The earlier reports on cellulose degrading microorganisms indicate that similar media (CMC medium) was used for the isolation (Ashok Shinde et al., 2020). Cellulolytic activity was determined based on formation of clear zone and cellulolytic index on CMC plate media (Andri Ferbiyanto et al., 2015). The optimum temperature for cellulase action with CMC as substrate varied between 77°C with ½ h incubation time and 58°C with 10 h as incubation time (Johny Eriksen et al., 1976). Optimum production of cellulase from *A. niger* was achieved at incubation time 120 h, pH 4, temperature 40°C (A.O. Sulyman et al., 2020).

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