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Comparative characterization of five cellulolytic bacterial strains isolated from soil samples of Patna region in Bihar

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Abstract : Five bacterial strains labeled as BS1, BS2, BS3, BS4 and BS5 isolated from soil samples collected locally from Patna region were selected for the present study. Exoglucanase and endoglucanase activities of the strains were assayed using dinitrosalicylic acid method. The strain BS5 showed highest exoglucanase activity in terms of liberated reducing sugar (7.4 µg/ml) followed by BS1 and BS3 (both releasing 6.8 µg/ml reducing sugar). BS 4 showed slightly more exoglucanase activity (5.7 µg/ml) than BS2 (5.5 µg/ml). The highest endoglucanase activity was exhibited by the strain BS4 (3.0 µg/ml) and least by BS2 (2.2 µg/ml). BS1, BS3 and BS5 possessed similar endoglucanase activity (all releasing 2.7 µg/ml reducing sugar). Colony characteristics and growth adaptability of the strains were investigated. The strains showed moderate to luxuriant growth on different ISP and non ISP media. Good growth was observed at pH 5-11 with optimum being 7 to 9. Slight to luxuriant growth of the selected strains were observed at the temperatures of 15, 26, 37 and 45 °C.

Key Words: Biomass degradation, cellulolytic bacterial strains, endoglucanase activity, exoglucanase activity.

INTRODUCTION

The cellulosic biomass, once thought to be an ever increasing unmanageable waste, is now considered as an important renewable source of energy. Cellulose hydrolysis results in the production of glucose, which in turn can be utilized for the production of a number of industrially important chemicals including ethanol[1]. The exhaustibility and the rising cost of fossil fuels have shifted global efforts to utilize renewable resources of plant biomass in the form of agricultural and municipal wastes for the production of alternative energy and in the process help in bioremediation of landfill. The saccharification of the cellulosic biomass involves the action of several cellulose degrading enzymes. Different fungi and bacteria have been used for production of cellulases using different substrates[2]. A major obstacle in the exploitation of cellulose is the fact that the production of cellulase is expensive, contributing as much as 50 % to the overall cost of hydrolysis[3]. Ever since the discovery of the enzyme cellulases from cellulose degrading microorganisms by Reese and his coworkers[4], the researchers worldwide have focused their attention towards newer microbial isolates, from which the cellulases can be extracted and can be used in different industrial processes.

The present study was undertaken to present a comparative study of five potential cellulolytic bacterial strains isolated from soil samples collected from Patna region in Bihar.

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MATERIALS AND METHODS

Screening and selection of cellulolytic microbial strains

Bacterial strains were isolated from soil samples collected locally from Patna region in Bihar by six fold serial dilution technique[5] on CMC Agar (carboxymethylcellulose 0.5 g/l, $NaNO_3$ 0.1 g/l, K_2HPO_4

0.1g/l, $MgSO_4$ 0.05g/l, yeast extract 0.05g/l, agar 15 g/l)[6] and were purified by repeated sub culturing. The pure strains were tested for their cellulolytic potential using 1% aqueous solution of Congo red (w/v) followed by 1N HCl after one week of incubation on CMC Agar plates and were selected on the basis of clear zone of cellulose hydrolysis on the plates around their colonies. The strains were maintained on Nutrient Agar (peptone, 5.0 g; beef extract, 3.0 g; sodium chloride, 5.0 g; agar, 15.0 g; distilled water, 1.0 litre) slants at 4^0C for further investigation.

Characterization of the strains

The strains were characterized according to the standard cultural, biochemical and physical characteristics[7] and the Gram stained slides were observed under compound microscope provided with camera (Carl Zeiss) for micro-morphological characterization. The results were compared with Bergey's Manual of Systematic Bacteriology[8]. The isolates were tested for their ability to grow at various pH (3-11), temperature (4-65 °C) and NaCl concentrations ranging between 1-10% (w/v). The cultures were inoculated separately onto plates of Nutrient Agar medium with pH adjusted to 3, 5, 7, 9 and 11 using 1N HCl and 1N NaOH as per the requirement and were observed for growth after four days of incubation at 4, 15, 26, 37, 45, 50, 55 and 60 °C. The tests were performed in triplicates and the observations were recorded. The cultures were characterized by growing on different ISP media (HiMedia) that included ISP 1, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and ISP 7; and non ISP media that included Cellulose Congo Red Agar (CCRA) medium (K_2HPO_4 0.50 g/l, $MgSO_4$ 0.25 g/l, cellulose powder 1.88 g/l, Congo red 0.20 g/l, Agar 15 g/l, Gelatine 2 g/l); SCA (previously mentioned); Casein Starch Peptone Yeast Malt Extract (CSPY-ME) medium (K_2HPO_4 0.5 g/l, Casein 3 g/l, Maize starch 10 g/l, Peptone 1 g/l, Yeast extracts 1 g/l, Malt extracts 10 g/l, Agar 15 g/l); Mcbeth medium (K_2HPO_4 1 g/l, $CaCO_3$ 2 g/l, Na_2SO_4 2 g/l, $MgSO_4.7H_2O$ 1 g/l, $[(NH_4)_2SO_4]$ 2 g/l, CMC 1.0 %, Agar 15 g/l) and Stanier's basal medium [$(NH_4)_2SO_4$ 1g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.2 g/l, $CaCl_2$ 0.1 g/l, $FeCl_3$ 0.02 g/l]. Carbohydrate utilization was determined by growing the isolates onto plates of mineral salt medium supplemented separately with 0.5% (w/v) of sucrose, lactose, dextrose, fructose, mannitol and inositol; and incubating for four days at 37 °C. The growth of the cultures was recorded. Plates were evaluated visually for poor growth (+), moderate growth (++) or luxuriant growth (+++).

Enzyme preparation

The strains were inoculated separately in duplicates in 50 ml of sterilized CMC broth medium at pH 7.0 and incubated for four days at 37 °C for cellulase production. The culture broths were centrifuged at 10,000 rpm for 15 minutes at the room temperature. The supernatant collected was used as enzyme for cellulase assay.

Enzyme assay

The enzyme activity was assayed according to the instructions of IUPAC[9]. The FPase activity (exoglucanase) was measured by mixing 0.5 ml of enzyme solution with 50 mg of Whatman No. 1 filter paper discs and 1.0 mL of citrate buffer (pH 6.0) in a test tube; and incubating at 50 °C for 60 min. For CMC-ase (endoglucanase) activity, 0.5 ml of enzyme solution was added to 0.5 ml of 1% (w/v) of CMC prepared in sodium citrate buffer (pH 6.0) in a test tube and incubated at 50 °C for 30 min. In both the above mentioned procedures, the reactions were stopped by adding 1.0 mL 3, 5-dinitro salicylic acid (DNSA) reagent. The mixture was boiled for 5 min and liberated reducing sugars were quantified by noting down the optical density at 540 nm using UV/Vis spectrophotometer (Thermo Scientific) and comparing it with the standard curve for glucose[10] to determine the amount of reducing sugar (mg/ ml) produced during cellulose hydrolysis.

RESULTS AND DISCUSSION

Screening and selection of cellulolytic microbial strains

Five cellulase producing strains labeled as BS1, BS2, BS3, BS4 and BS5 were selected for the present study on the basis of their ability to produce clear zone of cellulose hydrolysis on CMC Agar plates.

Characterization of the strains

The selected cellulose degraders were critically examined for their macro and micro morphology. On CMC Agar medium, the colonies of all the strains showed powdery texture and the color of mycelium ranged from white to greyish white; Gram's staining and microscopic view revealed gram-positive, filamentous structure. The observations are recorded in Table-1.

The biochemical characteristics of the strains that included Indole production test, MR-VP test, Citrate

utilization test, Starch hydrolysis test, Casein hydrolysis test, Catalase test, Hydrogen sulphide (H_2S) production test and fermentation test are presented in Table-2.

The physical characteristics of the strains regarding their ability to grow on the specified pH and temperatures revealed good growth at pH 5-11 with optimum being 7 to 9 and slight to luxuriant growth at the temperatures of 15, 26, 37 and 45 °C. The strains could not withstand highly acidic medium (pH 3) as shown in Table-3 and temperatures below 15 °C and above 45 °C except strain BS2 as shown in and Table-4.

The colony characteristics of the selected strains regarding their growth, color of aerial mycelium, color of substrate mycelium and pigmentation on different ISP media are given in Table-5.

Cultural characteristics of the selected strains were observed on different media, viz. Cellulose Congo Red Agar, CSPY-ME medium, Starch Casein Agar, Mcbeth medium and Stanier's basal medium (Fig. 1-5). It was found that Starch Casein Agar proved to be the best medium on which all the strains grew luxuriantly whereas Stanier's basal medium was the least favorable medium for the growth of the cultures. Rest of the specified media used in the present investigation showed moderate growth of the cultures. For the strain BS5, CSPYME medium proved to be most suitable growth medium.

Enzyme assay

The strain BS5 showed highest exoglucanase activity in terms of liberated reducing sugar (7.4 µg/ml) followed by BS1 and BS3 (both releasing 6.8 µg/ml reducing sugar). BS 4 showed slightly more exoglucanase activity (5.7 µg/ml) than BS2 (5.5 µg/ml). The highest endoglucanase activity was exhibited by the strain BS4 (3.0 µg/ml) and least by BS2 (2.2 µg/ml). BS1, BS3 and BS5 possessed similar endoglucanase activity (all releasing 2.7 µg/ml reducing sugar). The data are presented in Figure-6.

The cellulose degrading strains BS1, S2, BS3, BS4 and BS5 showed a wide range tolerance to various pH, temperature and a wide adaptability to different media making it a suitable candidate to be utilized as commercial producer of the enzyme.

Table 1: Macro and micro morphology of the selected strains

Colony morphology	BS1	BS2	BS3	BS4	BS5
Texture	Powdery	Powdery	Powdery	Powdery	Powdery
Colour of aerial mycelium	White	Greyish white	White	White	White
Colour of substrate mycelium	Nil	Nil	Nil	Orange	Nil
Gram's reaction	(+)ve	(+)ve	(+)ve	(+)ve	(+)ve
Shape	Filamentous	Filamentous	Filamentous	Filamentous	Filamentous

Table 2: Biochemical characteristics of the selected strains

Biochemical reactions	BS1	BS2	BS3	BS4	BS5
Indole production	-	-	-	-	-
MR-VP test	-	+	-	-	-
Citrate utilization test	+	-	-	-	+
Starch hydrolysis test	+	+	+	+	+
Casein hydrolysis test	-	-	-	-	+
Catalase test	+	+	+	+	+
H_2S production test	-	+	-	-	+
Fermentation of Sucrose	A^-G^+	A^+G^+	A^+G^-	A^-G^-	A^-G^-
Fermentation of Dextrose	A^+G^-	A^-G^-	A^-G^+	A^-G^-	A^-G^-
Fermentation of Lactose	A^-G^-	A^-G^-	A^-G^+	A^-G^-	A^-G^-

+ positive; - negative; A Acid production; G Gas production

Table 3: Effect of pH on growth of the selected isolates

pH	BS1	BS2	BS3	BS4	BS5
3	-	-	-	-	-
5	++	++	++	+	+
7	+++	+++	+++	+++	+++
9	+++	+++	+++	+++	+++
11	++	+++	++	+	+

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

Table 4: Effect of temperature on the growth of the selected isolates

Temperature in °C	BS1	BS2	BS3	BS4	BS5
4	-	+	-	-	-
15	+	++	++	+	+
26	+++	+++	++	+	+
37	+++	+++	+++	+++	+++
45	+++	+++	+++	++	++
50	-	++	-	-	-
55	-	+	-	-	-
60	-	+	-	-	-

- No growth; + poor growth; ++ moderate growth; +++ luxuriant growth

Table 5: Colony characteristics of the selected strains on different ISP media

Medium	BS1	BS2	BS3	BS4	BS5
ISP 1					
Growth	+	+++	+++	+++	+++
Color of aerial mycelium	White	Greyish white	Greyish white	White	White
Color of substrate mycelium	Nil	Nil	Nil	Nil	Nil
Pigmentation	Nil	Nil	Nil	Nil	Nil
ISP 2					
Growth	+++	+++	+++	+++	+++
Color of aerial mycelium	White	Grey	Greyish white	White	White
Color of substrate mycelium	Nil	Black	Brownish	Nil	Nil
Pigmentation	Nil	Nil	Nil	Nil	Nil
ISP 3					
Growth	+++	+++	+++	+++	+++
Color of aerial mycelium	Grey	Grey	Grey	White	White
Color of substrate mycelium	Brownish black	Black	Black	Nil	Nil
Pigmentation	Brownish	Nil	Nil	Nil	Nil
ISP 4					
Growth	+++	+++	+++	+++	+++
Color of aerial mycelium	Grey	Grey	Greenish Grey	White	White
Color of substrate mycelium	Nil	Black	Nil	Brown	Nil
Pigmentation	Nil	Nil	Nil	Nil	Nil
ISP 5					
Growth	+	++	++	+++	++
Color of aerial mycelium	White	Grey	Grey	White	Grey
Color of substrate mycelium	Nil	Nil	Blackish	Nil	Nil
Pigmentation	Nil	Nil	Nil	Nil	Nil

Table 5 continued....

Table 5 continued....

ISP 6					
Growth	+++	+++	+++	+	++
Color of aerial mycelium	White	Greyish white	White	White	White
Color of substrate mycelium	Nil	Brownish	Nil	Nil	Nil
Pigmentation	Nil	Nil	Nil	Nil	Nil
ISP 7					
Growth	+++	+++	+++	+++	++
Color of aerial mycelium	Grey	Grey	Grey	White	White
Color of substrate mycelium	Greyish	Black	Greyish	Nil	Peach
Pigmentation	Nil	Nil	Nil	Nil	Nil

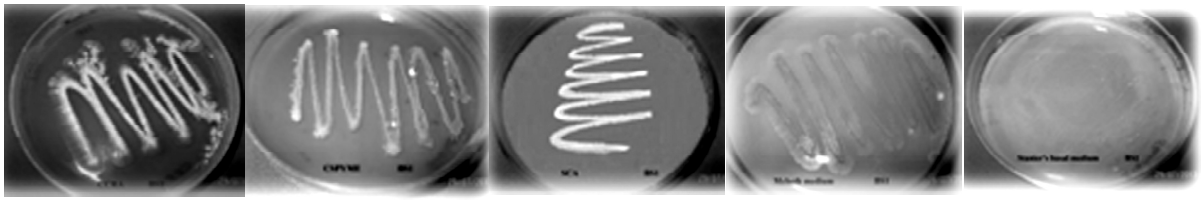

Fig. 1. Growth of strain BS1 on (a) CCRA; (b) CSPYME; (c) SCA; (d) Mcbeth medium and (e) Stanier's basal medium

Fig. 2. Growth of strain BS2 on (a) CCRA; (b) CSPYME; (c) SCA; (d) Mcbeth medium and (e) Stanier's basal medium

Fig. 3. Growth of strain BS3 on (a) CCRA; (b) CSPYME; (c) SCA; (d) Mcbeth medium and (e) Stanier's basal medium

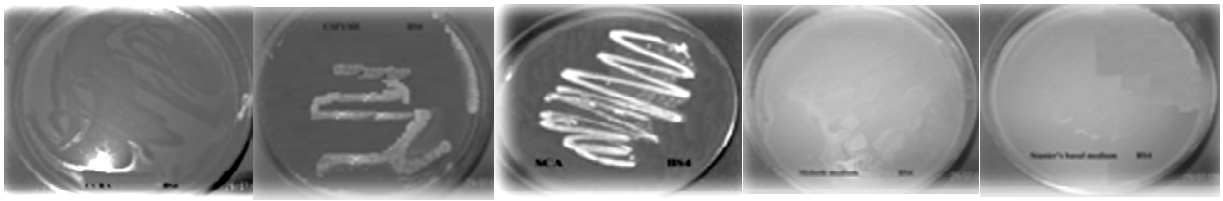

Fig. 4. Growth of strain BS4 on (a) CCRA; (b) CSPYME; (c) SCA; (d) Mcbeth medium and (e) Stanier's basal medium

Fig. 5. Growth of strain BS5 on (a) CCRA; (b) CSPYME; (c) SCA; (d) Mcbeth medium and (e) Stanier's basal medium

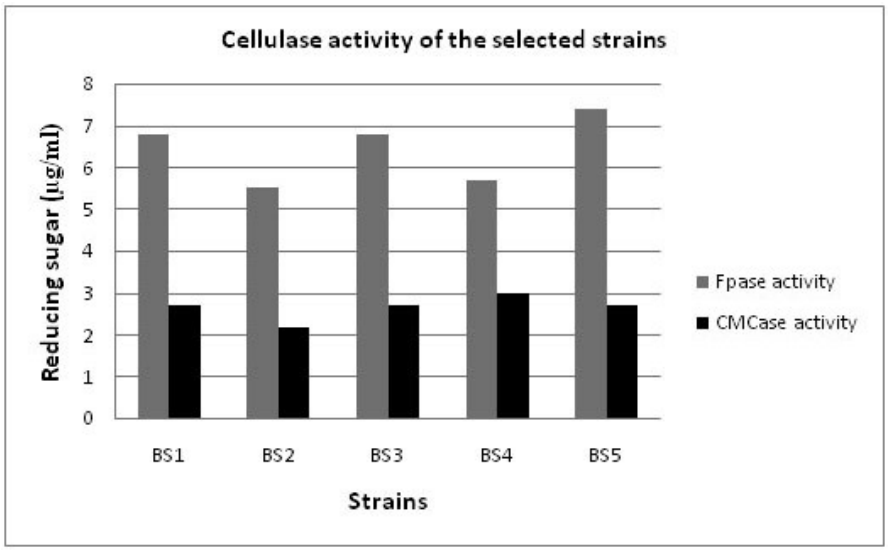

Fig. 6. Histogram showing FPase and CMCase activities of the strains BS1, BS2, BS3, BS4 and BS5

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