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ISOLATION OF EFFICIENT CELLULOSE DEGRADERS FROM MUNICIPAL WASTES

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Cellulose degrading bacteria were isolated locally by survey and collection of the soil samples from the municipal wastes. In course of screening for cellulolytic activity using a congo red test, thirty-five cellulose degraders were selected, which differed in their potential to degrade the cellulose. On secondary screening, five isolates with good cellulolytic activity were selected. The selected isolates were investigated regarding their culture characteristics and biochemical reactions, and identified as per Bergeys Manual of Systematic Bacteriology to their genus level. Two of the isolates belonged to the genus *Streptomyces*; one each to the genus *Streptococcus*; *Alcaligenes* and *Pseudomonas*. Preliminary work revealed that the isolates were mesophiles with *Streptomyces* sp. being more tolerant to higher temperature (45^{0}C), slightly acidophilic to neutral and showed maximum cellulase production at 37 ^{0}C.

Key words: Cellulose degraders, municipal waste, *Streptomyces*, *Streptomyces* cellulose degraders

The cellulosic biomass, once thought to be an ever increasing unmanageable waste (Smith *et. al*. 1997), is now considered as an important renewable resource for the microbial production of food, fuel and chemicals (Cauglan 1985). Despite being an abundant and low cost renewable organic matter in nature (Lynd *et.al.* 2002), cellulose can be utilized as a source of energy and for the production of useful end products only after its hydrolysis to glucose (Obuekwe and Okungbowa 1986). The pioneering studies of Mandels and Reese (1964) and Halliwell (1965) on the *in vitro* saccharification property of culture filtrates have stimulated researches in the microbial hydrolysis of cellulosic wastes (Mukerji *et. al*. 1984). In this perspective, maintenance and enumeration of potential cellulose degraders is beneficial in two broad ways as it will not only degrade cellulosic wastes and help in bioremediation of landfills but also offer a potential partial solution to the problem of the world's dependence on petroleum for energy. Because the production yield and stability of bacterial cellulases are comparatively lesser than fungal cellulases, a detailed study on exploitation of this enzyme from *Streptomyces* species have been hindered (Chellapandi *et. al*. 2008). Actinomycete's, one of the known cellulase-producers, has attracted considerable research interest due to its potential application in recovery of fermentable sugars from cellulose that can be of benefit for human consumption and to the ease of their growth (Jang and Cheng 2003). Although, molecular engineering is leading towards developing microorganisms which can produce more efficient cellulases, the traditional techniques of isolation still play an important role, as the isolate may have the ability to contribute in a more efficient degradation of cellulosic wastes in that particular environment. The present study is intended to isolate and purify the efficient strains of cellulose degrading bacteria and actinomycetes that may prove useful in cost-effective biomass degradation and landfill

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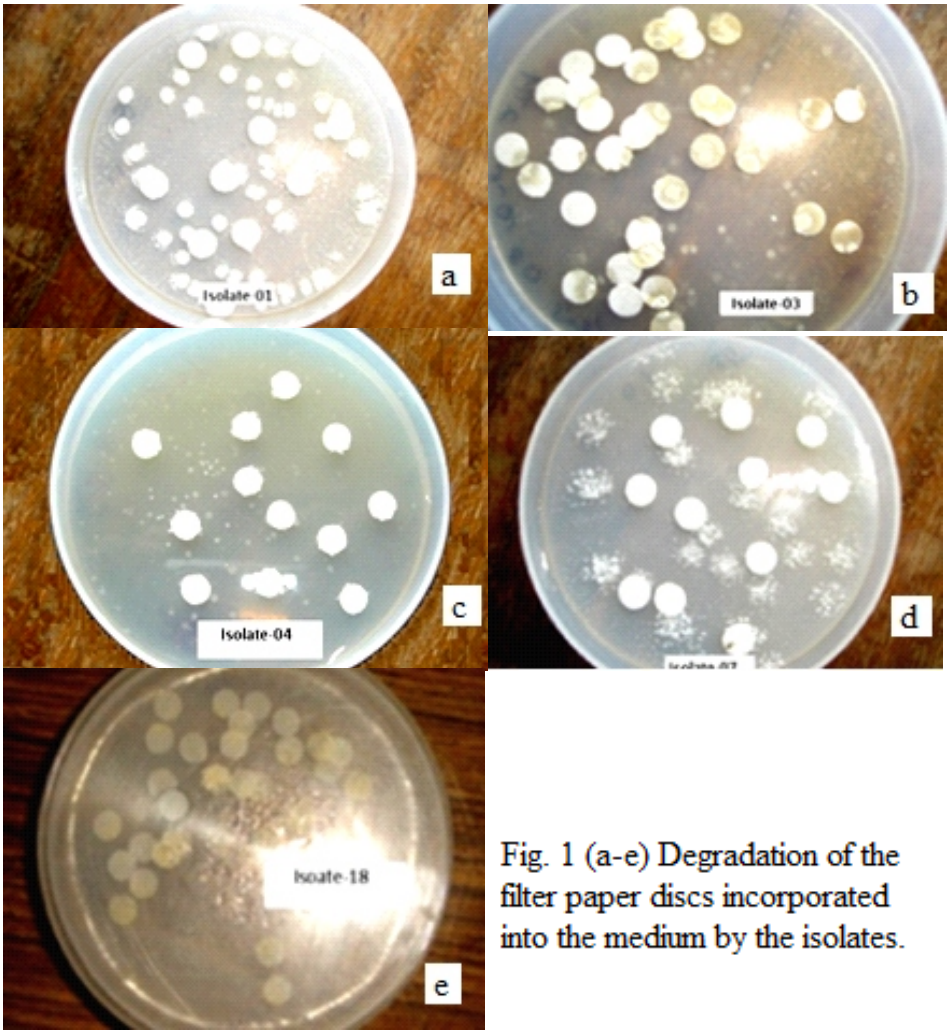

Fig. 1 (a-e) Degradation of the filter paper discs incorporated into the medium by the isolates.

bioremediation.

MATERIALS AND METHODS

Sources of media and analytical chemicals: Chemicals used for the preparation of the media and analyses were of the highest purity grade and obtained from HiMedia, Loba Chemie, Merck and Qualigens.

The cultures used in this study were isolated from soil samples collected from municipal wastes from different localities of Patna in Bihar.

Nutrient Agar was used for isolation and preservation of the cellulose degraders. Milk Agar, Methyl Redbroth Voges Proskauer broth, Nitrate broth, Phenol red dextrose broth, Phenol red lactose broth, Phenol red sucrose broth, Simmons Motility Agar, Simmons Motility Agar with tryptophan as substrate, Simmons citrate Agar, Starch Agar, Trypticase Soy Agar and Urea broth were used for specific biochemical tests (Cappuccino and Sherman 2005). Stanier's basal medium; CMC agar (Kasana *et. al.* 2008) and Modified Cellulose agar replacing carboxymethylcellulose in CMC agar with cellulose were used for cellulose degrading efficiency test.

Isolation, purification and maintenance of isolates: 100 mg of each of the soil samples were serially diluted in 10 ml of sterilized

Table 1. Cultural characteristics of the selected isolates

Colony morphology	Isolate 01	Isolate 03	Isolate 04	Isolate 07	Isolate 18
Configuration	Round	Erose	Erose	Round	Erose
Colour	White	Dirty white	Dirty white	White	Dirty white
Margin	Radiating	Smooth	Smooth	Smooth	Smooth
Elevation	Flat	Slight	Slight	Convex	Flat
Surface	Powdery	Slimy	Slimy	Powdery	Slimy
Pigments	Reverse- Peach	X	X	Reverse- orange	X
Gram's Reaction	(+)ve	(+)ve	(-)ve	(+)ve	(-)ve
Shape	Filamentous	Cocci	Coccobacillus	Filamentous	Rods

Table 2. The biochemical activities of the selected isolates

Biochemical Tests	Isolate 01	Isolate 03	Isolate 04	Isolate 07	Isolate 18
Amylase Test	+	-	-	+	-
Caseinase Test	-	-	-	+	-
Catalase Test	+	-	+	+	+
Citrate Utilizaion Test	-	-	+	+	+
Fermentation of carbohydrate	-	-	-	-	-
Gelatinase Test	-	-	-	+	-
Hydrogen Sulphide Test	-	-	-	+	-
Indole Test	-	-	-	-	-
Methyl Red Test	-	+	-	-	+
Nitrate Reduction Test	+	-	-	+	+
Urease Test	-	-	-	-	-
Voges- Proskauer Test	-	-	-	-	-

+ Positive, - Negative

normal saline (0.85%) and direct plating of six fold serial dilutions in triplicates was done on Nutrient Agar and kept in the incubator at 37° C for 24-48 hours. Different colonies of bacteria and actinomycetes thus obtained were purified by streaking (Dubey and Maheshwari 2004) and maintained on Nutrient agar slants at 4 ^{0}C with periodic sub culturing.

Screening of cellulose degrading microorganisms from the isolates: A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye on the colonies of the isolates grown on CMC Agar and also by their ability to degrade Whatman No. 1 filter paper discs incorporated into the Stanier's Basal medium. The plates were incubated at 37 ^{0}C for 5 days to allow for the secretion of cellulase. The agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min to visualize the

Table 3. Effect of different temperatures on the growth of the selected isolates

Growth at Temperature	Isolate-01	Isolate-03	Isolate-04	Isolate-07	Isolate-18
4^0C	-	-	-	-	-
10^0C	+	-	-	+	-
15^0C	+	-	-	+	-
25^0C	+	-	-	+	-
30^0C	++	++	++	++	+
37^0C	+++	+++	+++	+++	+++
42^0C	+++	++	++	+++	++
45^0C	++	-	-	+	-
55^0C	-	-	-	-	-
65^0C	-	-	-	-	-

- No growth ++ Moderate growth
+ Poor growth +++ Luxuriant growth

Table 4. Effect of different NaCl concentration on growth of the selected isolates

Growth at NaCl (in molar concentration)	Isolate-01	Isolate-03	Isolate-04	Isolate-07	Isolate-18
0.3M	+	++	++	++	++
0.5M	+	+	+	++	+
0.7M	+	+	+	++	-
0.9M	++	+	+	++	-
1.0M	++	-	-	++	-
1.2M	++	-	-	++	-
1.4M	++	-	-	+	-
1.5M	+	-	-	+	-
1.7M	-	-	-	-	-

- No growth ++ Moderate growth
+ Poor growth +++ Luxuriant growth

hydrolysis zone. The Congo red solution was then poured off and the plates were further treated by flooding with 1N HCl for 15 min. To indicate the cellulase activity of the organisms, diameters of clear zone around colonies on CMC agar were measured. Five types of colonies were selected on the basis of the area of the clear zone surrounding the colonies.

Characterization of the selected isolates:

Colonies were identified on the genus level by Gram's staining method and different biochemical tests as per Bergey's Manual of Systematic Bacteriology which includes Indole test, methyl red test, Voges-Proskauer test, citrate utilization test, catalase test, urease test, starch test, gelatin hydrolysis test, sugar fermentation test, caseinase test, hydrogen sulphide test and nitrate reduction test. Fresh cultures were used for all the tests.

Table 5. Effect of different pH on growth of the selected isolates

Growth at pH	Isolate-01	Isolate-03	Isolate-04	Isolate-07	Isolate-18
5.0	-	++	+	-	-
5.7	+++	+++	+++	++	++
6.8	+++	+++	+++	+++	+++
8.0	++	++	++	++	+
9.0	++	++	+	++	+
11.0	+	++	+	++	+

- No growth ++ Moderate growth
+ Poor growth +++ Luxuriant growth

Table 6. (a) Effect of different temperatures on zone of hydrolysis on Modified Cellulose agar

Temperature	01	03	04	07	18
37^0C	4.0	2.0	2.0	3.0	2.0
42^0C	3.0	2.0	1.5	2.0	1.5
55^0 C	x	x	x	x	x

x: no hydrolysis

Effects of various growth parameters on the isolates: The influence of NaCl concentration, pH and temperature on the growth of the isolates was studied. The experiments were done in triplicates.

The influence of temperature was studied by incubation of the media at 4^0C, 10 ^{0}C, 15^0C, 25^0C, 30 ^{0}C, 37^0C, 42^0C, 55^0C and 65^0C, for 24 hours. Similarly the influence of NaCl was studied using Nutrient agar with different NaCl concentrations (0.3M, 0.5M, 0.7M, 0.9M, 1.0M, 1.2M, 1.4M, 1.5M and 1.7M approx.) prepared by dissolving 2gm, 3gm, 4gm, 5gm, 6gm, 7gm, 8gm, 9gm and 10gm of NaCl in 100 ml of sterilized distilled water. The influence of pH was observed by adjusting pH of the media by using pH meter (Systronics) to 5.0, 5.7, 6.8, 8.0, 9.0 and 11.0; and incubating for 24 hours.

Optimization of temperature for maximum enzyme production: The selected isolates were plated in triplicates on Modified Cellulose agar and CMC agar and incubated at 37^0C, 42^0C and 55^0C for 6 days. The diameter of zone of hydrolysis (in mm), using Congo red dye, was measured by zone-scale (HiMedia) after every two days.

OBSERVATIONS AND RESULTS

Isolation and screening of cellulose degrading microorganisms from the isolates: The purified bacterial strains isolated from the soil samples were inoculated into CMC agar medium with pH 7.0 and inoculated at 37^0C for 5 days. Screening of isolated bacteria was conducted by using the Congo red test as a preliminary study for identifying cellulose degraders. Since the sole carbon source in CMC agar was caboxymethylcellulose, therefore, the result of the test was strong evidence that cellulase was produced in order to degrade cellulose. A total of the thirty-five cellulose degraders were obtained after screening. These isolates affected the hydrolysis of cellulose to different extents. Among them, the best five strains designated as 01, 03, 04, 07 and 18 were selected on the basis of area of zone clearing and filter paper degradation as shown in Fig. 1(a-e).

Morphological characterization of the selected isolates: The selected cellulose degraders were critically examined for their morphology as recorded in Table 1. The

colonies of the Isolate-01 were round, white and powdery. The margins were radiating and no elevations were observed. Yellow to brown pigmentation was observed on the reverse side of the colonies. The microscopic view showed Gram-positive filaments with chains of spores in whorls. The colonies of the Isolate-03 were erose, dirty white and slimy. The margins were smooth and slight elevations were observed. The microscopic view showed Gram-positive cocci. The colonies of the Isolate-04 were erose, dirty white and slimy. The margins were smooth and slight elevations were observed. The microscopic view showed Gram-negative coccobacilli. The colonies of the Isolate-07 were round, white and powdery. The margins were radiating and convex elevations were observed. Orange pigmentation was observed on the reverse side of the colonies. The microscopic view showed Gram-positive filaments. The colonies of the Isolate-18 were erose, dirty white and slimy. The margins were smooth and no elevations were observed. The microscopic view showed Gram-negative rods. The cultural and microscopic observations revealed that the isolates 03, 04 and 18 were bacterial strains whereas the isolates 01 and the 07 were actinomycetes.

Biochemical characterization of the selected isolates: The biochemical tests were performed on the five isolates and are recorded in Table 2. Isolate 01 showed positive result for amylase test, catalase test, nitrate reduction test and negative for all the rest of the specified biochemical tests ; isolate 03 showed the negative result for all the specified tests except the methyl red test; isolate 04 positive result for catalase test and citrate utilization test and negative for all the rest of the specified tests; isolate 07 showed positive result for amylase test, caseinase test, catalase test, citrate utilization test, gelatinase test, hydrogen sulphide test, nitrate reductase test and negative for fermentation of carbohydrates, indole test, methyl red test, urease test and Voges-Proskauer test. Isolate 18 showed positive results for catalase test, citrate utilization test, methyl red test nitrate reduction test and negative for all the rest of the specified biochemical test. The results of these tests were compared to known results for that organism to confirm its identification. For identification, Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994) and Bergey's Manual of Systematic Bacteriology (Williams *et al*. 1989) have been referred. With the help of morphology, microscopic observations and biochemical tests, the selected isolates were identified to their genus level. However, in some of the isolates, variations from the expected biochemical results were observed which may be attributed to strain differences within the given species. Isolate-01 was identified as *Streptomyces* species; Isolate-03 as *Streptococcus* species; Isolate-04 as *Alcaligenes* species; Isolate-07 as *Streptomyces* species; and Isolate-18 as *Pseudomonas* species.

Effect of various growth parameters on the isolates: Growth parameters of the isolates were compared with respect to pH, temperature and saline tolerance. The results recorded in Table 3 showed that the isolates 01 and 07 showed growth on wide range of temperatures (10^0C, 15^0C, 25^0C, 30^0C, 37^0C, 42^0C, 45^0C, 55^0C and 65^0C), though moderate to luxuriant growth was restricted between 30^0C and 42^0C. However, isolate 01 was able to grow upto a temperature of 45^0C. A comparatively narrow range (30^0C to 42^0C) was observed for the isolates 03, 04 and 18, which indicated that the strains are mesophilic, but isolate 01 has higher temperature tolerance.

Isolates 01 and 07 showed growth at all NaCl concentrations from 0.3M to 1.5M except 1.7M

, isolates 03 and 04 showed growth at 0.3M to 0.9M NaCl concentrations. Isolate 18 showed growths at 0.3M and 0.5M NaCl concentrations only (Table 4).

After 24 hours of incubation, there was no growth in any isolate except 03 and 04 at pH 5.0. At pH 6, 7, 8, 9, 10 and 11, all isolates showed growth (Table 5). The isolates showed luxuriant growth between pH 5.7 and 6.8. Hence the strains are slightly acidophilic to neutral.

Optimization of temperature for maximum enzyme production: The five selected isolates were plated in triplicates on Modified Cellulose Agar and CMC Agar and incubated at 37^0C, 42^0C and 55^0C for 6 days and the diameter of zones of hydrolysis (in mm) was observed. The diameters of clear zone around colonies on Modified Cellulose agar were 4.0 mm for the isolate 01, 3.0 mm for the isolate 07 and 2.0 mm each for the isolates 03, 04 and 18 at 37^0C. At 42^0C, it was 3.0 mm, 2.0mm, 1.5mm, 2.0 and 1.5mm for the isolates 01,02,04,07 and 18 respectively as shown in table 6(a). Same on CMC agar were 3.0 mm each for isolates 03and 2.0mm each for the isolates 04 and 18 at 37^0C. At 42^0C, it was 2.0 each for the isolates 01and 07, 1.5mm each for the isolates 1.0 and 07, 1.5 mm each for the isolates 01and 07, 1.5 mm each for the isolates 03 and 18, and 1.0mm for the isolate 03 and 18 and 1.0mm for the isolate 04 as shown in Table 6(b). None of the isolates showed hydrolysis at 55^0C on either of the media.

Out of the five selected cellulose degraders, isolates 01 and 07 were found to be efficient cellulose degraders on the basis of our present investigation. These isolates were sent to Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh for further identification up to species level. The isolate-01 was identified as ***Streptomyces albospinus*** **(MTCC No. 8768)**; and the isolate-07 was identified as ***Streptomyces somaliensis*** **(MTCC No. 8769)**.

DISCUSSION

A large proportion of vegetation added to soil is cellulose; therefore, decomposition of cellulose has a special significance in the biological cycle of carbon (Lederberg 1992). Introduction of cellulose degrading bacterial (CDB) inoculants is a beneficial microbiological tool to aid recovery of energy from degraded ecosystems (Balamurugan *et. al.* 2011). A wide variety of bacteria are known for their production of hydrolytic enzymes with streptomycetes being the best known enzyme producers (Vinogradova and Kushnir 2003). They are capable of secreting an array of different extracellular enzymes including cellulases, chitinases, and xylanases (Jaradat *et. al.* 2008). The present study was a preliminary investigation into the isolation and cultivation of cellulolytic bacteria and actinomycetes and is intended as a basis for future, in-depth studies. In this investigation, among the five screened cellulose degrading strains of bacteria and actinomycetes, the isolate 01 (***Streptomyces albospinus*****: MTCC No. 8768**) and the isolate 07 (***Streptomyces somaliensis*****: MTCC No. 8769**) proved to be promising cellulose degraders based on the results of our present study. To our knowledge, there is no record of cellulolytic activity of *Streptomyces albospinus* and *Streptomyces somaliensis* isolated from Indian soil and there is scope for further research and utilization of these strains for efficient cellulose degradation.

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