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RESEARCH ARTICLE

OPTIMIZATION OF PARAMETERS AND ASSESSMENT OF CELLULOLYTIC POTENTIAL OF SELECTED CELLULOSE DEGRADERS

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ABSTRACT

The present study was aimed at isolation of cellulolytic microbial strains from decaying agro wastes and optimizing the environmental parameters including culture media, temperature and pH for their mass cultivation. Also, their cellulolytic potential on locally available substrates was assessed. Five efficient cellulolytic microbial strains labeled as St-01, St-03, St-04, St-07 and St-18 were selected from a total of thirty five cellulose degraders. From the investigation, it was found that Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and Stanier's Basal medium were found to be the most suitable media for luxuriant growth of all the selected strains at pH 7 and temperature 37°C. The assessment of their cellulolytic potential on locally available substrates proved that the grated vegetable stalk was most suitable substrate for degradation compared to straw powder and wood powder. Under similar physical and chemical conditions, the highest cellulolytic potential was exhibited by the isolate St-01 followed by the isolate St-07. The isolate St-01 was identified as *Streptomyces albospinus* (MTCC No. 8768); and the isolate St-07 was identified as *Streptomyces somaliensis* (MTCC No. 8769) at Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh.

INTRODUCTION

Cellulose is the principal component of the cell walls in the plants where it provides the main structural feature. It is also one of the dominating waste materials in nature because of its recalcitrant nature, and thus, poses severe disposal problems. On hydrolysis, the cellulose produces simpler utilizable glucose units which can further be used as a substrate for the other bioprocesses. Cellulose hydrolysis can be done chemically as well as enzymatically. The technology for the chemical hydrolysis of cellulosic biomass to sugars is still not very cost-effective. Absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass is the main obstacle in the widespread utilization of this important resource. According to Lynd et al. (1996), if microorganisms can be developed that possess the required combination of substrate utilization and product formation properties, it may offer very large cost reductions. The cellulose is hydrolyzed by a multienzyme complex, the cellulase system. Cellulases are the group of hydrolytic enzymes including endo-β-1,4-glucanases, exo- β -1,4-glucanases and β-glucosidases, that act synergistically to convert crystalline cellulose to glucose (Lynd et al., 2002; Warren, 1996). The endoglucanases cleave at random at internal amorphous sites in the cellulose glucan chains, and the exoglucanases act processively to release cellobiose primarily from the chain ends (Teeri, 1997; Warren, 1996). The β-glucosidase is considered an integral part of this complex because its presence shows significant improvement in the saccharification process as it relives inhibition of cellulase action caused by accumulation of the cellobiose in the hydrolysate (Reese and Levinson, 1952). Economical production of cellulases is the key for feasible waste disposal and bioethanol production from cellulosic biomass using cellulase based processes. Therefore, by the selection of efficient cellulolytic microorganisms and cost-effective operational techniques, the production of such useful end products from the biodegradation of the low cost enormous stock of cellulose in nature, can be very beneficial. It will not only provide renewable source of substrate in the form of glucose, but also help in reducing the disposal problem of cellulosic wastes. For increased cellulase production, mass cultivation of cellulolytic microorganisms is needed on commercial scale which can be achieved by providing them optimum environmental parameters including suitable growth medium, optimum pH and temperature. In this pretext, the present investigation was aimed at exploring the microbial world to enumerate cellulolytic bacterial and actinomycete strains by collection of the soil samples locally from decaying agro wastes; and growing them on different media, temperature and pH to determine the optimum environmental conditions for their mass cultivation and cellulases production. Also, the cellulolytic potential of the selected isolates was investigated using locally available substrates.

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MATERIALS AND METHOD

Collection of soil samples: The soil samples were collected locally from decaying agro wastes sites from a minimum of five spots at each site from a depth of 3-4 inches and collected in sterilized polythene bags.

Isolation and purification of microbial strains: Modified Nutrient Agar (with cellulose as a substrate) medium was prepared, autoclaved, poured in petriplates and left to solidify. Collected soil samples were serially diluted in normal saline and plated on the medium with six fold dilutions in triplicates by standard method (Aneja, 1993) and incubated at 37°C for 24 h. The microbial colonies thus isolated were purified by serially diluting the inoculums and plating on freshly prepared sterilized solidified Modified Nutrient Agar plates with six fold inoculum dilution and incubated at 37°C for seven days for production of cellulase.

Screening of cellulose degrading microbial strains bacteria: After a period of seven days, the pure microbial cultures were tested for their cellulolytic potential. The cultures were flooded with an aqueous solution of 1 % Congo red for 15 min followed by flooding with 1N HCl for 15 min and then it was also poured out. The medium turned dark purple and a clear zone surrounding the colonies of the cellulolytic isolates on the plate was observed, while non cellulolytic strains did not show any clear zone around the colonies.

Morphological characterization of the selected cellulolytic microbial strains: The selected cellulolytic microbial strains were examined macro-morphologically by observing the colony characteristics in terms of colour, texture and pigment released by the colonies. The strains were Gram stained and examined micro-morphologically under compound microscope.

Biochemical characterization of the selected cellulolytic microbial strains: The selected isolates were biochemically characterized by performing different biochemical tests including amylase, caseinase, catalase, citrate utilization, fermentation of carbohydrate, gelatinase, hydrogen sulphide, IMViC, nitrate reduction test and urease tests. The results of the tests were compared with that of known organisms for their tentative identification up to genus level.

Growth of the selected cellulolytic microbial strains on different culture media: The selected strains were grown on different media, *viz*. Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate, Stanier's Basal medium, Starch Casein Agar, CSPY-ME medium and Cellulose Congo Red Agar to find the most suitable growth medium for mass cultivation of the strains. The media were prepared, autoclaved poured in tubes and left to solidify. The selected strains were inoculated on these media and incubated at 37°C for seven days.

Optimization of temperature and pH: For optimization of temperature, Nutrient Agar plates were prepared and plated in triplicates with six fold inoculums dilution of the seven selected cellulolytic strains, separately. The plates were incubated at temperatures 4°C, 10°C, 15°C, 25°C, 37°C, 42°C, 45°C, 55°C and 65°C for 48 h. The growth of the colonies was observed and optimum temperature for their growth was determined. For optimization of pH, Nutrient Agar medium was prepared and divided into six parts and the pH was adjusted to 5, 6, 7, 8, 9 and 10, respectively using 1N NaOH or 1N HCl as per the requirement. The selected strains were serially diluted in normal saline and each plated with six fold in triplicates on the Nutrient Agar plates with the specified pH value. The plates were incubated for 48 h at the optimum temperature determined in the previous stage of investigation. The growth of the colonies was observed and optimum pH for their growth was determined.

Assessment of cellulolytic potential of the selected cellulolytic microbial strains on locally available substrates: The cellulolytic activities of the isolates were estimated on different cellulosic substrates including straw powder, wood powder and grated vegetable stalk. Four sets of Five ml of the Nutrient broth containing 50 mg of the substrates as the carbon source were inoculated with the each of the isolates, separately and incubated at 37°C for a period of 2, 4, 6 and 8 days. The degradation of the substrate at an interval of two days was estimated by weighing the substrate on the aforesaid periods and subtracting it from the initial weight i.e. 50 mg.

RESULTS

Collection of soil samples: The soil samples were collected locally from decaying agro wastes from Rajvanshi Nagar, Raja Bazaar, Shastri Nagar and Danapur sites of Patna region in Bihar, India.

Isolation and purification of microbial strains: Altogether thirty-five cellulolytic microbial isolates were collected from the different sampling sites of Patna. It was found that 06 of the total cellulolytic microbial isolates were isolated from the Rajvanshi Nagar locality, 17 from the Raja Bazaar locality, 07 from the Shastri Nagar locality and 05 from the Danapur locality. The data is shown in Figure 1. Out of the thirty-five cellulolytic microbial isolates, five efficient isolates labeled as St-01, St-03, St-04, St-07 and St-18 were selected on the basis of zone of clearing for further investigation. Of these, St-01 was isolated from Rajvanshi Nagar locality, whereas St-03, St-04, St-07 and St-18 were isolated from Raja Bazaar locality.

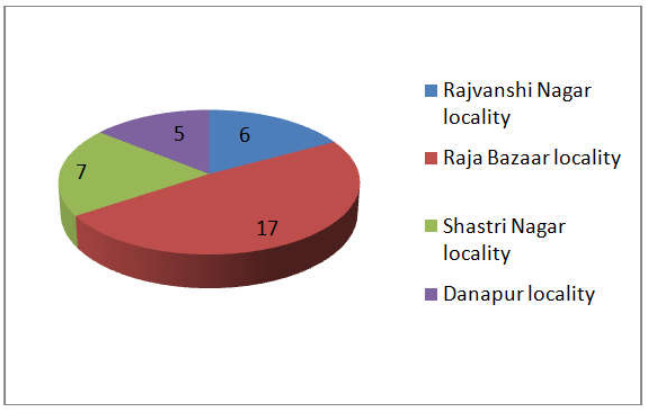

Fig. 1. Total number of isolated cellulolytic microbial strains and their locations

Morphological characterization of the selected cellulolytic microbial strains: The selected cellulolytic microbial strains were examined critically for their macro and micro morphology. The colonies of St-01 were white and powdery.

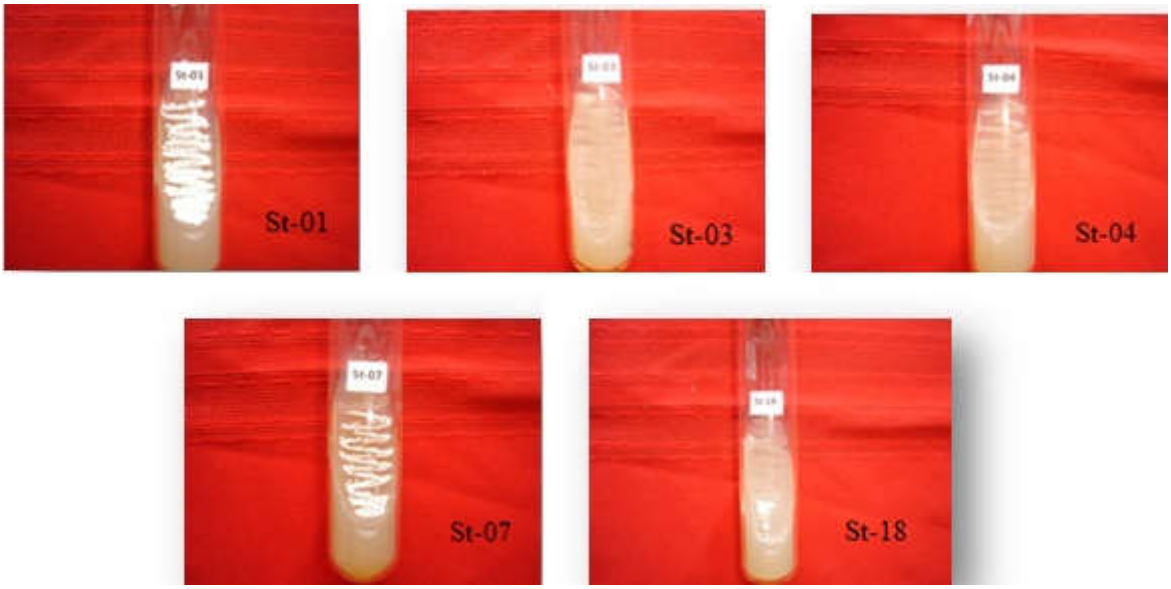

Fig. 2. Colonies of the selected cellulolytic microbial strains St-01, St-03, St-04, St-07 and St-18 on Nutrient Agar

Table 1. Biochemical characterization of the selected isolates

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

+ Positive, - Negative

Yellow to brown pigmentation was observed on the reverse side of the colonies. The slide showed Gram-positive filaments with chains of spores in whorls. The colonies of St-03 were dirty white and slimy with no pigmentation. The slide showed Gram-positive coccus. The colonies of St-04 were dirty white and slimy with no pigmentation. The slide showed Gram-negative coccobacillus. The colonies of St-07 were white and powdery. Orange pigmentation was observed on the reverse side of the colonies. The slide showed Gram-positive filaments. The colonies of St-18 were dirty white and slimy with no pigmentation. The slide showed Gram-negative rods. The colonies of the selected cellulolytic microbial strains St-01, St-03, St-04, St-07 and St-18 have been presented in Figure 2.

Biochemical characterization of the selected cellulolytic microbial strains: The selected isolates were biochemically characterized by performing different biochemical tests including amylase, caseinase, catalase, citrate utilization, fermentation of carbohydrate, gelatinase, hydrogen sulphide, IMViC, nitrate reduction test and urease tests and the results are shown in Table 1. Preliminary investigations on these isolates regarding their morphology, microscopic observations and biochemical reactions suggested that the isolates St-01 and St-07 resembled the genus *Streptomyces*; St-03 resembled the genus *Streptococcus*; St-04 resembled the genus *Alcaligenes* and St-18 resembled the genus *Pseudomonas*.

***Growth of the selected cellulolytic microbial strains on different culture media*:** The selected strains were grown on Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate, Stanier's Basal medium, Starch Casein Agar, CSPY-ME medium and Cellulose Congo Red Agar. The colonies were characterized for their potential of substrate utilization. The growth of the isolates on the specified media is shown in Figure 3-8. The isolate St-01 showed the luxuriant growth on Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate, Cellulose Congo Red Agar and Stanier's Basal media. The growth was moderate on Starch Casein Agar and poor on CSPY-ME media. The isolate St-03 showed the luxuriant growth on Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and CSPY-ME media. The growth was moderate on Stanier's Basal media whereas the isolate showed poor growth on Cellulose Congo Red Agar and Starch Casein Agar. The isolate St-04 showed the luxuriant growth on Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and CSPY-ME media. The growth was moderate on Nutrient Agar with cellulose powder as a substrate and poor on Starch Casein Agar and Stanier's Basal media. No observable growth was observed on Cellulose Congo Red Agar medium. The isolate St-07 showed luxuriant growth on Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and Stanier's Basal media.

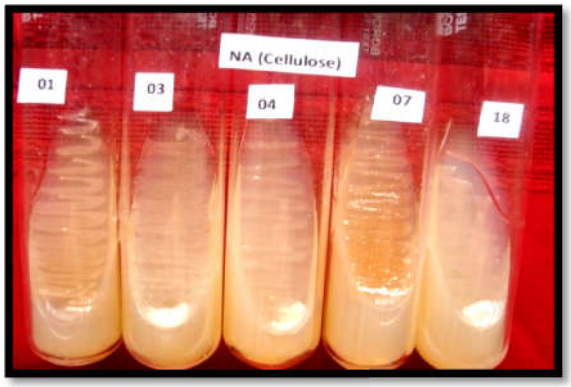

Fig. 3 Growth of selected isolates on Nutrient Agar with cellulose powder as a substrate

Fig. 4 Growth of selected isolates on Nutrient Agar with CMC as a substrate

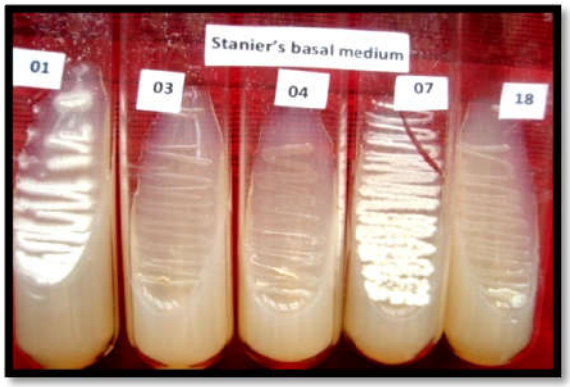

Fig. 5 Growth of selected isolates on Stanier's basal medium

Fig. 6 Growth of selected isolates on Starch Casein Agar

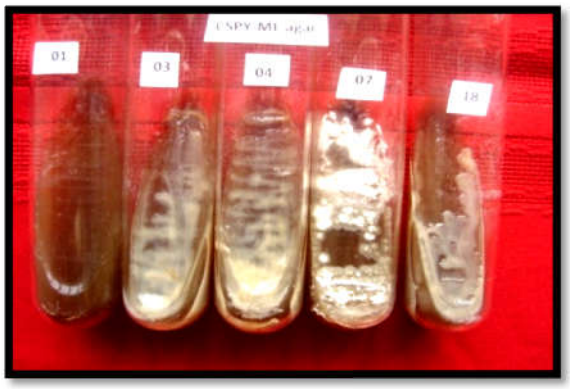

Fig. 7 Growth of selected isolates on CSPY-ME medium

Fig. 8 Growth of selected isolates on Cellulose Congo Red Agar

The growth was moderate on CSPY-ME while it was poor on Nutrient Agar with cellulose powder as a substrate, Cellulose Congo Red Agar and Starch Casein agar. The isolate St-18 showed luxuriant growth on Nutrient Agar with cellulose powder as a substrate and CSPY-ME. The growth was moderate on Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and Starch Casein Agar, while it was poor on Cellulose Congo Red Agar and Stanier's Basal medium. Thus, it was inferred that Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and Stanier's Basal medium were found to be the most suitable media for luxuriant growth of all the selected strains. Also, the isolates St-01 and St-07 showed higher range of substrate utilization in comparison to the other selected isolates.

Optimization of temperature and pH: The selected isolates were grown on specified temperatures and pH to optimize the environmental conditions for their optimum growth. The observations are recorded in Table 2 and Table 3, respectively. The range of the temperatures at which the isolates could grow was from 30-42°C as shown in Table 2. However, St-01 was able to grow even at 25°C and 45°C. St-07 showed growth at a wider range of temperature from 10°C to 45°C, though the growth was poor at the extreme ends. Comparing the growth of all the selected isolates at different temperatures, it was found that the optimum temperature for growth of all the selected cellulolytic isolates was 37°C. From the observations shown in Table 3, it is evident that all the selected isolates showed different degree of growth at all the specified pH showing their greater tolerance towards fluctuating environmental conditions. Only the isolates St-01, St-07 and

St-18 were not able to grow in highly acidic condition. The isolates preferred slightly acidic to neutral environment for their growth. The present findings suggest that pH value 7 was most suitable for the optimum growth of all the selected isolates.

Table 2. Effect of temperature on growth of the selected cellulolytic microbial strains

Temperature	St-01	St-03	St-04	St-07	St-18
4°C	-	-	-	-	-
10°C	-	-	-	+	-
15°C	-	-	-	+	-
25°C	+	-	-	+	-
30°C	++	++	++	++	+
37°C	+++	+++	+++	+++	+++
42°C	+++	++	++	+++	++
45°C	++	-	-	+	-
55°C	-	-	-	-	-
65°C	-	-	-	-	-

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

Table 3. Effect of pH on growth of the selected cellulolytic microbial strains

pH	St-01	St-03	St-04	St-07	St-18
5	-	++	+	-	-
6	+++	+++	+++	++	++
7	+++	+++	+++	+++	+++
8	++	++	++	++	+
9	++	++	+	++	+
10	+	++	+	++	+

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

Assessment of cellulolytic potential of the selected cellulolytic microbial strains on locally available substrates

The cellulolytic activities of the isolates were estimated on different cellulosic substrates including straw powder, wood powder and grated vegetable stalk. Measured amount of each of these substrates were used as sole carbon source by the selected isolates and their cellulolytic potential was estimated by calculating the substrate degradation in terms of weight loss over a period of eight days. The data are shown in Figure 9-11.

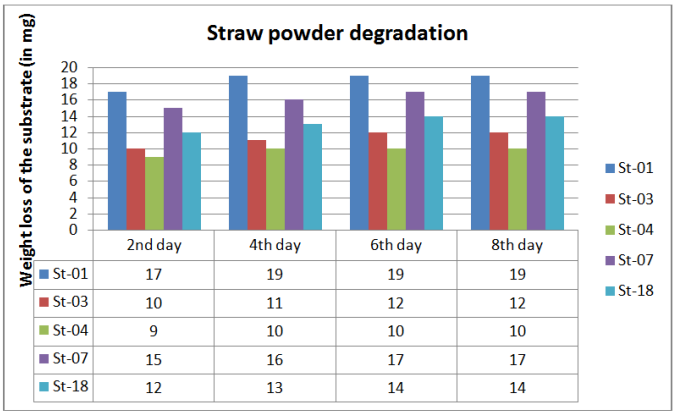

Fig. 9. Straw powder degradation by the selected cellulolytic strains over a period of 8 days

It was found that all the isolates showed maximum cellulolytic activities against grated vegetable stalks followed by straw powder and then wood powder. The potential of any cellulase system to hydrolyze various cellulosic substrates depends on the nature of the substrate used to produce the enzyme system (Chahal et al., 1976). For any particular bioprocess, the degradation of the substrate depends upon its chemical composition. From the present findings, it was inferred that St-01 was the most efficient cellulose degrader among the five selected isolates followed by the St-07. St-18 was third, St-03 fourth and St-04 fifth in positions regarding their cellulolytic potential.

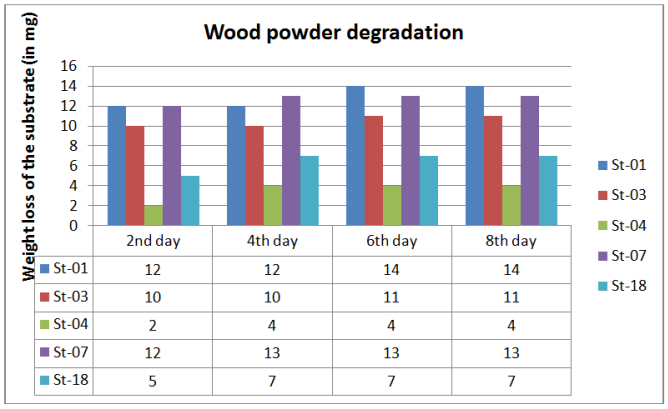

Fig. 10. Wood powder degradation by the selected cellulolytic strains over a period of 8 days

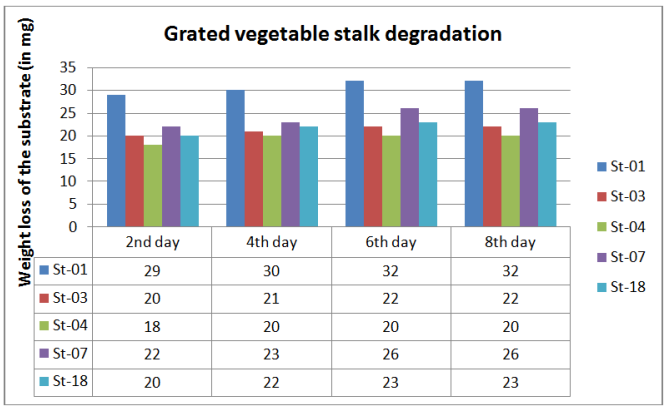

Fig. 11. Grated vegetable stalk degradation by the selected cellulolytic strains over a period of 8 days

DISCUSSION

Life on the earth depends on photosynthesis, which results in the production of plant biomass having cellulose as the major component. The amount and the ease of its availability make it a rich and renewable source of energy. In fact, plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Despite being an abundant renewable organic matter in nature, cellulose can be utilized as a source of energy and chemical feed stock only after its hydrolysis to glucose. The generated glucose can be used as a substrate for the production of a number of useful products including food and feed stock; chemicals and fuels like alcohol, butanol etc. Due to its recalcitrant, durable nature, cellulose accumulates in terrestrial environments; where a variety of cellulolytic microorganisms, that exists in virtually every niche, decompose it to yield soluble oligosaccharides and glucose. Keeping this fact in mind, the soils from the specific sites were explored for the isolation, identification and partial characterization of cellulolytic microbial strains with respect to cultural and biochemical activities. The investigation clearly indicated the presence of a wide variety of cellulose degrading microorganisms in decaying agro

wastes. Modified Nutrient Agar with cellulose as a substrate was used as medium to isolate the cellulose degrading microorganisms. A total of the thirty-five strains of the cellulose degraders were isolated from the different sampling sites. These isolates affected the hydrolysis of cellulose to different extents. Among them, the best five strains labeled as St-01, St-03, St-04, St-07 and St-18 were selected on the basis of zone clearing in congo red test. The cultural and microscopic observations revealed that the isolates St-03, St-04 and St-18 were bacterial strains whereas the isolates St-01 and St-07 were actinomycetes. Out of the five selected strains, isolates St-01 and St-07 were found to be very efficient cellulose degraders. The physical factors like the temperature and the pH influence the growth and enzymatic activities of the microorganisms. The temperature plays a major role in affecting the activity of bacterial enzymes. The enzymes are most active and the enzymatic reactions proceed at the maximum speed and efficiency at an optimum temperature that varies with the bacterium. Beyond the maximum and minimum extremes of temperature for the microorganisms, the enzyme becomes inactive. Low temperatures are less damaging than high temperatures, which denature proteins causing irreversible changes and total enzyme destruction. The test organisms were treated to different temperatures ranging from 4°C to 65°C. All of the isolates flourished between 30°C and 42°C and can be categorized as mesophilic. The isolate St-01 showed growth even at 25°C and 45°C. The isolate St-07 showed growth over a broad range of temperature ranging between 10°C and 45°C, though the growth was poor at the extreme ends. After investigation, it can be concluded that the optimum growth of the selected isolated occurred at 37°C. The pH of an organism's environment has the maximum influence on the bacterial growth. It limits the synthesis of the enzymes responsible for synthesizing the new protoplasm (Dubey and Maheshwari, 2004). The increase or decrease in hydrogen ion concentration of the medium slows down the rate of chemical reactions because of the destruction of cellular enzymes. Each species has a specific range of pH, which may be broad or limited, for maximum enzymatic activity. The isolates were subjected to different pH for optimization of their growth. The isolates exhibited optimum growth at pH 7, though they were capable of survival within a broad pH range. The cellulolytic activities of the different isolates were estimated on different cellulose substrates, namely, straw powder, wood powder and grated vegetable stalk. It was found that vegetable stalk was better substrate for degradation by the selected isolates.

Conclusion

From the present investigation, it was concluded that Nutrient Agar with cellulose powder as a substrate, Nutrient Agar with Carboxymethylcellulose (CMC) as a substrate and Stanier's Basal medium were found to be the most suitable media for luxuriant growth of all the selected strains at pH 7 and temperature 37°C.

The assessment of their cellulolytic potential on locally available substrates proved that the grated vegetable stalk was most suitable substrate for degradation compared to straw powder and wood powder. Under similar physical and chemical conditions, the highest cellulolytic potential was exhibited by the isolate St-01 followed by the isolate St-07. These isolates were sent to Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh for identification up to species level. The isolate St-01 was identified as *Streptomyces albospinus* (MTCC No. 8768); and the isolate St-07 was identified as *Streptomyces somaliensis* (MTCC No. 8769).

Acknowledgements

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