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Isolation and characterisation of bacterial strains with potential cellulolytic activities on different substrates

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Abstract : *The present study was aimed at isolation of cellulose degrading bacterial strains from soil samples and to investigate the optimum pH and temperature for their growth and cellulase activity. Two cellulolytic bacterial strains labelled as Strain-1 and Strain-2 were selected for investigation on the basis of clear zone around their colonies when tested with Congo red. The present study indicated that the selected bacterial strains were capable of growing on a wide range of temperature (4–50°C) and pH (5–11) with 26°C and 7 being the optimum temperature and pH, respectively for the CM Case activity of both the selected strains (0.0259 IU/ml for Strain-1 and 0.0266 IU/ml for Strain-2, respectively). Strain-1 released maximum amount of reducing sugar at 26°C (57µg/ml) and pH 11(64 µg/ml), while Strain-2 released maximum amount of reducing sugar at 37°C (65µg/ml) and pH 11(62µg/ml). The cellulolytic potential of the selected strains was assayed on the locally available untreated and pre-treated cellulosic wastes namely sugarcane bagasse, groundnut shell, corncob, and vegetable peel, under optimum working conditions. Highest cellulase activity was observed in the medium containing acid treated groundnut shell yielding 0.0259 IU/ml for Strain-1 and 0.0244 IU/ml for Strain-2, respectively.*

Key words: *Cellulose degradation, cellulolytic bacterial strains, CMCase activity, pre-treatment of cellulosic wastes*

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Introduction :

Lignocellulose is the most abundant carbohydrate in nature (Lee, 1997) and regarded as the most important renewable resource for bioconversion. Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes (Oberoi *et al*., 2008). The cellulases have great potential in saccharification of lignocellulosics to fermentable sugars which can be used for production of bioethanol, lactic acid, and single cell protein (Maki *et al*., 2009). Cellulase is a multi-enzyme system composed of several enzymes with numerous isozymes that hydrolyzes the β-1,4-glycosidic bonds in the polymer to release glucose units and have a wide range of industrial applications such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bio ethanol production (Bhat, 2000). Cellulases are widely spread in nature, predominantly produced by microorganisms, like molds, fungi and bacteria (Pérez *et al*., 2002). The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria for conversion to fuel. The purpose of the pre-treatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials (Songklanakarin, 2011). The barrier to the production and recovery of valuable materials from lignocellulosic waste is the structure of lignocelluloses which has evolved to resist degradation due to cross linking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages (Yan and Shuya, 2006). Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicelluloses and cellulose from lignocellulosic waste. Alkali (NaOH, $Ca(OH)_2$, NaOH-urea, Na_2CO_3) hydrolyses rice straw (Carrillo *et al*., 2005); spruce wood waste (Zhao *et al*., 2007); sugarcane, cassava, peanuts wastes (Thomsen and Belinda, 2007); corncob (Torre *et al*., 2008); organic fraction of municipal solid waste (Torres and Lloréns, 2008). The biggest obstacle in commercial success of enzyme production is the high cost of raw material used as substrate which could be overcome by resorting to microbial fermentation technology using low valued biological substrates including agro wastes, viz., rice straw, wheat straw, rice bran, wheat bran and baggasse etc. (Nigam and Singh, 1996; Ikram-ul-Haq *et al*., 2006).

The present study was, therefore, an attempt to isolate and characterize cellulose degrading bacterial strains from natural environment and compare its enzymatic activity on locally available untreated and pre-treated cellulosic wastes.

Accordingly, the major objectives were:

- Isolation and purification of bacterial strains from the soil samples
- Screening for efficient cellulolytic strains from the isolates
- Characterization of selected strains on the basis of different biochemical parameters
- Optimization of pH and temperature for growth and cellulolytic activity
- Comparative assessment of cellulolytic potential of the selected strains on the locally available untreated and pre-treated cellulosic wastes namely sugarcane bagasse, groundnut shell, corncob, and vegetable peel

Materials and Methods :

Isolation and screening of cellulose degrading bacterial strains : Compost soil was collected from a depth of 3-4 inches and dried in hot air oven at 55°C for 4 hours and sieved and plated with fivefold dilution on CMC agar (Peptone, 5g; NaCl, 5g; CMC, 3g; Agar, 20g; Distilled water, 1000ml; pH7.0) plates by serial dilution method. The plates were incubated for 48 h at 37°C. The bacterial colonies obtained were purified and screened by Congo red test to visualize the zone of cellulose hydrolysis. The selected strains were

preserved on slants of Nutrient Agar for further investigations with periodic sub culturing.

Morphological and biochemical characterization of the selected strains : For characterization of the selected strains, macro and micro morphology and the biochemical characteristics were studied. Biochemical tests were conducted by the standard protocol (Cappuccino and Sherman, 2005) and the strains were tentatively identified on the basis of characters as given in Bergey's Manual of Systematic Bacteriology (Williams, 1989).

Effect of temperature on growth : The effect of temperature on the growth of the cellulolytic bacterial strains was observed by inoculating the strains on CMC Agar plates and incubating at temperatures 4, 26, 37 and 50°C for 4 days and the observations were recorded.

Effect of pH on growth : The effect of pH on the growth of the cellulolytic bacterial strains was observed by inoculating the strains on CMC Agar plates with pH of the medium adjusted to 5, 7, 9 and 11 using 1N HCl or 1N NaOH as per the requirement and incubating at 37°C for 4 days. The observations were recorded.

Enzyme assay

Preparation of crude enzyme : The bacterial cultures were inoculated separately in the sterilized CMC broth and incubated for 4 days at 26°C for enzymes production. After incubation, the cultures were harvested by centrifugation at 5000 rpm for 10 minutes and the cell free culture supernatants were used as crude enzyme sources.

CMCase assay : CMCase activity was measured by taking a reaction mixture in a test tube containing 0.5 ml of 1% CMC in citrate buffer (pH 6) with 0.5ml of enzyme supernatant filtrate (Ghose, 1987). The reaction mixture was incubated at 50°C for 30 min and to that solution 1 ml of DNS reagent was added and boiled for 5 minutes in boiling water bath. The amount of reducing sugar produced was determined by DNS method (Miller, 1959) by taking absorbance at 540 nm and comparing it with the standard glucose curve. One unit of CMC-ase activity is defined as the amount of enzyme, which released 1 µmole of reducing sugar measured as glucose per min under the assay conditions.

Amount of reducing sugar released : For estimating the amount of reducing sugar (mg/ml) released, 1 ml of culture filtrate and 1 ml of DNS reagent were taken in a test tube, boiled for 5 minutes in boiling water bath. The absorbance was taken at 540 nm and compared with the standard glucose curve (Miller, 1959).

Effect of temperature and pH on CMCase enzyme activity and production of reducing sugar : To study the effect of temperature on enzyme production, tubes of CMC broth (pH 7) inoculated with the selected cellulolytic bacterial strains were incubated at temperatures 4, 26, 37 and 50°C for 4 days for enzyme production. The effect of pH on enzyme production was studied over a range of pH 5-11. Tubes containing CMC broth with specified pH were inoculated with the selected cellulolytic bacterial strains and incubated at 26°C for 4 days for enzyme production. The enzymes produced in both the cases were assayed (Ghose, 1987) and the amount of reducing sugar produced was estimated (Miller, 1959).

Preparation of cellulosic substrates : 1 kg of each of the cellulosic substrates that included sugarcane bagasse, groundnut shell, corncob, and vegetable peels were dipped in 5L of distilled water to remove any soluble sugars present in the substrates and then dried in hot air oven at 80°C for 36 hours. The substrates were then grounded and sieved through 100 micrometer mesh size and kept at room temperature. These untreated substrates were used as sources of cellulose for the enzyme production by the isolated cellulolytic bacterial strains.

Pretreatment of cellulosic substrates : 100g of each of the dried and powdered substrates was treated separately for 24 hours with 500 ml of

each of NaOH and HCl. After treatment, powdered substrates were repeatedly washed with distilled water till neutral pH was obtained. Then the substrates were dried overnight at 60°C. These treated substrates were also used as sources of cellulose to compare the enzyme production by the isolated cellulolytic bacterial strains.

Fermentation medium : Stainer's basal salt medium (pH 7.5) mixed separately with specified cellulosic substrates (10 g/l) was used as fermentation medium for cellulase production.

Cellulase production : The fermentation medium (10 ml) was dispensed in tubes, autoclaved and inoculated with the isolated bacterial strains. The tubes were incubated at 26°C for 4 days for cellulase production. The samples were centrifuged at 5000 rpm for 10 min and the supernatants obtained were used as crude enzymes to determine CMCase activities by the method described above (Ghose, 1987) and reducing sugar released by the method described above (Miller, 1959).

Results and Discussion :

Isolation and screening of cellulose degrading strains : Among the four isolated strains of bacteria screened for their cellulolytic activity using 1% (w/v) Congo red and 1N HCl, two strains labelled as Strain-1 and Strain-2 were selected for investigation on the basis of the visible clear zone of cellulose degradation on the CMC plates (Figure1).

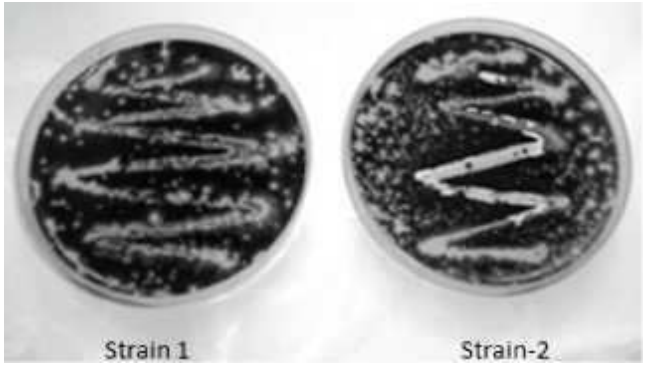

Fig 1. Strains showing clear zone of cellulose degradation on the CMC plates after Congo red test.

Morphology of the isolated cellulose degrading strains : The selected cellulose degraders were critically examined for their macro and micro morphology and the observations are recorded in Table 1.

Table 1. Colony characteristics of Strain-1 and Strain-2 on CMC medium

Sl. No.	Colony Characteristics	Strain-1	Strain-2
1.	Color	Creamish	White
2.	Diffusible Pigment	ND	ND
3.	Texture	Butyrous	Powdery
4.	Gram's staining	Positive	Positive
5.	Shape	Rod	Filamentous

ND = Non-Detectable.

Biochemical characterization of Strain-1 and Strain-2 : Strain-1 and Strain-2 were characterised biochemically and the results are recorded in Table 2.

Table 2. Results of biochemical test of cellulolytic strains Strain-1 and Strain-2

Sl. No.	Biochemical Tests		Strain-1	Strain-2
1.	Indole production		–	–
2.	Methyl red		–	+
3.	Voges-Proskauer		–	–
4.	Citrate utilization		+	+
5.	Amylase production		+	+
6.	Casein hydrolysis		–	–
7.	Fermentation of	a. Glucose	A–, G–	A+, G–
		b. Lactose	A–, G–	A+, G–
		c. Sucrose	A+, G–	A+, G–
8.	Catalase		+	+
9.	Gelatin hydrolysis		–	–

+ = Positive; **–** = Negative; A = Acid production; G **=** Gas production

Effect of temperature on growth of strains : Strain-1 and Strain-2 were grown at the specified temperatures. Strain-1 showed optimal growth only at 26°C whereas Strain-2 showed wider adaptability to different temperatures except 4°C. The data are presented in Table 3.

Table 3. Effect of temperature on the growth of Strain-1 and Strain-2

Sl. No.	Temperature (°C)	Strain-1	Strain-2
1.	4	+	+
2.	26	+++	+++
3.	37	+	+++
4.	50	–	+++

– No growth; + Poor growth; ++ Moderate growth; +++ Luxuriant growth

Effect of pH on growth of strains : Strain-1 and Strain-2 were grown at the specified pH. Both the strains showed luxuriant growth at all the specified pH except pH 5. The data are presented in Table 4.

Table 4. Effect of pH on the growth of Strain-1 and Strain-2 at 26°C

Sl. No.	pH	Strain-1	Strain-2
1.	5	+	+
2.	7	+++	+++
3.	9	+++	+++
4.	11	+++	+++

– No growth; + Poor growth; ++ Moderate growth; +++ Luxuriant

Effect of temperature and pH on CMCase enzyme activity and production of reducing sugar : Effect of temperature on enzyme production was studied and it was found that the CMCase activity was optimum at temperature 26°C for both the strains (0.0259 IU/ml of Strain-1 and 0.0266 IU/ml of Strain-2, respectively). The CMCase activity of Strain-1 was 0.0215 IU/ml, 0.0199 IU/ml and 0.0222 IU/ml at 4, 37 and 50°C, respectively. The same for Strain-2 was 0.0192 IU/ml, 0.0240 IU/ml and 0.0196 IU/ml at 4, 37 and 50°C, respectively. The data presenting the effect of temperature on enzyme production is shown in Figure 2. CMCase activity of Strain-1 and Strain-2 at different pH was studied and it was revealed that alkaline pH (9 and 11) optimized enzyme production in Strain-1 (0.0285 IU/ml) whereas optimum enzyme production (0.0266 IU/ml) in Strain-2 was observed at neutral pH 7. The CMCase activity of Strain-1 was 0.0270 IU/ml and 0.0259 IU/ml at pH 5 and 7, respectively. The same for Strain-2 was 0.0240 IU/ml at pH 5and 9 both; and 0.0244 IU/ml at pH 11, respectively. The data presenting the effect of pH on enzyme production is shown in Figure 3. The amount of reducing sugar released at different temperatures and pH was estimated and it was found that Strain-1 released maximum amount of reducing sugar at 26°C (57µg/ml) and pH 11(64 µg/ml). The amount of reducing sugar released of Strain-1 was 47 µg/ ml, 49 µg/ml and 47 µg/ml at 4, 37 and 50°C, respectively and 58 µg/ml, 57 µg/ml and 60 µg/ml at pH 5, 7 and 9 respectively. Strain-2 released maximum amount of reducing sugar at 37°C (65 µg/ml) and pH 11(62 µg/ml) with 47 µg/ml, 60 µg/ml and 47 µg/ml at 4, 26 and 50°C, respectively and 60 µg/ml at pH 5, 7 and 9. The amount of reducing sugar released at different temperatures and pH are given in Figure 4 and 5, respectively.

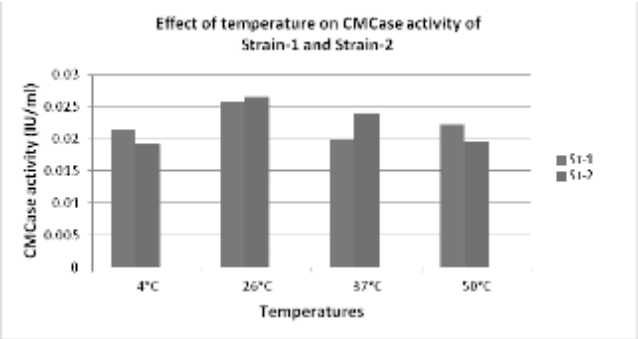

Fig 2. Effect of temperature on CMCase activity of Strain-1 and Strain-2.

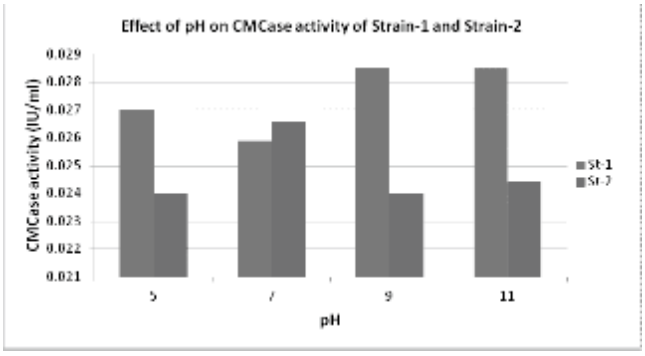

Fig 3. Effect of pH on CMCase activity of Strain-1 and Strain-2

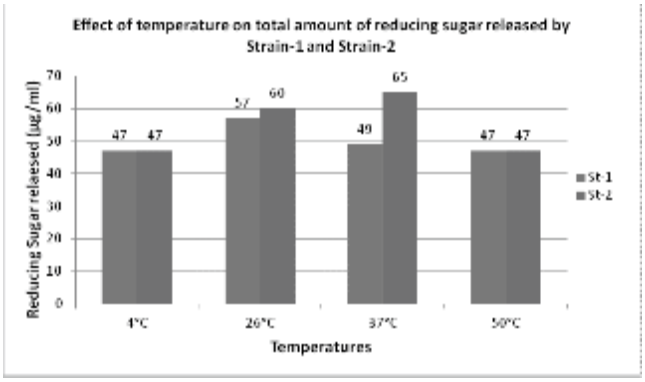

Fig 4. Effect of temperature on the amount of reducing sugar released by Strain-1 and Strain-2

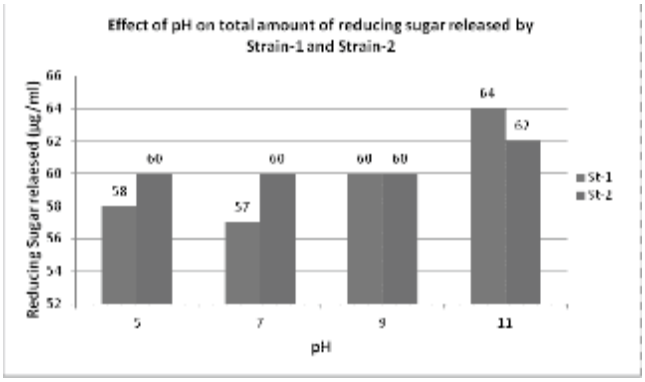

Fig 5. Effect of pH on total amount of reducing sugar released by Strain-1 and Strain-2.

Assay of CMCase activities and estimation of reducing sugar by Strain-1 and Strain-2 using different cellulosic substrates : In untreated condition maximum CMCase activity was observed in vegetable peel for both the strains (0.0163 IU/ml for Strain-1 and 0.0181 IU/ml for Strain-2). After alkali treatment the readings were higher for corncob hydrolysis by Strain-1 (0.1920 IU/ml) and sugarcane bagasse hydrolysis by Strain-2 (0.0215 IU/ml); and after treatment with acid, groundnut shell was found as the most suitable source for enzyme production (0.0259 IU/ml by Strain-1 and 0.0244 IU/ml by Strain-2). Reducing sugar released was maximum in corncob hydrolysis in untreated (53 µg/ml by Strain-1 and 54 µg/ml by Strain-2); NaOH treated (170 µg/ml by Strain-1 and 78 µg/ml by Strain-2) and HCl treated (390 µg/ml by Strain-1 and 410 µg/ml by Strain-2) conditions for both strains. All data regarding assay of CMCase activities and estimation of reducing sugar by Strain-1 and Strain-2 using different cellulosic substrates (untreated and pre-treated) are given in Figures 6-9.

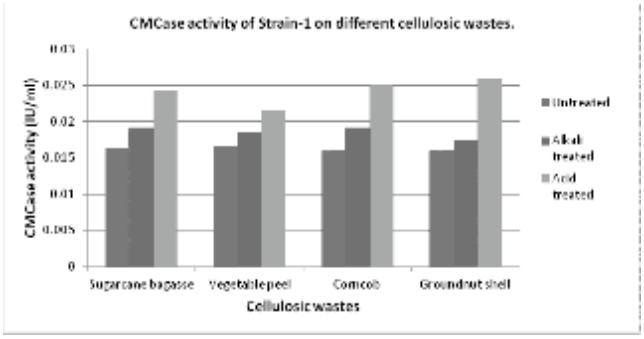

Fig 6. CMCase activity of Strain-1 on different cellulosic wastes.

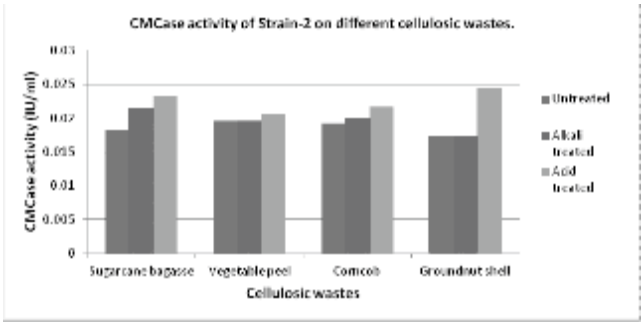

Fig 7. CMCase activity of Strain-2 on different cellulosic wastes.

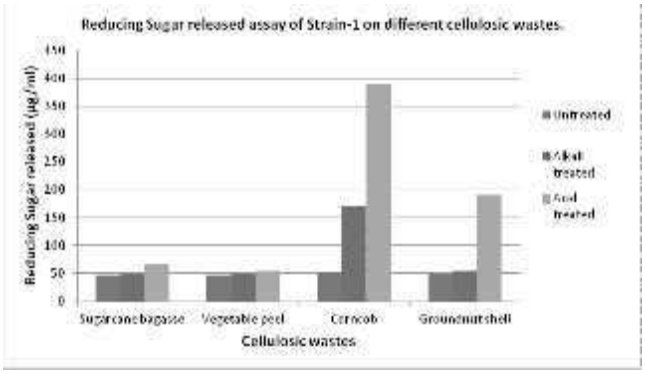

Fig 8. Reducing Sugar released assay of Strain-1 on different cellulosic wastes.

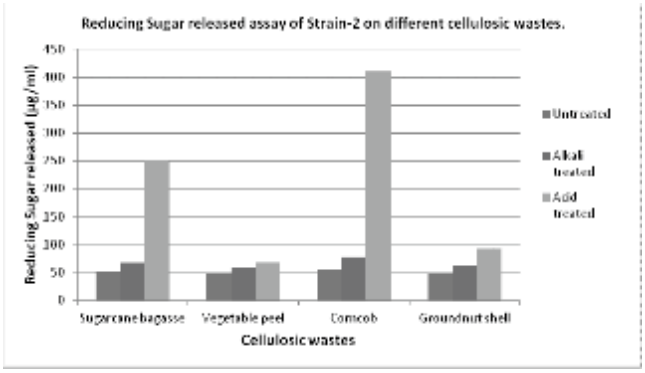

Fig 9. Reducing Sugar released assay of Strain-2 on different cellulosic wastes.

Bacteria are well known agents of decomposition of organic matter in general and of cellulosic substrates in particular (Lynd et al., 2002). Compost was selected as a source for obtaining desirable cellulase producing organisms, because these are rich sources of diverse group of cellulolytic microorganisms. Screening of the cellulolytic bacterial isolates was performed based on the formation of clear zone surrounding the colonies on the CMC plates. On the basis of Gram staining and biochemical characterization and comparing the same with Bergey's Manual of Determinative Bacteriology, Strain-1 was tentatively identified as Bacillus species and Strain-2 as strain of Actinomycetes but for confirmation 16S rRNA sequencing has to be done.

The effect of environmental factors such as temperature and pH are important factors that influence enzyme activities (Cappuccino and Sherman, 2005). The present study indicated that the isolated bacterial strains were capable of growing on a wide range of temperature (4 –50°C) and pH (5–11).

CMCase activity was optimum at temperature 26°C for both the strains. This differed from the findings of Immanuel et al. (2006) who reported 40°C as optimum temperature for endoglucanase activity of Bacillus spp. Reports of Li et al. (2008) and Hirosawa et al. (2006) were also different who reported 50-60°C as optimum temperature for cellulase production by Bacillus subtilis. Alkaline pH (9 and 11) was found to be optimum for enzyme production by Strain-1 which also differed from the reports of Immanuel et al. (2006) who recorded pH 7 as optimum for endoglucanase activity but matched with the report of Song et al. (1985) who observed optimal cellulase production at pH 9 by Clostridium acetobutylium. The optimum enzyme production by Strain-2 was observed at neutral pH 7 which was in accordance with that of the report of Immanuel (2006).

Agricultural residues like corncob, sugarcane bagasse, groundnut shell, vegetable peel etc. represent an abundant, inexpensive and readily available source of renewable lignocellulosic biomass. In the present study, these locally available cellulosic wastes were used as substrates for the enzyme production by the selected strains Strain-1 and Strain-2.

The effect of different substrates on cellulase production was examined and it was found that the highest cellulase activity was found in the medium containing acid treated groundnut shell (0.0259 IU/ml by Strain-1 and 0.0244 IU/ml by Strain-2). The amount of reducing sugar released was found highest in medium containing acid treated corncob

for both the strains. Both the cellulase activity and reducing sugar content were observed higher in treated substrates as compared to untreated substrates. The present study indicated that acid treatment was more effective than alkali treatment in cellulose hydrolysis but still alkali treatment is preferred as its disposal after the treatment is less harmful in comparison to the acid disposal at the commercial level.

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