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Isolation, screening, characterization and optimization of cellulase production of cellulytic bacteria from farm yard manure

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ABSTRACT
The cellulase producing bacteria were isolated from Defence Institute of Bioenergy Research (Haldwani) farm yard manure (FYM) collected. A total of 72 isolates were isolated and screened, of which ten isolates showed measurable hydrolyzing zone on Congo agar media plates. Isolates were tentatively characterized on the basis of morphological and biochemical characteristics. LCDB 10 identified as *Pseudomonas aeruginosa* exhibited the maximum zone of clearance of 4 mm, implying maximum enzyme activity however LCDB 1, LCDB 2, LCDB 4 and LCDB 9 exhibited the minimum zone of clearance i.e. 2 mm respectively. The enzyme assays for two enzymes, filter paper cellulase (FPC) and cellulase were examined. The extracellular cellulase activities ranged from 0.39 to 0.72 U/mL for FPC and 0.2 to 0.40 U/mL for CMC cellulase assay. However the intracellular FPCase activities range from 0.41 to 0.69 U/mL and 0.23 to 0.72 U/mL for CMC cellulase assay. Optimization of different parameters i.e. temperature and pH was carried out for the production of cellulose. The optimum pH and temperature for cellulose activity produced by strain LCDB 10 were pH 9 and 50° C respectively. The enzyme was found to be stable at a wide range of pH ranging from 5-9 for LCDB 10. However for other strains it was found that the cellulase activity was declined with increasing pH.

Keywords: Farm yard manure, cellulose degrading bacteria, cellulase, endoglucanase

Significance statement
Cellulase enzyme has a huge significance, as it presents 8% of the total industrial enzyme demand. Keeping that in mind the present study is conducted to isolate the bacteria from farm yard manure having cellulytic potential by evaluating their cellulase enzyme production activity.

1. INTRODUCTION
Climate change and depleting fuel reserves have caused an increased awareness and potential for renewable fuels like biofuels. The inherent major concern associated with the biofuels is related to the use of materials otherwise exploited as food, with the consequent reduction in food supply and increase in its costs. Lignocellulosic biomasses such as agricultural residues and energy crops are the most abundant material present on earth. Therefore lignocelluloses degradation is important for maintaining the global cycle and help in reducing the cost of crude oil and environmental pollution [1]. The polysaccharide component in lignocellulosic masses includes cellulose and hemicelluloses, which amounts to 60 - 80% of the total system, and remaining solids are predominantly lignins [2], though they may also include extraneous components including extractives and non-extractives.

Understandably, polysaccharides can be fermented to yield ethanol, but these polysaccharides are required to

undergo extensive pre-treatment to remove lignin [3]. Certain microorganisms possess the abilities to hydrolyze all three of cellulose, hemicellulose and lignin [4]. For industrial exploitation of these organisms, these must first be hunted at the habitats where these substrates are present abundantly. About one fifth of fresh water and soil samples yield cellulose degrading bacteria after enrichment [5]. This is due to existence of microenvironments where different growth conditions for cellulose degrading bacteria are present. These bacteria are generally found in 'well-mannured' soils and have been discovered for decades which have capacity to convert cellulose into simple sugars. Despite that, the need for newly isolated cellulose degrading microorganism is still relevant. In this study, several efficient cellulase-producing microorganisms were isolated from FYM. The purpose was to characterize lignocellulosic abilities of all cellulase-positive isolates and identify those isolates that display greatest variety of activity towards lignocellulosic biomass for possibly more practical use.

2. MATERIALS AND METHODS

2.1 Collection, Isolation and screening of samples

The soil samples used in this study was collected from Defence Institute of Bioenergy Research (Haldwani) from farm yard manure. Cellulolytic bacterial strains were isolated from soil by using serial dilutions and pour plate technique. The medium used for isolation of cellulolytic bacteria contained 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$and 0.25 % $(NH_4)_2SO_4$ kept at pH 7.0 for 48 h of incubation at 30˚C [6]. Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at 4ºC for further identification and screening for cellulase production.

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 h, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. NaCl (1M) was thoroughly used for counter-staining the plates. The bacterial colonies having clear degradation zone were selected for identification and cellulase production.

Identification was based on morphological i.e. shape size and gram staining and biochemical tests viz., Methyl red, Voges prausker, Urease, Gelatin hydrolysis, Indole, citrate utilization test, Malonate, Starch hydrolysis, ONPG, Nitrate reduction, Arginine, Trehalose, utilization of Sucrose, Glucose, Mannitol, Arabinose, Fructose, Xylose, Maltose, Raffinose, Lactose, Gram staining, Growth at temperatures 4, 10, 25, 30, 35, 45 and 55 ºC and growth in varied concentrations of NaCl (0.4M, 0.8 M, 1.3 M, 1.7M).

2.2 Cellulase enzyme production

Newly isolated strains were screened for cellulase enzyme production in submerged fermentation process using a modified Mandels medium whose composition was KH_2PO_4 1.5, $Na_2HPO_4 \cdot 7H_2O$ 2.5, $(NH_4)_2SO_4$ 1.5, $MgSO_4 \cdot 7H_2O$ 0.3, $CaCl_2$ 0.1, $FeSO_4 \cdot 7H_2O$ 0.005, $MnSO_4$ 0.0016, $ZnCl_2$ 0.0017, and $CoCl_2$ 0.002 in g/L pH 7.0. The 50 ml of medium was inoculated with 1 ml of selected bacterial isolates and 10 gm/L of CMC-Na incubated in a shaker at 35˚C for 24 h at 140 rpm. The broth was centrifuged at 14000 × g for 10 min at 4˚C to remove the unwanted material and the clear supernatant obtained after centrifugation served as crude enzyme source. Both the extracellular and intracellular enzyme activity was determined. The extracellular enzyme activity was determined by taking the clear supernatant and for intracellular enzyme activity the cells obtained after centrifugation were crushed in liquid nitrogen, centrifuged and supernatant left served as the intracellular enzyme source.

2.3 Estimation of Enzyme assay

Carboxymethyl cellulase (CMCase) and filter paper cellulase (FPCase) activities were determined using 3, 5-dinitrosalicylic acid (DNS) method. 500 μL of enzyme mixture was incubated with 50 mg of Whatman number 1 filter paper (1.0 × 6.0 cm) in 1 mL of 100 Mm of buffer for determining the FPCase activity. The buffer used for dissolving or resuspending the substrates was 100 mM sodium citrate buffer (pH 5.8) and incubated for 1 hr for FPCase assay. Reaction was stopped by adding 3 mL of DNS reagent and heated in boiling water for 5 min for color development and absorbance was measured at 540 nm. One FPU is defined as the quantity (in mg) of reducing sugar liberated in one hour under the standard assay conditions. Reducing sugars produced in one hour was calculated by comparing A_{540} with that of standard curve.

FPU/ml units ml^{-1} = mg glucose released x 0.185 was calculated [7].

The CMCase activity was determined by the colorimetric method as described by Miller [8] using the dinitrosalicylic acid (DNS) reagent. The principle of this method is based on the determination of the colour developed after the reaction between the reducing sugars liberated from cellulose by DNS reagent. Bacterial culture was taken in 2 ml microfuge tubes and centrifuged at 13,000 rpm for 5 min. Sample was prepared by mixing 250 µl of culture supernatant in 50 mM sodium phosphate buffer (pH 7.0) and 250 µl of distilled water. The mixture was incubated at 40˚C in a water bath for 30 min. Similarly the glucose standards (0.2-5 mg of glucose per ml) enzyme blanks, substrate blanks and the spectro zero were prepared. After incubation, 1.5 ml DNS-reagent and the tubes were placed in a boiling water bath for 5 min and allowed to cool. The O.D. of the samples was immediately measured at 540 nm. One enzyme unit was equivalent to 1 µmol of glucose equivalents released per min [8]. Carboxy methyl cellulase activity unit was calculated using the formula-

Carboxy Methyl Cellulase /ml units ml^{-1} = mg glucose released x 0.37 was calculated [9].

2.4 Effect of pH and temperature on activity and stability of crude cellulases

The optimum pH for the crude enzyme was determined by incubating crude enzyme prepared in appropriate buffers 50 mM acetate buffer (for pH 3.0 and 4.0), 50 mM Tris-base buffer (for pH 6.0) and 50 mM sodium phosphate buffer (for pH 8.0-10.0). Crude enzyme mixture in those buffers was incubated for 30 min at 50˚C. Cellulase activity was assayed by DNS method. The pH stability was determined by incubating crude enzyme mixture in above-mentioned buffers at room temperature for 30 min and enzyme stability was determined by using DNS method.

The effect of temperature on activity of cellulase activity was determined by incubating crude enzyme in sodium citrate buffer (pH 5.8) at temperatures between 30 to 60 °C. Enzyme activity was assayed by DNS method at different temperatures as described above.

2.5 Statistical analysis

The program CropStat for Windows (7.2.2007.2 module), developed by the Biometrics unit, IRRI, Philippines was used for analysis of variance (ANOVA) of experiments laid out in Completely Randomized Design (CRD). The treatment means were compared by using least significant difference test (LSD) at a significance level of $P \leq 0.05$.

3. RESULTS AND DISCUSSION

A total of 72 isolates were isolated from FYM, of which, ten isolates showed cellulose degrading activity using CMC agar medium. Clear zones appeared around growing bacterial colonies indicating cellulose hydrolysis. Ten bacterial isolates giving measurable halo zones in CMC agar medium after Congo red staining (Figure 1) were selected and named as lignocelluloses degrading bacteria (LCDB) and numbered 1 to 10 (LCDB 1-10). The hydrolyzing zone diameter and colony diameter are listed in Table 1. The diameter of maximum zone of clearance was found in LCDB-10 i.e. 4 mm and minimum was found to be of 2 mm. Congo red has been widely used for the screening of cellulose degrading microorganisms. In the present study some strains showed a good detectable large hydrolyzing zone but the activities of FPAase and CMCase was found to be lower suggesting that either the concentration of enzyme produced by the strains was very low or the ability of the strain to secrete CMCase was weak. Sadhu and Maiti and Liang et al also reported that the diameter of hydrolyzing does not truly reflect the real cellulase activity [10, 11].

Soil provides ideal growth conditions for cellulose degrading bacteria. Present research is focused on to the isolation characterization of bacterial isolates from the soil sample for cellulase enzyme production and also determining the optimum conditions required for maximum cellulase activity. Researchers studying on cellulolytic activity have isolated various bacteria from different environmental sources like soils from forest or farming land to that of cities, intestine/gut of animals, etc. [6, 9, 12-18].

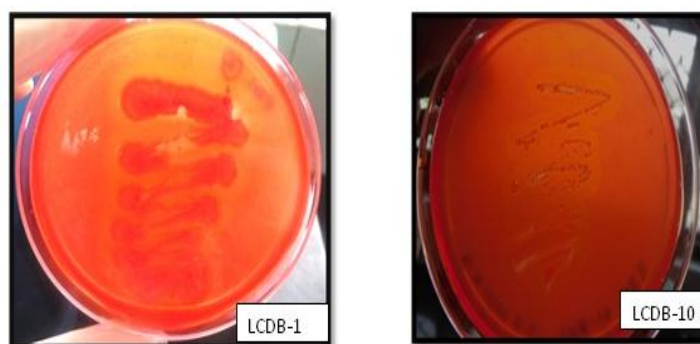

Figure 1: Congo red staining of isolates LCDB 1 and LCDB 10

Table 1: Diameter of clearance zone after Congo red staining of isolated bacteria.

Isolated strain labeled as	Zone of clearance (-growth); mm	Temp (˚C)	Time (h)	O.D at 620 at 30ºC after 24 hr
LCDB-1	2	35	24	0.615
LCDB-2	2	35	24	0.505
LCDB-3	3	35	24	0.505
LCDB-4	2	35	24	0.333
LCDB-5	3	35	48	0.212
LCDB-6	3	35	24	0.409
LCDB-7	3	35	24	0.388
LCDB-8	3	35	24	0.306
LCDB-9	2	35	24	0.743
LCDB-10	4	35	24	0.246

Table 2: Biochemical characterization of the isolates showing cellulose activity.

TEST	MICROBES ISOLATED									
	LCDB1	**LCDB2**	**LCDB3**	**LCDB4**	**LCDB5**	**LCDB6**	**LCDB7**	**LCDB8**	**LCDB9**	**LCDB10**
Methyl red	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
V.P Test	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
Urease	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve
Malonate	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
Citrate utilization	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
Starch hydrolysis	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
ONPG	-ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve
Nitrate reduction	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
Arginine	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Trehalose		+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
Utilization of carbon source										
SUCROSE	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve
GLUCOSE	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
MANNITOL	-ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
ARABINOSE	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	-ve
FRUCTOSE	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
XYLOSE	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve
MALTOSE	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
RAFFINOSE	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve
LACTOSE	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
Growth at temp										
4°C	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10°C	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
25°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
35°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
45°C	+ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve
55°C	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
Growth at NaCl										
2.5%	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
5%	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
7.5%	-ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
10%	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Gram staining	Negative	negative	negative	positive	negative	negative	negative	negative	negative	negative

Different microorganism could be determined based on their morphological and biochemical characteristics. Biochemical tests were performed for all the isolated bacteria. Different isolates gave different result for the parameters investigated i.e., colony characteristics, shape, size, Gram's reaction, catalase production, urease production, Voges-Proskauer (V-P) reaction, Nitrate reduction, citrate utilization, carbohydrate metabolism (acid-gas production), starch hydrolysis and growth at different pH and temperature, etc. (Table 2). All the isolates were gram negative except LCDB-4 which was found to be gram positive (Figure 2 and Table 2). On the basis of their morphological biochemical characters (Table 2), LCDB-1 to LCDB-10 have been identified as *Proteus vulgaris*, *Citrobacter freundii, Xanthomonas* sp., *Bacillus cereus, Citrobacter amalonaticus, Citrobacter diversus, Citrobacter freundii, Salmonella* sp., *Enterobacter cloaceae* and *Pseudomonas aeruginosa* respectively.

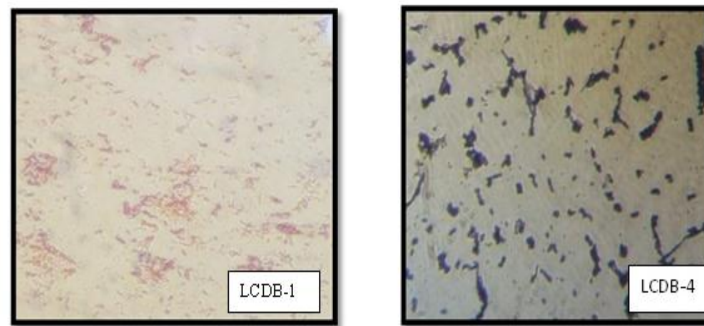

Figure 2: Gram staining of isolates LCDB 1 and LCDB 4.

3.1 Enzyme activity of isolated bacterial strains:

Cellulases are one of the most widely used enzymes required for the preparation of fermented foods and used in paper, pulp and textile industries. Cellulose, main building blocks of plants have major fraction of organic carbon in soil and microorganisms are accountable for recycling this organic carbon to the environment through the degradation of cellulosic materials [19]. As the diameter of the clearing zone does not reflect the true enzyme activity all the isolates were screened again by calorimetric method to determine the most potent isolates for FPCase and CMCase production.

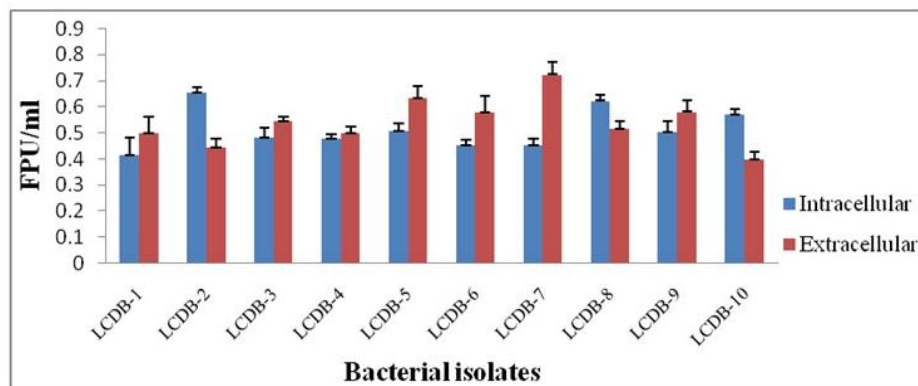

Figure 3: Extracellular and intracellular endoglucanase enzyme activity of isolated cellulolytic bacteria.

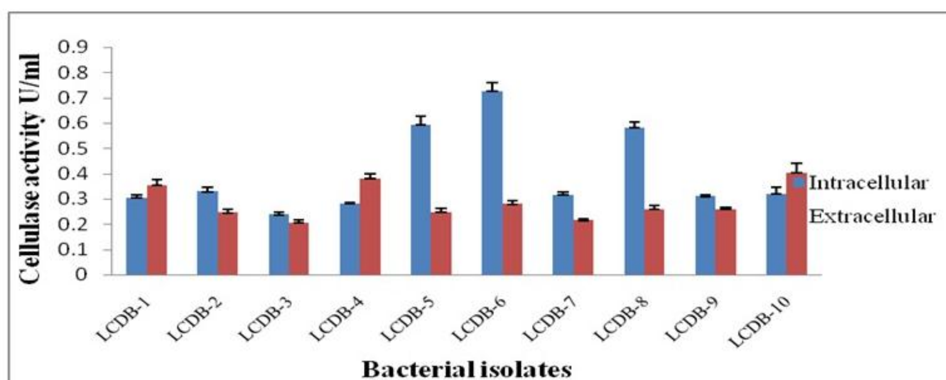

Figure 4: Extracellular and intracellular exoglucanase enzyme activity of isolated cellulolytic bacteria.

A total of ten isolates were checked for their enzyme production and cellulytic activity. FPCase for the isolated cellulolytic strains was analyzed using Whatmann filter paper No.1 as substrate, due to its ready availability, low cost and higher acceptability in scientific community [20]. The filter paper degradation was observed separately in all the isolates, as shown in Figure 3. Extracellular FPCase activity was found to be highest in *Citrobacter freundii* (0.72 U/ml) and minimum in *Pseudomonas aeruginosa* (0.39 U/ml) while intracellular filter paper cellulase acitivity was highest in *Citrobacter freundii* (0.65 U/ml) and lowest in *Proteus vulgaris* (0.41 U/ml) (Figure 3). All cellulolytic strains produced cellulase using CMC as substrate. A comparison of enzyme activities among the strains revealed that maximum extracellular cellulytic activity was determined to be in *Pseudomonas aeruginosa* and minimum in *Xanthomonas* sp. i.e., 0.40 U/ml/min and 0.20 CMC/ml/min respectively. However intracellular activity was higher in *Citrobacter diversus* (0.72 U/ml) and lowest in *Xanthomonas* sp. (0.23 U/ml) (Figure 4).

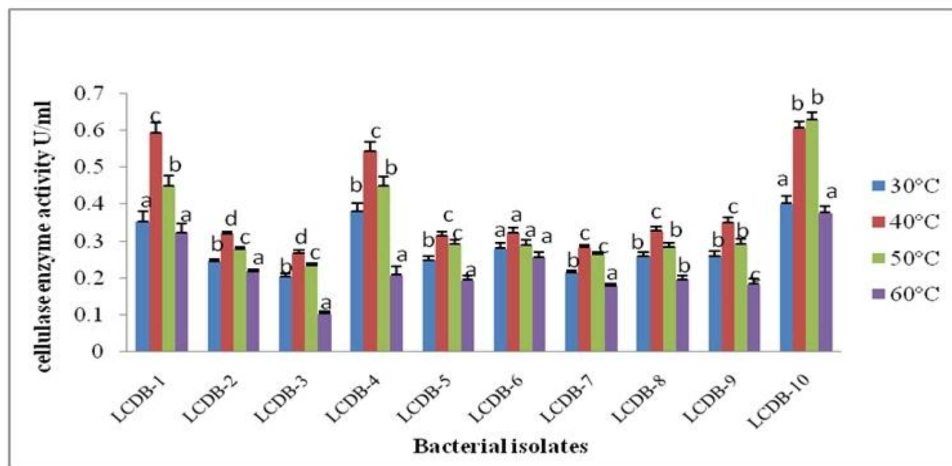

Figure 5: Cellulase enzyme activity of isolated cellulolytic bacteria at different temperature. (Values indicated by the same letter are not significantly different at $P \leq 0.05$)

3.2 Effect of pH and temperature on extracellular cellulase enzyme activity:

Cellulase enzyme production yields appear to depend on a complex relationship involving a variety of factors like substrate, substrate concentration, temperature, pH, incubation period, inorganic ions, etc. and vary from species to species [5]. Large scale production of cellulase for commercial purposes requires identification of high yielding bacterial isolates and optimization of process conditions. Optimum temperature for the cellulase activity was determined by measuring the released glucose molecules by cellulase enzyme through DNS method at temperatures 30-60 °C and was found that increasing temperature up to 40°C also increased enzyme activity significantly but further increasing the temperature caused decline in enzyme activity except in *Pseudomonas aeruginosa* whose activity was increased up to 50°C (Figure 5). The optimum temperature of crude cellulase of the present isolates (LCDB 1-9) was 40°C. *Pseudomonas aeruginosa* showed higher activity at 50°C with no significant difference at temperature 40 and 50°C. Cellulase activities increase with increasing temperature up to 40°C. Maximum enzyme activity was recorded at this temperature however further increasing the temperature caused decline in enzyme activity. One possible reason for this might be that the enzyme rapidly denatured with increased temperature.

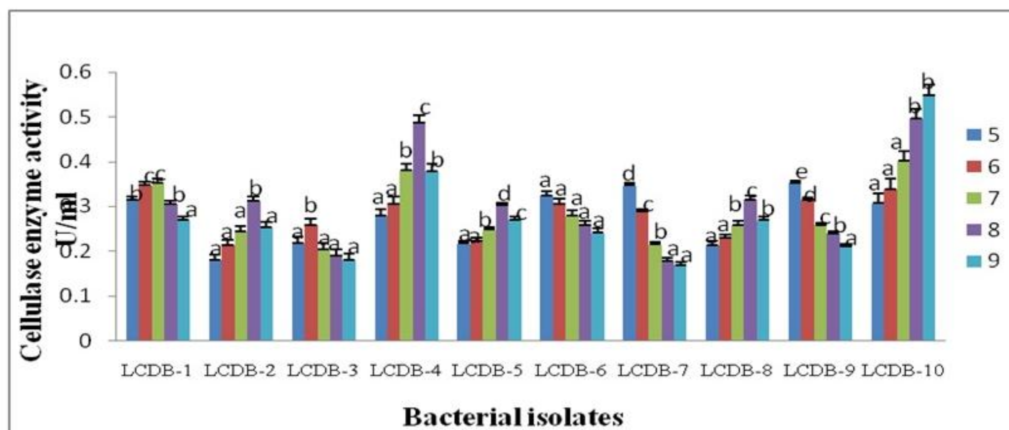

Figure 6: Cellulase enzyme activity of isolated cellulolytic bacteria at different pH (Values indicated by the same letter are not significantly different at $P \leq 0.05$)

Carbohydrate digestive enzymes usually work best at near to the neutral pH or under slightly alkaline conditions. Any change in pH can potentially cause changes in the enzyme active site [6]. Appropriate pH conditions promote the growth of strains and enhance the cellulase yield. This may be related to the negative feedback mechanism of enzymes. Effect of pH on the crude CMCase activity of isolates was examined at various pH ranging from pH 5.0 to 9.0 (Figure 6). *Bacillus cereus* and *Pseudomonas aeruginosa* showed maximum enzyme activity. Minimum enzyme activity was found to be at pH 5. The optimum pH for cellulase enzyme activity of *Bacillus cereus* is pH 8 while *Pseudomonas aeruginosa* showed linear increase in enzyme activity up to pH 9. In *Citrobacter diversus, Citrobacter freundii* and *Enterobacter cloaceae* increasing the pH from 5-9 there was a linear decline in enzyme activity (Figure 6). All the bacterial isolates gave different response at different pH in response to their cellulase enzyme activity.

4. CONCLUSION

The development of rapid and reliable methods for the screening of cellulases from microorganisms will allow a greater number of novel bacterial cellulases to be isolated with purpose for industrial use. Several microorganisms capable of converting cellulose into simple carbohydrates had been discovered for decades. However, demands from the enzyme industry for newly isolated cellulolytic microbes which can better convert cellulose in to value added products still active and there lies the importance of this study. The bacterial isolates showing potential to convert cellulose into reducing sugar could be used in many applications like production of many value added products and also be used for fermentation for the production of bioethanol.

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Conflict of Interest

Authors have no conflict of interests.

Compliance with Ethical Standards

All procedures performed in were in accordance with the ethical standards of the institutional and/or national research committee.

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