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RESEARCH ARTICLE

CELLULASE PRODUCTION FROM NEWLY ISOLATED BACTERIAL STRAINS FROM LOCAL HABITAT

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ABSTRACT

The study aimed at isolating and screening cellulolytic bacteria from local habitats for the production of cellulase. A number of cellulolytic bacteria were isolated from soil, melon and gut of herbivore (goat) by applying serial dilutions and culture methods. Two of the isolates, CB-2 from soil and CB-3 from melon produced sufficient amounts of cellulase on screening and were further investigated upon. Applying different biochemical tests, CB-2 and CB-3 identified as *Bacillus subtilis* were used for the flask-scale production of cellulase through submerged fermentation. A parametric study was also conducted to evaluate the optimized culture conditions such as temperature, pH, concentration of the carbon source, nitrogen source and inoculum size and concentration of the carbon source for maximum enzymatic yield. Results revealed the highest cellulolytic activity (CMCase) with 120.321 U/ml and FPase activity with 1.076U/ml by CB-2 strain followed by CB-3. Optimum temperature and pH of the medium for cellulase production was 37.5°C, pH and 9 respectively, with 2% untreated cotton stalk as carbon source, yeast as organic nitrogen source and ammonium sulphate as inorganic nitrogen source with 3% inoculum size. By applying ANOVA, a significant difference ($p<0.05$) among values was found.

INTRODUCTION

Cellulose is the earth's most abundant natural biopolymer. It is present as the basic structural component of plants, compositionally making up 30-35% of the total biomass. 150 billion tons of dry carbon is suggested to be produced through photosynthesis, 50% of which is cellulose **(**Abo-State *et al.,* 2010; Abadie and Renee, 2008**).** In order to exploit and make use of this cellulose, the presence of cellulolytic enzymes, produced mainly by microbial strains, is necessary for converting it into simpler units (Bakare *et al.,* 2005**).** Microorganisms such as fungi and bacteria are the main contributory organisms for the production of cellulase enzyme. Fungi have been found to exhibit efficient cellulase activities, but there is an increasing interest in the production of cellulase through bacteria. This is attributed to their faster growth rates (Shaikh *et al.,* 2013). Among the various types of cellulase, bacterial extracellular cellulases are the most significant as compared to the ones found in protozoans, viruses and fungi (Lonsane and Ramesh 2011). Moreover, bacterial species present an attractive potential for the exploitation of cellulase and hemicellulase due to their rapid growth rate, enzyme complexity and extreme habitat variability (Maki *et al*., 2009). For many years, cellulose degrading bacteria have been isolated and characterized from a variety of sources such as soil, decayed plant materials, hot springs, organic matters, faeces of ruminants and composts (Doi, 2008). The celluloytic properties of certain bacterial species viz *Cellulomonas spp*, *Pseudomonas spp*, *Bacillus spp* and *Micrococcus* have also been reported (Nakamura and Kappamura, 1982). Furthermore, there is evidence suggesting that cellulase is inducible and can be produced from different carbon sources.

Pakistan is primarily an agricultural country with the potential to produce enormous cellulosic biomass. To exploit this resource, the production of cellulolytic enzymes from microbial strains is a vital factor. Due to the complexity of enzymatic systems, as well as an immense industrial potential, bacterial cellulases are being feverishly explored.

The present study was designed for the isolation and identification of novel bacterial strains from local habitats, which could easily acclimatize to industrial conditions for cellulase production. Moreover, the study was focused on screening of bacterial isolates for cellulase activity, optimization and evaluation of culture conditions for maximum enzyme yield.

MATERIALS AND METHODS

Sample collection

Samples were collected from various locations for the isolation of cellulolytic bacterial strains. Soil samples were collected in sterilized plastic bags from the gardens of PCSIR Labs Complex, Ferozepur Road, Lahore. They were collected from areas where abundant amounts of leaf litter was in the process of decomposition. Melon samples were collected from the waste piles at fruit mandi, Bhatak, Qanchi Lahore. Goat gut samples were collected from the slaughter houses of Lahore.

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Isolation and purification of bacterial strains

The bacterial strains isolated from the collected samples were subjected to serial dilutions under sterilized conditions. The medium used for the isolation of the cellulolytic bacterial strains had the following composition (g/l): CMC, 10; Tryptone, 2; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4$, 0.2; $CaCl_2$, 0.001; $FeSO_4$, 0.004, agar, 15 and pH adjusted to 7 (Ariffin *et al*., 2006). One ml of culture was taken from the first five serial dilutions of all the samples. This was streaked onto plates in triplicate under sterilized conditions. These plates were incubated at 37°C in an incubator (EYLA, Japan) for 3 to 5 days. The bacterial colonies obtained were purified by repeated streaking method and preserved at 4ºC for further screening.

Secondary screening and identification of bacterial strains

Bacterial suspensions of the isolated strains labeled CB-1, CB-2 from soil, CB-3, CB-4 from melon and CB-5, CB-6 and CB-7 from the gut samples were made using sterilized water (10^{12}/ml). One ml of each bacterial suspension was poured in the bores made on the plates. The medium used was 1% sterilized CMC, and the plates were incubated at 37ºC for 72 h. The incubated plates were thereafter flooded with 1% Congo red solution for 20 min and then washed with 1N NaCl solution, in order to observe any cellulolytic activity. The formation of a zone of hydrolysis was indicative of cellulose degradation. The bacterial strains which showed larger zones were selected for identification and flask scale submerged fermentation. Identification of the isolated strains was done through gram's staining, motility and biochemical tests, as described by Bergey's Manual of Determinative Bacteriology (1994).

Flask study of isolated bacterial strains

The best zone producing strains (CB-2 and CB-3) were screened by conducting flask-scale submerged fermentation. The fermentation medium was composed of (g/l): 2 K_2HPO_4, 0.2 $MgSO_4$, 10 peptone, 2 $(NH_4)_2SO_4$, 0.001 $CaCl_2$, 0.004 $FeSO_4$, and 20 cotton stalks as the carbon source. The medium was autoclaved at 121°C for 15 min prior to use. The flasks were inoculated with 3% bacterial suspension having a cell distribution of 10^{12}/ml. The inoculated flasks were then incubated at 37.5ºC in a rotary shaker at 140 rpm. After incubation, the clear centrifugal supernatant obtained was used for the determination of any enzymatic activity.

Optimization of fermentation parameters

Parameters such as the temperature (25, 27.5, 30, 32.5, 35, 35.7 and 40°C), pH (6, 7, 8.0, 9.0, 10, 11 and 12.0), inoculum size (1, 2, 3, 4 and 5%) organic nitrogen source (peptone, yeast extract, urea) and inorganic nitrogen source (ammonium chloride, ammonium sulphate and ammonium nitrate) were optimized in order to increase the yield of cellulase enzyme by CB-2 and CB-3.

Evaluation of various plant biomass samples as carbon sources

Different samples of plant biomass, such as Sarkanda (*Saccharum spontaneous*), Kallar grass (*Leptochloa fusca*), and Cotton Stalks (*Gossypium hirsutum*) were evaluated to determine their potential as substrates or carbon sources for the production of enzymatic cellulase.

Evaluation of enzyme activity

Carboxymethyl cellulase (CMCase) activity

For determining CMCase activity in the culture filtrate, 0.5 ml of crude enzyme sample was incubated with 1ml CMC (0.05 M Citrate buffer, pH 5) at 50°C for 30 min. The reaction was then halted by the addition of 1.5 ml of DNS for 10 min in a boiling water bath. The reaction mixture was allowed to cool and the reducing sugars released were estimated through Miller's Method (1959). One unit of enzyme activity was defined as the amount of release of one micro mole of reducing sugars equivalent to glucose under the assay conditions.

Cellulase activity through filter paper assay (FPase) activity

For estimating FPase activity, 500µl of the culture filtrate was added to a test tube containing a strip of Whatman No.1 filter paper (1 x 6 cm). The test tube was incubated at 50°C for 30 min. 1.5 ml of DNS were then added, and after 10 min of boiling, the absorbance was measured at 550 nm. The reducing sugars liberated were estimated using Miller's Method (1959). One international unit of cellulase activity was defined as the amount of enzyme that forms 1µmol glucose (reducing sugars as glucose) per minute during the hydrolysis reaction.

One Way Analysis of Variance (ANOVA)

ANOVA was applied using Statistical Package for Social Science (SPSS), for examining any significant differences within various conditions. Three replicates were used for each condition. A significant difference of $p<0.05$ was found.

RESULTS AND DISCUSSION

The seven isolated strains, two (CB-1, CB-2) from soil, two (CB-3, CB-4) from melon and three (CB-5, CB-6, CB-7) from goat gut waste, were screened for cellulase production on CMC agar plates (Table 1). CB-2 (soil) and CB-3 (melon) were found to give maximum zones of hydrolysis. They were further characterized by using morphological, culture, biochemical and gram staining techniques according to Bergey's Manual (Table 2). Both the strains were identified as *Bacillus subtilis*. It has been reported that soil microorganisms are responsible for the recycling of organic carbon in the environment (Wang *et al*., 2008). A habitat containing rich cellulosic substrates would thus be the best source for the isolation of such cellulolytic microorganisms (Das *et al*., 2010). In the present study, the strain CB-2 was abundantly covered with plant biomass and cellulose. Cellulose is the main component of plant cell walls and is a major source of organic carbon in the soil. Maximum cellulose hydrolysis was observed on CMC plates (Fig. 1). In another study, cellulolytic bacteria were isolated from forest and farm soils, and their ability to decompose cellulose was determined (Hatami *et al*., 2008).

Table 1 Zone of hydrolysis of newly isolated strains

Isolated Strains	Zone of Hydrolysis (mm)
CB-1	3.50
CB-2	12.80
CB -3	10.40
CB -4	4.0
CB -5	2.50
CB -6	3.60
CB -7	2.75

This study supports the isolation of cellulolytic strains from soil. The researcher used cow dung to isolate eight strains of bacteria. The bacteria that produced the maximum amount of cellulase were found to be the *Bacillus sp.* (Das *et al*., 2010). Research for isolating potential cellulase producing microorganisms from diverse habitats is still ongoing (Ray *et al*., 2007).

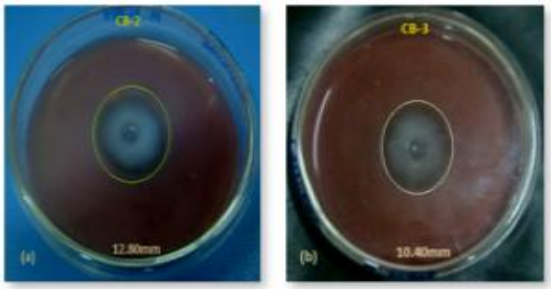

Figure 1 Isolated bacterial strain CB-2 (a) and CB-3 (b) zone of hydrolysis on CMC agar

Table 2 Biochemical characterization of isolated strains CB-2 and CB-3 from soil and melon

Characteristics	CB-2	CB-3
Morphology	Rod	Rod
Gram staining	+	+
Motility	+	+
Glucose	+	+
Arabinose	+	+
Maltose	-	-
Mannitol	+	+
Xylose	-	-
Sucrose	+	+
Lactose	+	+
Galactose	-	-
Citrate utilization	+	+
Urease	–	–
Catalase	+	+
Indole	–	–
VP	+	+
Oxidase	+	+

The effects of using different carbon sources (cotton stalk, kallar grass and sarkanda) on cellulase productivity were also evaluated. Results showed that the isolated strains (CB-2 and CB-3) exhibited different responses, and the maximum units of enzyme activity were found to be on cotton stalk. CB-2 gave CMCase 72.77 U/ml and FPase 0.421 U/ml, and CB-3 gave CMCase 48.405 U/ml and FPase 0.231 U/ml (Fig. 2). Different types of plant biomass were also evaluated as potential carbon substrates. It has been reported in earlier studies that agro-industrial residues, namely rice bran, rice straw, sugarcane, baggase and wheat bran, could be used as substrates for cellulase production. Results revealed that *Bacillus subtilis* (CBTK 106), *Bacillus subtilis* (BL62) and *Bacillus pumilus* exhibited maximum cellulase productivity when wheat bran, banana fruit stalk and soybean were added to the production media (Heck *et al*., 2002, Poorna and Prema, 2007). Many other researchers have also reported studies on the use of lignocellulosic material as a source of carbon during the production of cellulase (Ojumu *et al*., 2003). Agricultural lignocellulosic wastes have been used as sources of carbohydrates to produce various important products, including enzymes, ethanol, glucose and single cell protein (Solomon *et al*., 1999). Cotton stalk was found to be the richest source of organic carbon, and microorganisms produced the greatest amount of cellulase in its presence. However, the concentration of carbon was a vital factor, and it was found that CB-2 gave maximum units of CMCase (102.342 U/ml) and FPase (1.076 U/ml). CB-3 gave CMCase (89.901U/ml) and FPase (0.2920 U/ml) units at 2% substrate concentration (Table 3). Previous studies [thesis] have depicted that the optimum carbon concentration for obtaining maximum cellulase yield is 1%. This differed from the results of this study, according to which the highest yield was obtained at 2% substrate concentration.

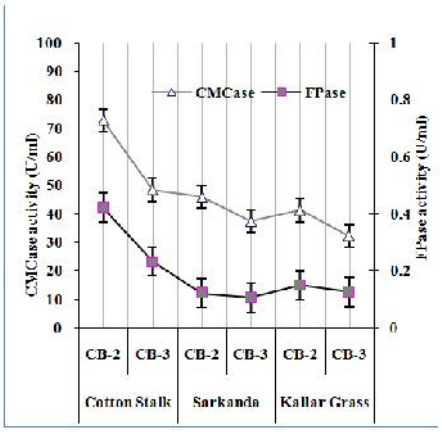

Figure 2 Study on plant biomasses to produce cellulase from the newly isolated CB-2 and CB-3 strains.

Table 3 Effect of various concentrations of cotton stalk in submerged fermentation to produce cellulase at 37.5^{o}C for 3 days at 140 rpm by CB-2 and CB-3.

Cotton stalk concentration (%)	Cellulase Enzyme (U/ml)			
	Strain CB-2		Strain CB-3	
	CMCase	FPase	CMCase	FPase
1	48.302±0.73	0.282±0.1	35.191±0.2	0.195±0.1
1.5	73.861±0.98	0.614±0.1	58.090±0.3	0.581±0.1
2	102.342±1.0	1.076±0.1	89.901±0.6	0.920±0.1
2.5	80.102±0.73	0.921±0.1	62.281±0.2	0.518±0.1
3	67.921±0.53	0.391±122	41.501±0.2	0.451±0.1

Values are the average of three replicates. ± denotes the standard deviation among triplicates

Temperature plays a vital role in the metabolism of the microorganism. The significant effects of changes in temperature from optimum line on enzyme productivity were thus investigated. The effect of temperature changes on cellulase productivity indicated that both the strains gave maximum units of CMCase and FPase at 37.5^{o}C (Fig. 3). Both CB-2 and CB-3 also showed optimum growth and enzyme productivity at 37.5^{o}C. Similar results have been reported for the growth of *Bacillus subtilis* at 30-37^{o}C (Shabeb *et al*., 2010). Other studies have reported that maximum cellulase productivity was achieved at 40^{o}C by lactic acid bacteria, namely *Bacillus amyloliquefaciens* UMAS 1002, *Arachnoitus sp.* and *Bacillus* strain DLG (Robson and Chambliss, 1984; Ahmed *et al*., 2004, Nakasaki and Adachi, 2003). Similar reports have also been described for Bacillus sp, *Clostridium cellulyticum* and *Pseudomonas flourescens* (Heck *et al*., 2002; Bakare *et al*., 2005).

It is generally necessary to optimize the size of inoculums. A low inoculum density gives insufficient biomass, and if the density is high, excessive biomass is produced. This results in the depletion of nutrients from the media,

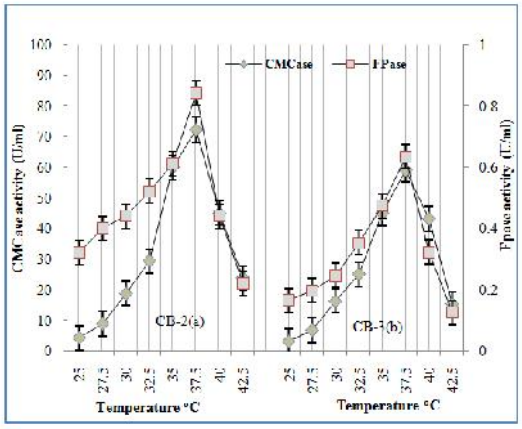

Figure 3(a), (b) Effect of various temperatures in submerged fermentation to produce cellulase at 140 rpm for 3 days by

adversely affecting cellulase enzyme production. This study indicated that both CB-2 and CB-3 gave maximum units of enzyme activity at 3% inoculum size (CMCase 75.451 U/ml and FPase 0.845 U/ml, CMCase 65.247 U/ml and FPase 0.781 U/ml, respectively) (Fig. 4). These findings coincided with earlier work in which maximum cellulase production by two cellulolytic *Bacillus sp.* was reported at similar inoculums sizes (Taleb *et al*., 2009). Another research reported that the enzyme production increased gradually with an increase in inoculum size, but decreased beyond 3% (Ray *et al*., 2007). Shaikh *et al.*, 2013 also reported that the maximum cellulase activity of newly isolated strains CDB27 (*Pseudomonas sp*) and CDB30 (*Bacillus sp*), was achieved at 2% inoculum size.

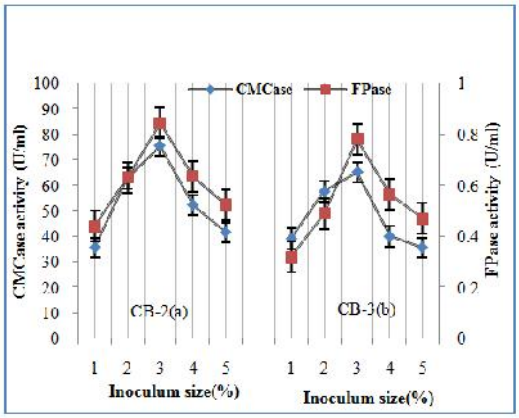

Figure 4 (a), (b) Effect of various inoculum sizes in submerged fermentation to produce cellulase at 37.5°C for 3 days at 140 rpm by CB-2 and CB-3

The optimization of working parameters such as carbon source, cellulose quality, pH value, temperature, inducers, medium additives, aeration, is important in the production of cellulase (Immanuel *et al*., 2006). Juhasz *et al.*, 2004 reported that among all process parameters, pH was of major interest for cellulase production. The metabolic activities of the microorganisms were found to be sensitive to changes in pH. The optimum pH at which maximum enzyme units were obtained was 9 for both strains (CB-2 and CB-3). However, further changes in pH showed a decrease in enzymatic yield (Fig. 5). These results were similar to the findings reported regarding *Bacillus* species by Kim *et al*., (2005). These bacteria are known to be alkalophilic, hence they grow maximally in alkaline conditions (Denizci *et al.*, 2004; Johnvesly *et al.*, 2002).

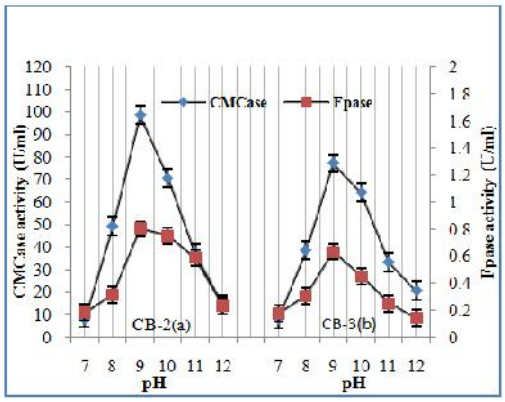

Figure 5(a), (b) Study at various pH values by submerged fermentation to produce cellulase at 37.5°C for 3 days at 140 rpm by CB-2 and CB-3

Nitrogen is an important nutrient for the growth of microorganisms. In this study, both organic and inorganic nitrogen sources were used and optimized. Results revealed that CB-2 (120.321 U/ml CMCase and 0.981 U/ml FPase) and CB-3 (89.309 U/ml CMCase and 0.891 U/ml FPase) gave maximum units of enzyme in medium containing 0.2% ammonium sulphate as the inorganic nitrogen source (Table 4). Moreover, the results were similar to the findings of many other researches. These studies elaborated that *Bacillus pumillus*, *Ruminococcus albus*, *Bacillus sp*., *Bacillus sp*.B21 and *Streptomyces sp*. BRC2 showed maximum cellulase productivity when ammonium sulphate was added to the production media (Kotchoni *et al*., 2003; Heck *et al*., 2002; Poorna and Prema, 2007; Chellapandi and Himanshu, 2008). Kalaiselvi and Jayalakshmi (2013) also reported that various organic and inorganic nitrogen sources coupled with peptone gave maximum enzyme activity, when followed by the addition of ammonium sulphate.

Table 4 Effect of organic and inorganic nitrogen sources on the cellulase production by CB-2 (It is not CB-20) and CB-3

Organic Nitrogen	**Cellulase Enzyme (U/ml)**			
	Strain CB-2		**Strain CB-3**	
	CMCase	**FPase**	**CMCase**	**FPase**
Peptone	65.450±0.33	0.821±0.1	48.812±0.3	0.721±0.1
Urea	59.960±0.22	0.612±0.1	46.942±0.4	0.751±0.1
Yeast extract	80.920±0.53	0.951±0.1	71.808±0.5	0.821±0.1

Inorganic Nitrogen	**Cellulase Enzyme (U/ml)**			
	Strain CB-2		**Strain CB-3**	
	CMCase	FPase	CMCase	FPase
NH^4Cl	68.581±1.10	0.752±0.1	48.008±1.2	0.581±0.1
(NH4)2SO4	120.321±1.2	0.981±0.1	89.309±1.3	0.891±0.1
NH4NO3	59.210±1.03	0.551±0.1	41.981±1.0	0.409±0.1

Values are the average of three replicates. ± denotes the standard deviation among triplicates

CONCLUSION

The CB-2 and CB-3 isolates from soil and melon were identified as *Bacillus subtilis.* Their cultures have produced the highest level of cellulolytic activity at 37.5^{o}C, pH 9 with 2% untreated cotton stalk as carbon source, yeast as organic nitrogen source and ammonium sulphate as inorganic nitrogen source with 3% inoculum size. Of the two strains, CB-2 was considered to be the better enzyme producing strain.

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