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Partial characterization of Cellulase Production by *Jeotgalibacillus marinus* MTCC 6233

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Cellulases form a very important group of enzymes which find application in a wide array of processes including production of bioethanol from lignocellulosic materials, biopulping, textile processing, as additives in animal feeds and laundry detergents etc. In the present research investigation, *Jeotgalibacillus marinus* MTCC 6233 was isolated from decomposing cellulosic waste material. The isolate was further screened and characterized for production of cellulases. The bacterial strain was found to be a potent producer of endoglucanase, exoglucanase as well as β –glucosidase. Highest production of all the three cellulolytic enzymes was observed at 6th day of incubation. Activity of all the three enzymes was found to be optimum at pH 6 and temperature 50^{0} C. Endoglucanase activity was found to be inhibited by 2,4-Di Nitro Phenol (2,4-DNP) and $HgCl_2$. Thus, the isolate *Jeotgalibacillus marinus* MTCC 6233 can be further assessed for these enzymatic activities and may prove to be a potential candidate for production of various cellulolytic enzymes at commercial scale.

Key words: *Jeotgalibacillus marinus;* cellulose; endoglucanase (EC 3.2.1.4); exoglucanase (EC 3.2.1.94); β –glucosidase **.**

The cellulase system of microorganisms is considered to comprise of three hydrolytic enzymes (i) the endo-(1, 4)-β-D-glucanase (synonyms : endoglucanase, endocellulase, carboxymethyl cellulase [EC 3.2.1.4]), which cleaves β-linkages at random, commonly in the amorphous parts of cellulose; (ii) the exo-(1, 4)-β-D glucanase (synonyms : cellobiohydrolases, exocellulase, microcrystalline cellulase, avicelase [EC 3.2.1.91]), which releases cellobiose from either the nonreducing or the reducing end, generally from crystalline part of cellulose; and (iii) the β-glucosidase (synonym : cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short chain oligosaccharides (Bhat and Bhat, 1997).

Cellulase enzymes have been reported to be quite useful for bioconversion of lignocellulosics to for production of energy, food, useful chemicals (Solomon *et al.*, 1999; Dinesh *et al.*, 1989). Cellulolytic enzymes are produced by wide variety of bacteria and fungi, aerobes and anaerobes, mesophiles and thermophiles (Coughlan and Ljungdah, 1988; Ljungdahl and Eriksson, 1985). However relatively few fungi and bacteria produced high level of extracellular cellulases capable of solubilizing crystalline cellulose extensively (Wood, 1975; Wood, 1985; Johnson *et al*., 1982).

Bacteria, especially the members of *Bacillus* spp., are reported to produce and secrete

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large quantities of extracellular enzymes which has placed them amongst the most important industrial enzymes producers (Schallmey *et al.*, 2004). This study reports *Jeotgalibacillus marinus* MTCC 6233 as producer of cellulase enzymes on synthetic cellulose and cellulose derivatives.

MATERIAL AND METHODS

Organism

Bacterial strain was isolated from decomposing organic cellulosic waste. Bacteria were isolated by enrichment method (Hans and Srinivasan, 1968) and maintained on Nutrient Agar Medium (NAM) slants at 4^0C. Bacterial Identification was done by Microbial Type Culture Collection(MTCC), Institute of Microbial Technology(IMTECH) Chandigarh based on epigenetic characters.

Screening of Cellulases

Cellulase (endoglucanase) activity is determined by method of Teather and Wood (1982) with slight modification. In this method, Mandels and Reese agar medium supplemented with CMC (1.2% w/v) was streaked with bacterial test organism and incubated at 37±2°C for 48 hrs. After incubation period, 0.5% Congo red was taken to reveal pale reddish zone around the bacterial colony. The zone around the bacterial colony was measured in mm. A control consisting of Mandels and Reese agar medium without CMC was also run simultaneously with the test.

CMCase (β–1→ 4 glucanase) activity of the test organism was evaluated using Fenske Ostwald Viscometer (efflux time for water = 15 sec). Mandel and Reese broth medium supplemented with CMC (1.2 % w/v) was prepared and 25ml of this medium was dispensed in 150 ml of flask and autoclaved at 15 psi for 15 min. The flask was then inoculated with 2 ml of test bacterial suspension in nutrient broth which gave the absorbance of 1.2 at 660 nm in spectrophotometer. The flasks were then incubated in BOD incubator at 37 ± 2^0C for 2 days. After incubation, aliquot (5 ml) is taken and centrifuged at 9500 rpm for 20 min. The supernatant obtained after centrifugation is used for viscometric analysis. Reaction mixture consisted of CMC solution (1.2% w/v), citrate buffer (pH 4.8) and crude enzyme of test organism in the ratio of 4:1:2. The mixture was poured into the small arm and efflux time (ET_O) was immediately determined. Viscometer was then kept at water bath at 50^0C. The efflux time was then determined after 60 min of incubation. A control consisting of enzyme, boiled at 100^0C, was also run simultaneously with the test. Efflux time of reaction mixture was taken after 60 min and percent loss in viscosity of CMC is calculated using the formula of Capelline (1966).

Inoculum Preparation

The bacterial inoculum consisted of 2 ml of 24 hour old bacterial culture grown on nutrient broth giving absorbance of 1.2 at 660 nm.

Production medium and Preparation of Enzyme sample

Extracellular cellulases complex viz. endoglucanase, exoglucanase and â -glucosidase activity of bacterial cultures were determined (Mandels and Reese, 1957). The Production medium for test organism for endoglucanase activity contained Carboxy Methyl Cellulose (CMC), while the production medium for exoglucanase and β–1→4 glucasidase was the modified Mandel and Reese medium containing cellulose powder as sole source of carbon in place of CMC (Ozaki and Ito, 1991). The composition of Mandel and Reese medium was as follows (g/L): Proteose peptone : 1.0; $(NH_4)_2SO_4$: 1.4; KH_2PO_4 : 2.0; NH_2-CO-NH_2 : 0.3; $MgSO_4.7H_2O$: 0.3; $CaCl_2$: 0.3; $FeSO_4.7H_2O$: 0.005; $MnSO_4.H_2O$: 0.0016; $ZnCl_2$: 0.0017; Carbon source (CMC or cellulose powder) : 10 ; Distilled water : 1000 ml; pH : 5.3. 25 ml of Mandels and Reese broth was dispensed in each 150 ml of flask. All the flasks were then sterilized at 15 psi for 15 minutes. Bacterial inoculum was added in Mandels and Reese broth containing CMC and cellulose powder separately. The experiment was done in duplicates and inoculated flasks were incubated at 37 ± 1^0C for bacteria in an incubator under stationary condition. 5ml aliquots were removed periodically from flasks under aseptic condition after 2^{nd}, 4^{th}, 6^{th} day of incubation. The samples were filtered through Whatmann filter paper no. 1 and the filtrate obtained was centrifuged at 9500 rpm for 20 minutes. The supernatant obtained was used as enzymatic source.

Cellulase Enzyme Assay

Endoglucanase and exoglucanases activity was measured in terms of International Unit (IU), which is equivalent to micromoles (µm) of glucose released min^{-1} ml^{-1}(Mandels, 1974)

Effect of pH on Activity of Cellulase Enzymes

The effect of pH was determined by measuring activity at standard assay over a pH range 3-10. Substrates solutions containing CMC (1%) for endoglucanase and salicin (1%) for â-glucosidase activity were prepared in three buffers *viz*. citrate buffer (pH 3-5), Potassium Phosphate buffer (6-8), and Borax-NaOH buffer (9-10). Exoglucanase activity was also assayed in buffers of different pH 3-10 with filter paper of 1X6 cm as substrate. Enzyme of test organism was mixed with substrate and cellulase assay was performed. Cellulase activity was determined in IU ml^{-1}.

Effect of Temperature on Activity of Cellulase Enzymes

The enzyme activities were determined by carrying out the standard assays at various temperatures between 30-90^{0}C at optimum pH obtained. Cellulase activity was determined in IU ml^{-1}.

Effect of Chemical Reagents on Endoglucanase Activity

The effect of various chemical reagents was evaluated on the endoglucanase activity of fungal and bacterial cultures as per the method of Ozaki and Ito (1991) with slight modification. EDTA, 2,4-DNP ,$HgCl_2$ and $SnCl_2$ solutions of three concentration *viz*. 0.1mM, 1mM and 5mM were prepared. The crude enzyme (1ml) of test organism and each chemical reagent (1 ml) were mixed and incubated at 30^{0}C for 30 min. and an aliquot (0.5 ml) was used for the assay. The endoglucanase activity was measured in IU ml^{-1}. A control was run consist of enzyme without reagent.

RESULTS AND DISCUSSION

In the present investigation bacterial strain was isolated from decomposing cellulosic waste and after purification was screened for production of cellulases. The isolate produced 12 mm zone of hydrolysis in Congo red test. It also showed 93% loss in viscosity in viscometric test. So, both the preliminary tests indicated positive result for cellulose production. The isolate showed appreciable growth (26 mm) on synthetic cellulosic substrate (Carboxy Methyl Cellulose) but relatively less growth on cellulose powder (1.7 mm) on 6th day of incubation (Table-1). The bacterial strain was further identified through epigenetic characters by MTCC, IMTECH Chandigarh and microorganism identified to be *Jeotgalibacillus marinus* MTCC 6233. Result of Biochemical characterization has been depicted in Table 2. The genus *Bacillus* is a heterogeneous collection of aerobic or facultative anaerobic endospore-forming bacteria that are ubiquitous in many environments. The sequencing of 16S rDNA led to the identification of five phylogenetically distinct groups within the genus including *Jeotgallibacillus*, *Marinibacillus* (Yoon *et al*., 2010). *Bacillus* species are phenotypically and genotypically heterogeneous (Claus and Berkeley, 1986). Several workers have also reported various members of *Bacillus* as promising sources of cellulolytic enzymes viz. *Bacillus subtilis* CK-2 (Aa *et al*., 1994), *Bacillus* HR 68 (Mawadza *et al.*, 1996), *B. sphaericus* JS1 (Singh *et al*., 2004), *Bacillus* and *Geobacillus* strains (Rastogi *et al.*, 2010) and *B. subtilis* (Deka *et al.*, 2011).

The cellulolytic activity of *Jeotgalibacillus marinus* MTCC 6233 was assessed with respect to endoglucanase, exoglucanase and β-glucosidase. In *Jeotgalibacillus marinus* MTCC 6233 maximum endo-, exoglucanase and b-glucasidase activity was observed on 6th day of incubation (Table-3). The extracellular cellulase systems consist of

Table 1. Screening of Cellulolytic Bacterial Culture

Bacterial Cultures	Isolate Number	Congo red Test (in mm)	Viscometric Test (% loss in viscosity)	Growth on Different Carbon Source					
				Carboxy Methyl Cellulose (CMC) (in mm)			Cellulose Powder (in mm)		
				2nd day	4th day	6th day	2nd day	4th day	6th day
Jeotgalibacillus marinus	O1	12	93	20	25	26	1	1.5	1.7

Table 2. Morphological and Biochemical Characterization of *Jeotgalibacillus marinus* MTCC 6233

Characters	Isolate no (O1)
Morphological and Cultural Characteristics	
Gram Staining	Positive
Cell Shape/ form	Rods
Cell size	1.0-1.4 X 1.4-2.1 µm
Density	Opaque
Colony Shape	ND
Colony Margin	Irregular
Colony Elevation	Convex
Colony Structure/ Configration	Lobate
Colony Colour	White creamish
Pigments	Nil
Motility	-
Endospore Staining	+
Flourescence	-
Growth on NaCl	
2%	+
5%	+
7%	+
9%	+
Growth on pH	
5	+
8	+
9	+
Growth on Temperature	
4^0C	-
15^0C	-
25^0C	+
37^0C	+
42^0C	+
50^0C	-
Anaerobic Growth	-
Pigments	Nil
Mac Conkey Agar Growth	-
Catalase	+
Oxidase	+
Indole	-
Methyl Red	-
Voges Prosekeur	-
Citrate	-
Growth on Furozolidone Agar	-
Starch	-
Casein	+
Gelatin	+
Oxidation Fermentation (OF)	O
Nitrate reduction	-
Urease	-
Hydrogen Sulphide	-
Acid Production From	
Arabinose	-
Xylose	-
Rhamnose	-
Galactose	-
Glucose	+
Salicin	-
Lactose	-
Sucrose	-
Raffinose	-
Inositol	-
Mannitol	+
Identified Bacteria	*Jeotgalibacillus marinus*

Jeotgalibacillus marinus Accession No. 6233 has been confirmed by Microbial Type Culture Collection and Gene Bank (MTCC) at Institute of Microbial Technology, (IMTECH), Chandigarh, India

endoglucanases, exoglucanases and β-glucosidase in free and bound form (Wood, 1992). In comparison to fungi, most bacteria produce mainly endoglucanase enzymes capable of solubilizing amorphous and swollen cellulose (Wood, 1985). In the present investigation also the endoglucanase activity was found to be higher (0.606 $IUml^{-1}$) as compared to exoglucanase (0.101 FPU ml^{-1})and b-glucosidase (0.067 $IUml^{-1}$). It is a generally accepted view that both bacteria and fungi are subjected to an inducer - repressor system with catabolite repression involved in the regulation mechanism of cellulase systems (Lao and Wilson, 1994). In catabolite repression the end products of cellulose hydrolysis interact with the cellular proteins to form a complex which interact with particular gene at the transcription level and represses cellulase synthesis (Lewin, 1987). The proof for catabolite repression lies in the fact that no cellulase is produced during the growth of a microorganism on glucose, glycerol and other carbon sources related to glycolytic metabolism (Bhat and Bhat, 1997).

The cellulase activity in fungi and bacteria is greatly dependent on factors like pH, temperature, incubation period, nutritional salt, substrates concentration etc. The results related to the impact of pH on activity of cellulolytic enzymes of *Jeotgalibacillus marinus* MTCC 6233 is presented in Fig. 1. Endoglucanase activity was observed at a broader pH range of 4-9 with pH 6 showing the highest value. In case of exoglucanase and β-glucosidase, the activities were found to be there in the pH range of 4-8 and

3-8 respectively, with optimum activities at pH 5 and 6 respectively. It has been suggested by many workers that pH may affect the velocity of reaction by changing the attraction between enzyme and substrate or products (Gascoigne and Gascoigne, 1960). The effect of different pH on the velocity of enzymatic reaction is mainly due to changed affinity of enzyme with its substrate, pH effects the ionization state of reaction mixture.

Temperature is another important parameter for enzyme activity as lower temperature retard and higher temperature often inactivates the enzyme proteins by denaturing. The cellulase enzymes of bacterial culture were subjected to different temperature range to evaluate optimum temperature for cellulolytic activities. The data obtained from the experiment are depicted in Fig 2. It is evident from the results that *Jeotgalibacillus marinus* MTCC 6233 has very narrow range of temperature tolerance for endoglucanase, exoglucanase and β-glucosidase activity i.e. from 30-50^{0}C. The maximum activity of all the three enzymes was observed at 50^{0}C. Any further increase in the temperature was found to be detrimental for the cellulase activity. The optimum temperature of an enzyme varies with factors such as enzyme purity, substrate, presence of activator or inhibitors, its usefulness for the purpose of characterizing enzyme is limited However, Ozaki and Ito (1991) have reported the optimum pH 5.2 and 45^{0}C temperature for cellulase activity in *Bacillus* sp. KSM-330. Robson and Chambliss (1984) observed optimum pH 4.8 and temperature 58^{0}C for cellulase activity in *Bacillus* isolate. The capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers (Deka *et al*., 2011).

Table 3. Cellulase Enzymes of *Jeotgalibacillus marinus* MTCC 6233

Incubation time (in days)	Endoglucanase Activity (CMCase)	Exoglucanase Activity (FPase)	β-glucosidase Activity
	(IU ml^{-1})	(FPU ml^{-1})	(IU ml^{-1})
2	0.190	0.044	0.048
4	0.404	0.086	0.065
6	0.606	0.101	0.067

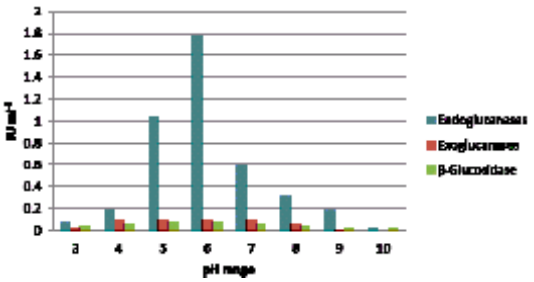

Fig. 1. Effect of pH range on Activity of Cellulase Enzymes of *Jeotgallibacillus marinus* MTCC 6233

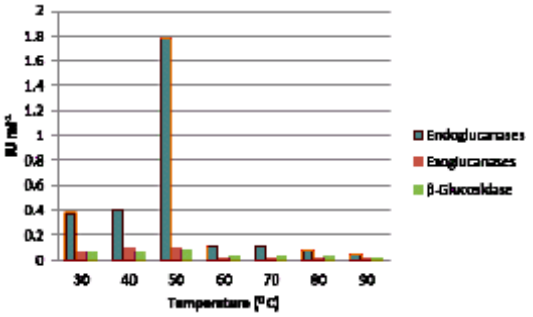

Fig. 2. Effect of Temperature range on Activity of Cellulase Enzymes of *Jeotgallibacillus marinus* MTCC 6233

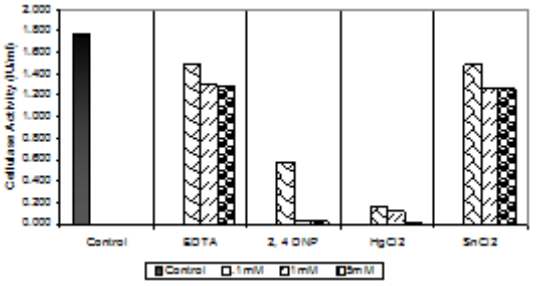

Fig. 3. Effect of Chemical Reagents on Endoglucanase Activity of *Jeotgallibacillus marinus* MTCC 6233

The effect of chemical reagents viz. EDTA, 2, 4- DNP, $HgCl_2$ and $SnCl_2$ on the endoglucanase activity *Jeotgalibacillus marinus* MTCC 6233 was also assessed (Fig. 3). 2, 4- DNP and $HgCl_2$ were found to severely inhibit the enzyme activity. The lower concentration (0.1 mM) of 2, 4 DNP reduced the endoglucanase activity up to 67% and the higher concentration (5 mM) was found to inhibit more than 95% of the enzyme activity. Similarly in case of 0.1 mM and 5mM $HgCl_2$ solution, 95% and 100% inhibition of endoglucanase activity was observed. Previously also, there have been several attempts to identify the active sites of endoglucanases by chemical modification as well as inhibition studies (Dinur *et al.,* 1986). Chelator EDTA was found to affect endoglucanase activity in *Jeotgalibacillus marinus* MTCC 6233 moderately at 5 mM concentration (not more than 45% inhibition). The results probably suggest that although EDTA is a metal chelator, it is either not chelating a possible divalent(s) required for endoglucanase activity or chelating the ion(s) to a lesser extent. Robson and Chambliss (1984) reported that EDTA at concentration 50 mM inhibited only 10 to 20% of TNP-CMC degrading activity in *Bacillus* sp. DLG and concluded that these compounds were not chelating cations required TNP-CMC degrading activity. Kotchoni *et al*. (2003) also reported 20% inhibition in endoglucanase activity at 1 mM of EDTA in wild strain of *Bacillus pumillus* BpCRI6. However in our finding 27.08% of endoglucanase activity was inhibited in *Jeotgalibacillus marinus* MTCC 6233 at the same concentration. The most notable inhibition was caused by 2,4- DNP and $HgCl_2$ in *Jeotgalibacillus marinus* MTCC 6233 inhibited almost complete endoglucanase activity at 5 mM concentration. 2,4 -DNP has been reported as an uncoupler for oxidative phosphorylation (Ali and Sayed, 1992) indicating that a series of phosphorylation steps may play a crucial role in signaling transduction leading to cellulase synthesis. 2,4 DNP is a phenolic compound and phenol compound are well known repressor of cellulases enzymes (Varadi, 1972).

Metal shows both stimulatory as well as inhibitory effect on microorganisms. Although some ions like mercury and silver are required in very minute concentration, they tend to become are toxic even at very small concentrations. In our results $HgCl_2$ was found to inhibit almost complete endoglucanase activity in the test organism. The sensitivity toward $HgCl_2$ may be attributed to the interactions of Hg^{2+} with trypthophan residue(s) at the active site of endoglucanase as suggested by Eriksson and Pettersson (1968) and Ozaki and Ito (1991). $SnCl_2$ also moderately inhibited the endoglucanase activity at high concentration. $SnCl_2$ is the member of p block, group IV metals (De, 1999). Lead, which is also the member of same block, was found to be inhibiting cellulase activity in *Trichoderma reesei* (Kim et al., 2001). $SnCl_2$ is also heavy metal like lead and probably might be inhibiting cellulase activity due to non-specific salt formation.

CONCLUSIONS

In the present finding, *Jeotgalibacillus marinus* MTCC 6233 exhibited relatively broad pH range and narrow temperature range for endoglucanase, exoglucanase and b-glucosidase activities, with maximum enzymatic activities at pH 6 and 50^0C. Although members of *Bacillus* sp.are known to produce an array of enzymes, this is the first ever report about the cellulolytic property of *Jeotgalibacillus marinus* MTCC 6233.

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