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REGULAR ARTICLE

CHARACTERIZATION OF A *BOSEA* SP. STRAIN SF5 (MTCC 10045) ISOLATED FROM COMPOST SOIL CAPABLE OF PRODUCING CELLULASE

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ABSTRACT

A cellulase producing bacterium, designated SF5 was isolated from compost soil. The strain was identified as *Bosea* sp. based on 16S rRNA gene sequence analysis and phenotypic characters including detail carbon sources utilization pattern. The effect of various carbohydrates such as Carboxy Methyl Cellulose (CMC) avicel, starch, maltose, sucrose, glucose, fructose and lactose (as carbon source) on cellulase production revealed that 0.75% CMC (with 8 days incubation) was optimum. Among the various nitrogen sources, 0.15% NH_4Cl gave optimal production of cellulase. The optimal conditions for the production of cellulase by strain SF5 were determined to be at 37 ºC temperature and at pH 7.0. The strain is also capable of producing xylanase and may have biotechnological potential.

Keywords: CMCase, Avicelase, FPase, β-glucosidase, *Bosea* sp.

INTRODUCTION

Cellulose is the most common abundant, renewable biopolymer on earth and domestic waste materials from agriculture representing about 1.5 x 10^{12} tons of the total annual biomass production through photosynthesis in the topics **(Klemm *et al*., 2002; Bhat, 2000)**. A promising strategy for efficient utilization of this renewable and inexhaustible resource is the microbial hydrolysis of it for bioconversion to value-added bi-products. During composting, the capacity of microorganisms to assimilate organic matter depends on their ability to produce the enzymes needed for degradation of lingocellulosic substrate **(Tuomela *et al*., 2000).** Enzymatic hydrolysis of cellulosic materials in compost could be accomplished through complex enzymes known as cellulase.

Cellulases are used in the textile industry for cotton softening; in the detergent market for colour care and cleaning; in the food industry for mashing; and in the pulp and paper industries for drainage improvement and fiber modification. The cellulase enzymes account for a significant share of the world enzyme market now. The cellulase is expected to expand dramatically when cellulases are used to hydrolyze pretreated cellulosic materials to sugars, which can be fermented to bioethanol and biobased products on large scale **(Cherry and Fidanstef, 2003)**.

Cellulose is commonly degraded by the hydrolytic action of a multicomponent enzyme system called cellulase. The enzymatic hydrolysis requires synergistic action of cellobiohydrolase or exoglucanase (E.C.3.2.1.91), endoglucanase or Carboxymethylcellulase (E.C. 3.2.1.4) and Cellobiase or β glucosidase (E.C.3.2.1.21) **(Bhat, 2000).**

Cellulase research has been concentrated mostly in fungi but there is increasing interest in cellulase production by bacteria due to their higher growth rate and thermostable and alkali stable properties.**(Miranda *et al*., 2009)**. The cellulase producing bacteria have been reported by different scientists such as *Thermomonospora curvata* **(Stutzenberger, 1971)**, *Clostridium* sp. **(Lee and Blackburn, 1975)**, *Streptomyces* sp. AT7 **(Amira *et al*., 1989)**, *Streptomyces* sp. F2621 **(Tuncer *et al*., 2004)**, *Streptomyces* BRC1 and BRC2 **(Chellapandi and Himanshu, 2008)**, *Micrococcus roseus***(Paul and Varma, 1993),** *Microbacterium sp.***(Sangrila *et al*., 2011),** *Micromonospora chalcae***(Gallagher *et al*., 1996)**, *Thermomonospora fusca***(Spiridonov and Wilson, 1998)** , *Cellulomonas flavigena***(Rajoka, 2004)**, *Anoxybacillus flavithermus* EHP1 **(Ibrahim and Ahmed, 2007),** *Acinetobacter anitratus***,** *Branhamella* sp.**(Ekperigin, 2007),** *Bacillus licheniformis* and *Bacillus* sp. **(Acharya and Chaudhary, 2011).**

The present paper characterized and identified the isolated SF5 strain from compost soil of Agricultural Farm, Burdwan University capable to produce cellulase. Attempts were made to investigate how the different media ingredient and cultural conditions would be influenced on enhancing cellulase yield.

MATERIAL AND METHODS

Bacterial strains

The bacterial strains were isolated from compost soil which mainly composed of cow dung and residual part of paddy straw near Farm Yard Manure production site of Agricultural Farm, Burdwan University, and West Bengal, India. Approximately, 1 g of soil was diluted with 100 ml sterile distilled water and homogenized by constant shaking. The sample was inoculated into Omeliansky's agar medium containing Carboxymethyl cellulose (CMC) and incubated. The cellulase activity was determined in carboxymethyl cellulose (CMC) agar plates treating with Congo red and NaCl **(Teather and Wood, 1982)**. After primary screening whose zone were appeared largest have selected for secondary screening in broth culture. According to potential cellulolytic activity in cell-free culture supernatant SF5 have been selected for high CMCase activity.

Culture medium and conditions of growth

The Omeliansky's medium (1902) was used for bacterial growth [(in grams per liter), $(NH_4)_2SO_4$, 1.0; K_2HPO_4, 1.0; $MgSO_4.7\ H_2O$, 0.5; NaCl traces; carboxymethyl cellulose (CMC) 1% for cellulase or xylan 1 % for xylanase; pH 7.0 ± 0.2) (Omeliansky, 1902). Two loops of SF5 strain was inoculated into test tubes containing 5 ml of sterile water and mixed well by cyclo-mixer. Thereafter, 0.5 ml of culture was inoculated into 100 ml Erlenmeyer flask containing 20 ml of production medium with various saccharides. The culture was rotated (220 rpm) in a rotary shaker at 37ºC for various periods. Samples were removed after 8 d and centrifuged. The supernatants were analyzed after centrifugation as crude enzyme preparation.

Scanning electron microscopic (SEM) study of the bacteria

The bacteria are grown overnight in Omeliansky's medium and washed with phosphate buffer (0.2 M, pH 7.2) and fixed with 2% glutaraldehyde After centrifugation the pellet of the bacteria was dehydrated with different concentration of ethanol up to 100%, washed with acetone and makes it ready for grid preparation to enable the study of bacteria under Scanning Electron Microscope (model no. HITACHI S-530).

Phenotypic studies

Phenotypic studies such as colony morphology, Gram staining, some biochemical tests were carried out as per standard methods **(Benson, 1990).**

BIOLOG Method

Oxidation of different substrates was determined using the Biolog Microlog system (release 4.2). Suspension of active bacterial culture (Grown on TSA at 25 C° for 72 hours) of defined density (as per manufacture's instruction) was inoculated into microtitre plate. An inoculum of 150 µl was dispensed into Biolog GN2 Microplates using a multichannel micropipette. The oxidation of various carbon sources as indicated by reduction of tetrazolium violet dye that results in production of purple color was monitored and recorded by using a Biolog Microstation that is coupled to a computer. The latter has release 4.2 software for comparison of various patterns of oxidation as per Biolog database.

DNA Extraction and molecular phylogenetic analyses using 16S rRNA gene Sequence

DNA was extracted as described by **Shivaji *et al.* (1989).** PCR amplification of the 16S rRNA gene was carried out using universal primers 8–27f and 1492r as described by **Pandey *et al.* (2002).** Amplified PCR product was purified and sequenced as described by **Suresh *et al.* (2006)**. A continuous stretch of 1,451-nucleotide long gene sequences of 16S rRNA gene was used to search for similar sequences from RDP database Release 10 http://rdp.cme.msu.edu/) using various online tools (CLASSIFIER, SEQMATCH). After confirmation of generic affiliation, sequences from type strains of different species were

retrieved from GenBank. All these sequences were aligned by CLUSTAL_X programme **(Thompson et al., 1997)** and edited manually. Similarity values were determined after pair wise alignment by CLUSTAL_X programme followed by manual calculation. A phylogenetic tree showing relationship between SF5 and other reference strains was constructed by neighbor-joining (NJ) method **(Saitou and Nei, 1987)** with **Jukes and Cantor (1969)** correction, using TREECON software for Windows **(Van de Peer and Wachter, 1997).**

Enzyme assay

The filter paper (FPase) activity of cellulase was measured as described by **Ghosh (1987).** The CMCase activity was assayed based on that of **Miller (1959)**. 0.5 ml of culture supernatant fluid was incubated with 0.5 ml of 1% CMC in 0.05 M sodium acetate buffer, pH 4.8 at 40ºC for 1 h. Avicelase and β-glucosidase activity were determined under similar conditions, except that 1% avicel and 1% salicin were used as substrate. The reduced sugar released was analyzed using the dinitrosalicylic (DNS) method, using glucose as the standard sugar. A unit of enzyme activity was defined as the amount required producing 1µmole of reducing sugars per min. The specific activity is the number of units of enzyme activity per milligram of enzyme protein. The soluble protein concentration was determined by method of **Lowry *et al.* (1951)** using BSA as standard .

Statistical Analysis

Values are the mean ± SEM of 3 replicates. All data were subjected to students t- test analysis with significance level of $P<0.05$ using SPSS soft were package.

RESULTS AND DISCUSSION

The cellulase producing SF5 strain was isolated from compost soil. The strain is also capable of producing lower amount xylanase. The cells of the strain SF5 were Gram Negative, motile, small rod shaped. Most of the cases observed in single rod but rarely in double (Figure 1A & B) The strain could not hydrolyze starch and is negative for MR-VP reaction, indole and H_2S production (Table 1). It is positive for catalase and citrate utilization. Details of carbon sources utilization pattern for the strain SF5 is provided in the Table 3.The 16S rRNA gene sequence of SF5 strain was deposited to NCBI for nucleotide accession

number. On the basis of 16S rDNA sequence analysis, it was affiliated to the genus *Bosea*.The strain showed closest sequence similarity with *Bosea thiooxidans* DSM 9653^T (AJ250796) (99.59%), followed by *Bosea eneae* CCUG 43111T (AF288300) (98.89%), *Bosea vestrisii* CCUG 43114T (AF288306) (98.83%), *Bosea minatitlanensis* ATCC 700918T (AF273081) (98.69%) and *Bosea massiliensis* CCUG 43117T (AF288309) (98.53%) (Table 2) From the phylogenetic tree (Figure 2) it is also evident that the strain SF5 formed a cluster along with *Bosea thiooxidans* with very boot strap value of confidence. Thus on the basis of 16S rRNA gene based phylogenetic analyses, the strain was identified as *Bosea* sp. However, in absence of detail phenotypic, chemotaxonomic and overall genome relatedness analysis, its species status is not deciphered **(Stackebrandt and Goebel, 1994).**

Cellulase production was found to be dependent upon the nature of the carbon source used in culture media. The choice of an appropriate substrate is of great importance for the successful production of cellulases. In the present study among the different carbon sources tested, CMC promoted maximal enzymes yield when compare to other followed by lactose, starch, maltose, avicel etc. From Table 4 it was observed that SF5 utilized different carbon sources for bacterial growth and cellulase production. Among these carbon sources glucose, sucrose, Fructose and galactose are not suitable for cellulase production by SF5, but CMC exhibited extensive role to higher enzyme production. Similar observation found in many bacteria like *Acinetobacter anitratus***(Ekperigin, 2007)**, *Branhamella* sp **(Ekperigin, 2007).** This may be a result of induction of the enzyme, as cellulose is known to be a universal inducer of cellulase synthesis. **Paul and Varma (1993)** had stated that induction of endoglucanase by CMC in *Micrococcus roseus*.

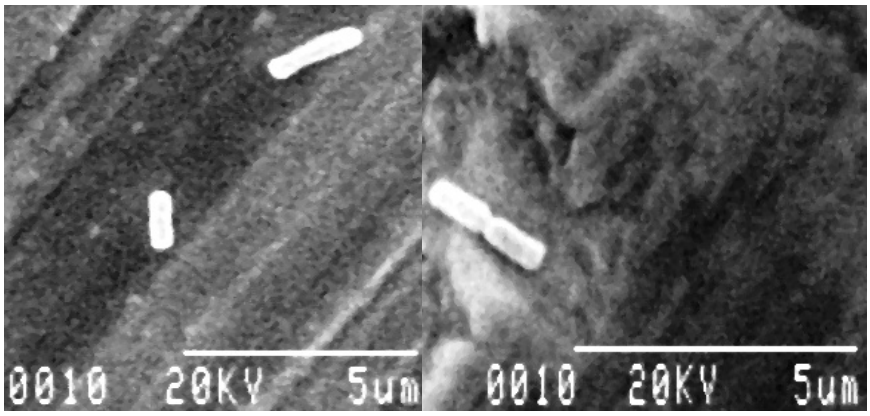
Incubation time is necessary for optimal production of enzymes. In the present study the enzyme production was increasing steadily from the beginning and reached the maximum at 8 d of incubation, then the level start decreasing (Figure 3A). It was observed that *Streptomyces* sp. BRC1 and BRC2 gradually raised endoglucanase synthesis and reached maximum activity at 3 d, after that enzyme activity slowly decreased **(Chellapandi and Himanshu, 2008)**. The different concentrations of CMC were tested for cellulase production, among which 0.75% CMC was optimum for this strain (Figure 3B). Above this concentration cellulase production was inhibited. Similarly cellulose production was inhibited by 1 % cellulose in *Thermomonospora curvata* isolated from municipal solid waste compost **(Stackebrandt and Goebel, 1994).**

Temperature is the important factors for growth of microorganism and production of enzyme. It was found from Figure 3C, the strain SF5 gradually raised cellulase synthesis and

reached maximum activity at 37ºC, after that cellulase production slowly decreased. The optimum temperature is 26 ºC in *Streptomyces* sp BRC1 and BRC2 **(Chellapandi and Himanshu, 2008)** but 52ºC in *Micromonospora chalcae***(Gallagher *etal*., 1996).** So the different bacterial strains able to produced this enzyme at different optimal temperature.

As shown in Figure 3D the pH optima for the cellulase productivity was 7.0 of the SF5 strain. Beyond this limit of pH, the enzyme productivity was decreased. Similar results have been found in *Streptomyces* sp F2621 **(Tuncer *et al.*, 2004),***Streptomyces* BRC1 and BRC2 **(Chellapandi and Himanshu, 2008)[**5], *Clostridium* sp. **(Lee and Blackburn, 1975),***Streptomyces* sp AT7 **(Amira *et al.*, 1989)** and *Anoxybacillus flavithermus* EHP1 **(Ibrahim and Ahmed, 2007).**

The enzyme production is affected significantly under different concentration of the organic and inorganic nitrogen sources. The production media incorporated with different inorganic nitrogen source, supported to bring more cellulase activity by *Bosea* sp. Among the different nitrogen sources tested, the enzyme activity was higher with NH_4Cl (Table 5). To find out the suitable concentration of NH_4Cl, different concentrations of NH_4Cl tested, among which 0.15% $NH_4Cl$was optimum for this strain (Figure 3E). Meat extract and tryptone (1%) served as intensive sources to *Streptomyces* sp. BRC1 and yeast extract (1%) suited for *Streptomyces* sp BRC2 **(Chellapandi and Himanshu, 2008).** Organic N source were found to be more suitable than inorganic N sources for optimizing cellulose production *Bacillus* sp. **(Rajoka, 2004; Acharya and Chaudhary, 2011)**. **Rajoka (2004)** reported KNO_3 and NH_4NO_3 as the best N sources for cellulose production in *Cellulomonas flavigena*. NH_4 compounds was considered as most favourable N sources for cellulase synthesis as noted in *Thermomonospora fusca***(Spiridonov and Wilson, 1998)**.



A B

Figure 1A and **B** are the Scanning electron micrographs of the strain SF5. Bar, 5 µm

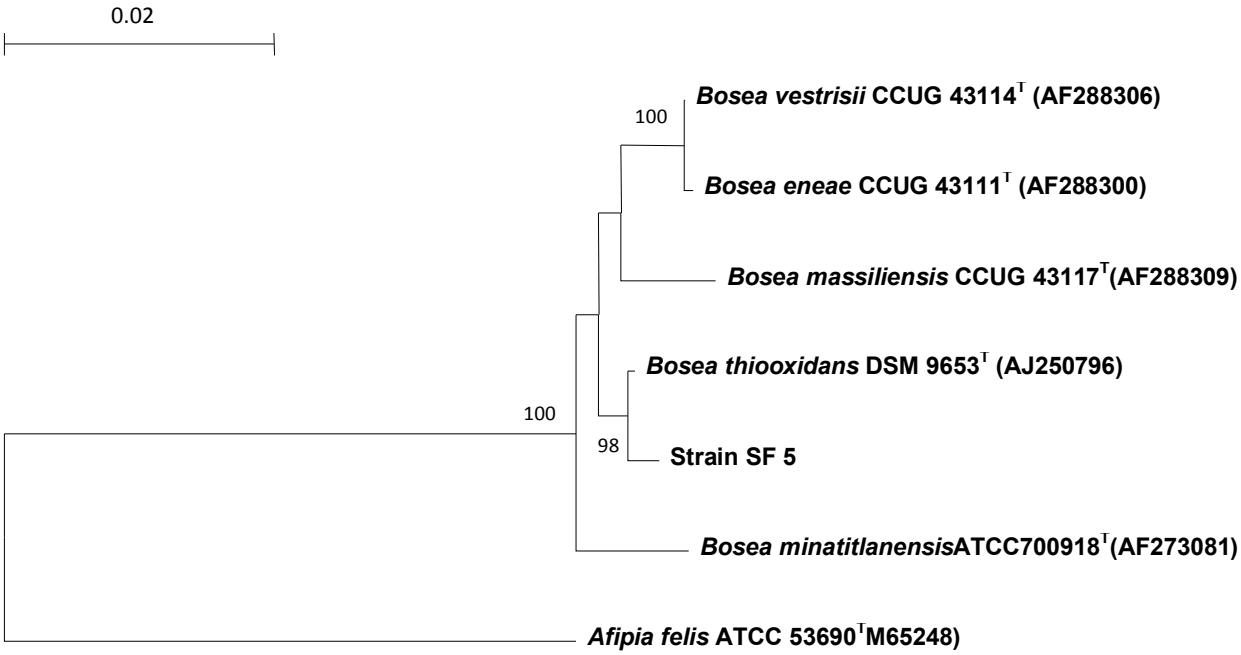


Figure 2 Phylogenetic tree (based on 16S rRNA gene sequence) showing relationship of the strain SF5 among other species of genus *Bosea*. Sequence of 16S rDNA from *Afipia felis* was taken as out-group. Number at the nodes indicates bootstrap values of 100 replications. The tree was generated using TREECON with Juke's & Cantor's correction. Bar 0.02 substitutions per site.

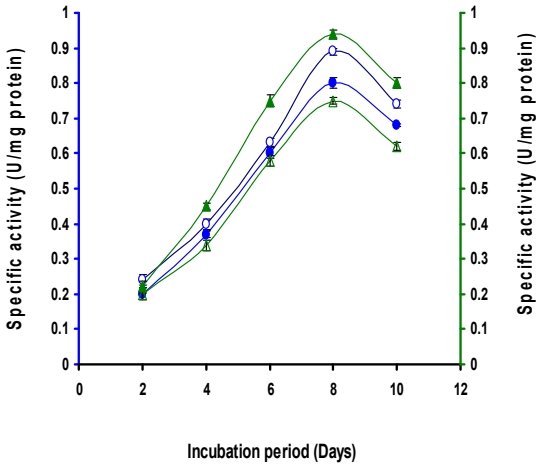


Figure 3A

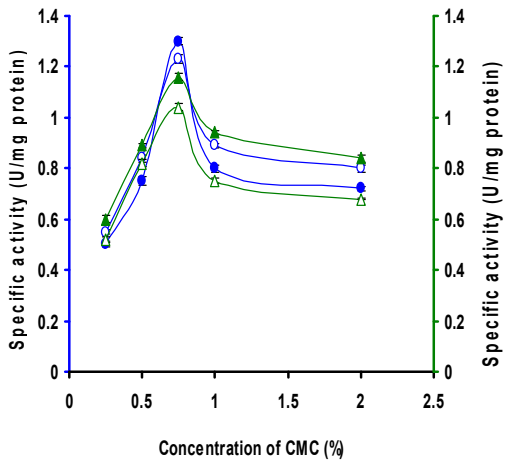


Figure 3B

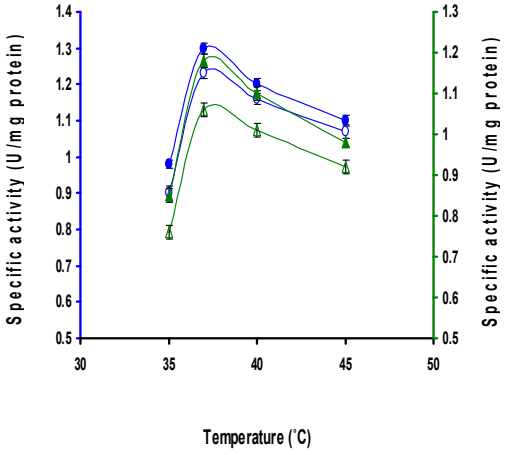


Figure 3C

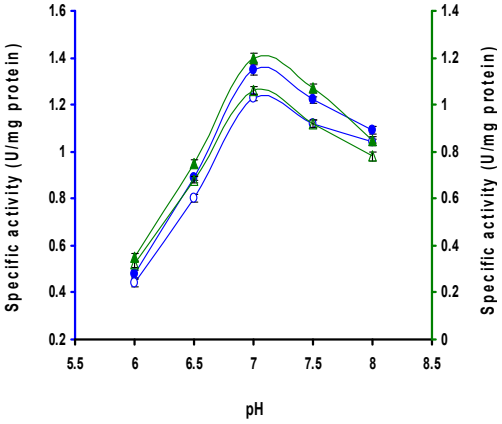


Figure 3D

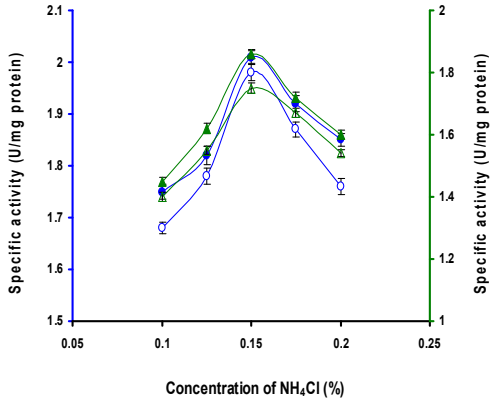


Figure 3E

Legend for figures:

Figure 3A. Time course of crude enzyme production in culture medium containing 1% CMC by *Bosea* sp. at 37ºC.

Figure 3B. Effect of CMC Concentration (in %) in culture medium on crude enzyme production at 37 ^{0} C after 8 d by *Bosea* sp.

Figure 3C.Effect of Temperature on crude enzyme production in culture medium containing 0.75% CMC at 37 ^{0} C after 8 d by *Bosea* sp.

Figure 3D.Effect of pH on crude enzyme production in culture medium containing 0.75% CMC at 37 ^{0} C after 8 d by *Bosea* sp.

Figure 3E. Effect of different concentrations of NH_4Cl on crude enzyme production in culture medium containing 0.75% CMC at 37 ^{0} C after 8 d by *Bosea* sp.

Symbols used: open circles, CMCase; closed circles, Avicelase ; open triangles, FPase ; closed triangles, β-glucosidase.

Table 1 Some morphological and physiological properties of SF5 strain

Colony morphology	Rounded
	Circular
	Smooth
	Cream colour
Cell morphology	Small rod
	Most of the cases in single rod, rarely in double
Gram Character	-
Indole production	-
V-P test	-
Gelatin hydrolysis	-
Hydrogen sulphide production	-
Citrate utilization	+
Oxidation of reduced sulphur compounds	No growth
Catalase	+
Methyl red	-
Urease	-
Starch hydrolysis	-

Table 2 The five 16 S rDNA sequence alignment matches with SF5 strain

Rank	Name Title	Authors	Strain	Accession	Pairwise similarity	Diff/Total nt	Mega BLAST score	BLAST TN score
1	*Bosea thiooxidans*	Das et al. 1996	DSM 9653(T)	AJ250796	99.654	5/1444	2823	2823
2	*Bosea vestrisii*	La Scola et al.2003	34635 (T)	AF288306	99.168	12/1442	2763	2763
3	*Bosea eneae*	La Scola et al.	34614 (T)	AF288300	99.098	13/1442	2755	2755
4	*Bosea minatitlanensis*	Ouattara et al.2003	AMX51 (T)	AF273081	98.754	18/1445	2722	2722
5	*Bosea massiliensis*	La Scola et al.2003	63287 (T)	AF288309	98.601	20/1430	2668	2662

Table 3 Oxidation of substrates using Biolog GP2 microplates

Oxidation of substrates	SF5	Oxidation of substrates	SF5	Oxidation of substrates	SF5
α-cyclodextrin	-	Maltotriose	-	acetic acid	+
β cyclodextrin	-	D-mannitol	-	α- hydroxybutyric acid	+
Dextrin	-	D-mannose	-	β- hydroxybutyric acid	+
Glycogen	-	D-melezitose	-	γ-hydroxybutyric acid	-
Inulin	-	D-Melibiose	-	p-hydroxyphenylacetic acid	-
Mannan	-	β-methyl-D-galactoside	-	α-ketoglutaric acid	-
tween-40	-	α -methyl-D-galactoside	-	α -ketovaleric acid	+
tween-80	-	3-methyl-D- glucose	-	Lactamide	+
N-acetyl-D-Glucosamine	-	α -methyl-D-glucoside	-	D-lactic acid Methyl Ester	-
N-Acetyl β-D-mannosamine	-	β -methyl-D-glucoside	-	L-lactic acid	+
Amygdain	-	α -methyl-D-mannoside	-	D-Malic Acid	-
L-arabinose	-	Palatinose	-	L-Malic Acid	-
D-arabitol	-	D-psicose	-	Pyruvic acid methyl ester	-
Arbutin	-	D-raffinose	-	Succinic Acid mono methyl ester	+
D-cellobiose	-	L-rhamnose	-	Propionic acid	-
D-cellobiose	-	D-ribose	-	pyruvic acid	-
D-fructose	-	Salicin	-	Succinamic Acid	-
L-fucose	-	Sedoheptulosan	-	Succinic Acid	-
D-galacturonic acid	-	D-sorbitol	-	N-Acetyle L Glutamic Acid	+
D-galactose	-	Stachyose	-	L-Alaninamide	+
Gentiobiose	-	Sucrose	-	D-Alanine	-
D-gluconic acid	-	D-tagatose	-	L-Alanine	-
α –D-glucose	-	D-trehalose	-	L-Alanyl-Glycine	-
m-inositol	-	Turanose	-	L-Asparagine	-
α -D- lactose	-	Xylitol	-	L-Glutamic Acid	-
Oxidation of substrates	**SF5**	**Oxidation of substrates**	**SF5**	**Oxidation of substrates**	**SF5**
Lactulose	-	D-xylose	+	Glycyl-L-Glutamic Acid	-
Maltose	-	L-Pyroglutamic Acid	-	Uridine	-
L-Serine	-	Adenosine	-	Adenosine 5'-monophosphate	-
Putrescine	-	2-deoxy adenosine	-	Thymidine-5'-Monophosphate	-
2-3 butenediol	-	Inosine	-	Uridine 5'-monophosphate	-
Glycerol	-	Thymidine	-	D-Fructose-6-Phosphate	-
α-D-Glucose-1-Phosphate	-	D-Glucose-6-Phosphate	-	D-L- α-glycerol phosphate	-

Legend: (+ = positive of substrate and - = negative of substrate utilization)

Table 4 Effect of different carbon sources (1% W/V) on cellulase production by *Bosea* sp. after 8d of incubation at 37 ^{0}C

Carbon Sources	Specific Activity (U/mg Protein)			
	CM Case	Avicelase	FPase	B - Glucosidase
Starch	0.40±0.0152	0.42±0.0152	0.39±0.010	0.40±0.0152
Mattose	0.38±0.0152	0.39±0.010	0.35±0.0115	0.37±0.010
Sucrose	0.12±0.0152	0.11±0.0152	0.10±0.0152	0.10±0.0152
Glucose	0.09±0.010	0.07±0.0088	0.07±0.0088	0.10±0.0152
CMC	0.75±0.0152	0.74±0.0115	0.75±0.0152	0.70±0.0185
Avicel	0.22±0.0152	0.30±0.0152	0.26±0.0152	0.22±0.0152
Lactose	0.58±0.0152	0.52±0.0152	0.55±0.0152	0.50±0.0152
Fructose	0.20±0.0057	0.18±0.0115	0.22±0.0152	0.20±0.0200
Galactose	0.20±0.0200	0.15±0.01732	0.18±0.01155	0.20±0.01732

Table 5 Effect of different nitrogen sources on growth and enzyme production

Strain	Nitrogen Source (0.1%)	Specific Activitμy (U/mg Protein)			
		CM Case	Avicelase	F Pase	β – Glucosidose
SF5	KNO_3	1.35±0.0208	1.23±0.0152	1.20±0.0173	1.06±0.0152
	$(NH_4)_2 SO_4$	0.95±0.0152	0.92±0.0152	0.80±0.0152	0.78±0.0208
	$NH_4 NO_3$	1.12±0.0152	1.07±0.01732	1.03±0.01732	0.98±0.0115
	NH_4Cl	1.75±0.0152	1.68±0.01155	1.45±0.01155	1.40±0.0152
	Peptone	0.80±0.0152	0.82±0.0152	0.75±0.0152	0.68±0.005
	Yeast extract	0.73±0.0200	0.77±0.0152	0.84±0.0152	0.75±0.0152
	Beef extract	ND	ND	ND	ND
	Casein	ND	ND	ND	ND
	Urea	ND	ND	ND	ND
	Tryptone	0.80±0.0152	0.70±0.01856	0.75±0.0152	0.69±0.0152

Legend: ND = Not Detectable

CONCLUSION

Majority of studies on cellulase production have been focused on fungi and bacteria. However, bacteria may serve as highly potent sources of industrially important enzymes for the conversion of cellulosic biomass due to their higher growth rate; more complex glycoside hydrolases with providing synergy and their extremely high natural diversity have the capability to produce thermostable, alkalistable enzymes. For this reason we have screened cellulase producing bacteria from different ecological niches and obtained this strain of *Bosea* sp. The literature survey indicated that except our strain (*Bosea* sp. strain SF5) no other spp. (strain) did not produced cellulase, so this character is novel for this strain and which may achieved from other cellulase producing bacteria by horizontal gene transfer. Purification and characterization of the cellulase enzyme of this strain are at present running in our laboratory after which biotechnological potential of this enzyme of this strain can be known.

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