ISOLATION, IDENTIFICATION AND OPTIMIZATION OF CELLULASE PRODUCING BACTERIA FROM FORESTS OF WESTERN GHATS, INDIA

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Abstract: Cellulose is the major component of plant biomass and most abundant biomass on the earth. Cellulase is an enzyme used for the bioconversion of cellulosic and lignocellulosic residues. Cellulase is used for commercial food processing in coffee and also used in textile industry and in laundry detergents as well as pulp and paper industry for various purposes. Traditionally, majority of studied are focused on fungi, lesser importance given to bacterial sources. To fill this lacuna, we have collected different soil samples from different sites in the reserve forests of Western Ghats of Nilgiri district in Tamil Nadu, India. Totally 154 bacterial strains were isolated from the soil samples. Based on the clear zones formation on the xylan red-agar medium only 3 bacterial strains were selected for the further studies and they were identified based on the 16S rRNA sequence. Solid state and submerged fermentations were done to identify strains that could produce cellulase. *Bacillus subtilis* (strain 2) produced maximum cellulase production, 5.02 IU/ml and 2.21 U/ml in solid state and submerged fermentations, respectively. All the strains produced cellulase during solid state as well as, submerged fermentation.

Key words: ***Bacillus,*** **Cellulase, Solid state fermentation, Submerged fermentation, Western Ghats.**

I. INTRODUCTION

Sustainable resources, which are in need of human beings, are derived from plant biomass. Cellulose is the major component of plant biomass and most abundant biomass on the earth (Tomme *et al*., 1995; Camassola and Dillon, 2007). The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Bhat, 2000). It has become a considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources (Shanmugapriya *et al*., 2012). Cellulase is an enzyme used for the bioconversion of cellulosic and lignocellulosic residues, produced by several microorganisms, commonly by bacteria and fungi (Shin *et al*., 2000; Immanuel *et al*., 2006). Cellulolytic activity is a multi-complex enzyme system and complete enzymatic hydrolysis of enzyme requires synergistic action of 3 enzymes; endo-β-glucanase (EC 3.2.1.4), exo- β - glucanase (EC 3.2.1.91) and β –glucosidase (EC3.2.1.21) (Shankar and Isaiarasu, 2011). The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria, have been isolated and characterized (Tomme *et al*., 1995).

For many years, cellulose degrading bacteria have been isolated and characterized for obtaining more effective cellulase from variety of sources such as soil, decayed plant materials, hot springs, organic matters, feces of ruminants and composts (Doi, 2008). Researchers keep on working to isolate microorganisms with higher cellulase activity (Ray *et al*., 2011). Among bacteria, species of *Bacillus* can produce number of extracellular polysaccharide hydrolyzing enzymes (Bhat and Bhat, 1997). However, these carboxymethyl cellulase (endoglucanase) enzymes cannot hydrolyze crystalline cellulose (Ozaki, 1991). These polysaccharide hydrolyzing enzymes also include alkaline cellulase with high potential as laundry detergent additives. There are, however, some studies on *Bacillus* and fungal endoglucanases which had shown

detectable activity on microcrystalline cellulose (Ogawa *et al*., 1991). *Bacillus subtilis* was one of the first bacteria studied by scientists. It was originally named *Vibrio subtilis* in 1835 by Christian Gottfried Ehrenberg and later renamed *Bacillus subtilis* by Ferdinand Cohn in 1872. *Bacillus subtilis* produces a natural surfactant and possess the ability to biodegrade hydrocarbons. *B. subtilis* is a facultative aerobe; spores can survive extreme heat (Cohn, 1872; Ehrenberg, 1985). A cellulase producing *Bacillus* strains have been isolated from soil and optimized the culture conditions for enzyme production (Li *et al*., 1998). With this background, the present study was attempted with the following objectives,

* To isolate and screen cellulolytic bacteria from different sites of forest soils of Western Ghats by plate assay method (carboxyl methyl cellulose).
* To identify efficient isolates by 16S rRNA analysis.
* Production of cellulase from potential isolates by submerged fermentation and solid state fermentation process.

II. MATERIALS AND METHODS

Isolation of bacteria

The humus soil samples were collected from different sites in the reserve forests of Western Ghats of Nilgiri district in Tamil Nadu, India. One gram of soil added to 10ml of sterile distilled water and mixed well (10^{-1}). 1ml of the sample was pipetted out from 10^{-1} tubes onto 9ml of sterile distilled water taken in another tube. The sample was then serially diluted up to $10^{-6.}$ Dilutions were poured in separate sterile petri plates containing nutrient agar medium and incubated at 37ºC for 2 days. Bacterial isolates having a zone of inhibition to other isolates were purified by repeated streaking. The pure isolates stored at 4ºC for further studies.

Screening of cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred in carboxy- methylcellulose (CMC) agar plates. The CMC medium containing (g/l) $MgSo_4$, 0.5; KCL, 0.5; K_2HPO_4, 1.0, CMC, 5.0; Agar-agar, 20; DH_2O, 1000ml; pH 7.0. After incubation for 48 hours, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counter staining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis (Andro *et al*., 1984). The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged and solid state system.

Molecular identification of cellulolytic bacteria

The molecular characterization was done by using 16S rRNA sequencing technique (Bajaj and Sharma, 2011). The genomic DNA was extracted from the given organism using standard procedures (Sambrook and Russel, 2001). The 16S rRNA sequence was blasted using NCBI blast similarity search tool. The phylogeny analysis of test sample sequenced with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminated poorly aligned positions and divergent regions (Talavera and Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 used as a Substitution model. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*., 2008).

Production of cellulase

Submerged fermentation (SmF)

The bacteria were cultured in Erlenmeyer flasks (250 ml) containing 50 ml of medium. The medium containing (g/L) NH_4Cl, 0.5; KH_2PO_4, 0.5; KCl, 0.75; K_2HPO_4, 2.5; $MgSO_4.7H_2O$, 7.0; Brich wood xylan, 10; NaCl, 30; DH_2O-1.0 and pH was adjusted into 5.5. The flasks were inoculated with 1ml of bacterial broth culture flasks were incubated at 30ºC under static conditions for 3 days. After incubation, the culture filtrate was harvested by filtration using Whatman No.1 filter paper and centrifuged at 10,000rpm for 15 min at 4°C. The clear supernatant was used for enzyme assays, stored in refrigerator at 4ºC.

Solid state Fermentation (SsF)

The bacteria were cultured in Erlenmeyer flasks (250 ml) containing 5g of wheat bran and rice bran separate flask and moistened with 15ml of mineral salts solution respectively. The mineral salt solution composition (g/L) $FeSO_4.7H_2O$, 0.01; KH_2PO_4, 0.5; NH_4Cl, 0.5; KCl, 0.75; K_2HPO_4, 2.5; $MgSO_4$, 7.0; NaCl, 30; DH_2O, 1.0; Trace metal, 1ml and P^H adjusted into 5.5. Trace metal solution containing $ZnCl_2$, 0.02; Na_2MO_4, 0.02; $CuCl_2$, 0.03; $CoCl_2$, 0.04; $C_4H_4KNaO_6.4H_2O$, 1.77; $MnCl_2.7H_2O$, 1.80; $FeSO_4$, 2.49; H_3BO_3, 2.8; and DH_2O-1.0. Similarly the above procedure was used to the enzyme extraction.

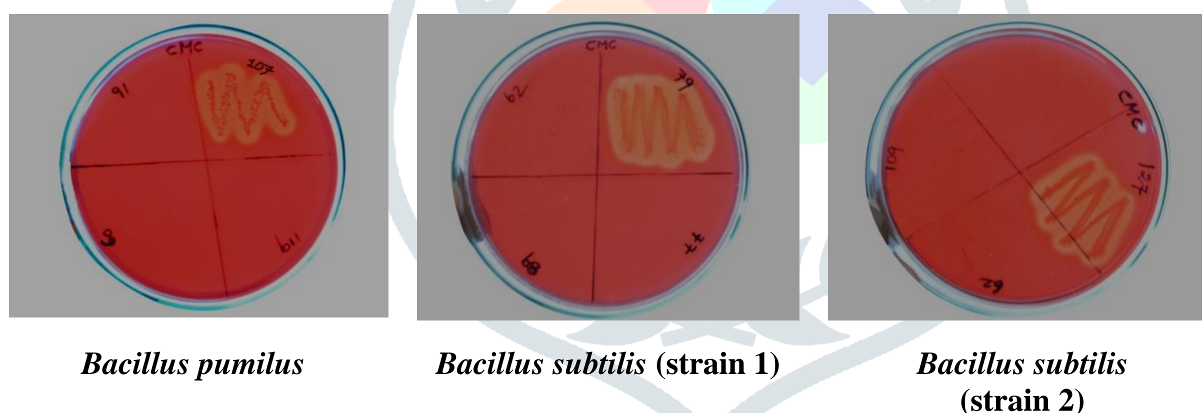
Cellulase Assay

0.5ml of 1% CMC solution was added to 0.5ml of suitably diluted culture supernatant of each bacterial strain. Reaction mixture was incubated for 30 minutes at 50˚C. After incubation, reaction was terminated by adding 3 ml DNS reagent and reaction mixture was boiled in water bath for 5 minutes. After coiling, the color developed was read at 540 nm against the blank. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1µmole of glucose equivalents per minute under the assay condition.

III. RESULTS AND DISCUSSION

Isolation and screening of cellulose- Degrading bacteria

Soil samples collected from the different sites in the reserve forests of Western Ghats of Nilgiris district in Tamil Nadu, India. A total of 154 bacterial strains were isolated from soil samples. The isolates were initially differentiated based on the morphological characteristics such as colony pattern, growth, colour and gram staining repeated strains is avoided. 28 isolates were differentiated after preliminary differentiation procedure. Only three isolates were showed hydrolyzing zones on CMC agar plates after Congo red staining **(Fig 1).** The hydrolyzing zone diameter and colony diameter are listed **(Table 1)**. These 3 isolates selected for the further studies.



Bacillus pumilus ***Bacillus subtilis* (strain 1)** ***Bacillus subtilis* (strain 2)**

Figure 1: Preliminary screening of cellulase activity by plate assay

Table 1: Enzyme hydrolyzing capacity in CMC agar plates

Bacterial Strain	Enzyme hydrolyzing diameter (cm)	Colony diameter (cm)
Bacillus pumilus	6.4±0.197	4.2±0.155
Bacillus subtilis (strain 1)	6.6±0.254	4.6±0.141
Bacillus subtilis (strain 2)	7.5±0.212	4.5±0.268

Identification of cellulose degrading bacteria

An analysis of the 16S rRNA gene of isolate revealed that this organism was closely related phylogenetically to members of the genus *Bacillus* rRNA group 5 (Rainly *et al.,* 1994). Similarly in the study DNA fragments containing partial 16S rRNA genes of the three isolates were amplified and

sequenced **(Fig 2).** The sequence obtained were matched with those available in genbank, which revealed maximum identity of these isolates and allowed identification of these cellulose-degrading bacterial strains **(Table 2).** The 3 identified bacterial isolates are *Bacillus subtilis* (strain 1), *Bacillus subtilis* (strain 2) and *Bacillus pumilus.*

Table 2: Cellulolytic bacterial strains identification based on BLAST result

Bacterial strain	Closest strain in Genbank	Closest strain accession number
Bacillus pumilus	*Bacillus pumilus* strain IHB B 6571	(KF475865.1)
Bacillus subtilis (strain 1)	*Bacillus subtilis* strain LS-1	(KC668310.1)
Bacillus subtilis (strain 2)	*Bacillus subtilis sub* sp. *inaquosorum* strain IHB B 6833	(KF668463.1)

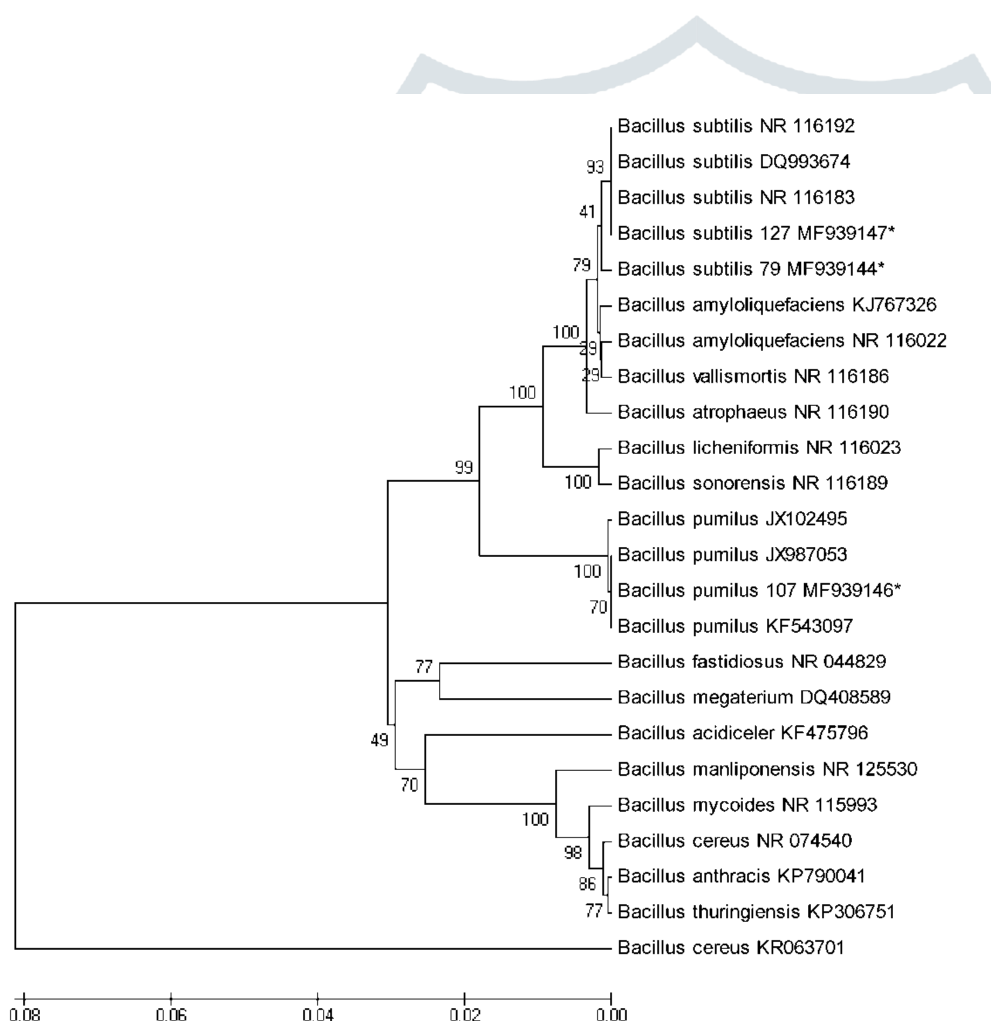


Figure 2: Phylogenetic dendrogram based on 16SrRNA sequence

Production of cellulase under solid state and submerged fermentation

The technique of solid-state fermentation involves the growth and metabolism of micro-organisms on moist solids in the absence or near absence of any free-flowing water. These fermentation systems, which are closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites (Qadeer *et al.*, 1980; Babu and Satyanarayana, 1995). Thus, solid state fermentation offers distinct advantages over submerged fermentation including economy of the space, simplicity of the media, no complex machinery, equipments and control systems, greater compactness of the fermentation vessel,

greater product yields, reduced energy demand, easier scale up of processes and easier control of contamination due to the low moisture level in the system (Raimbault, 1988; Aguilar *et al*., 2008).

Based on the preliminary cellulolytic activity, a total of 3 bacterial isolates belonged to genus *Bacillus* was selected for submerged fermentation (SmF) and Solid State fermentation (SsF) studies. The selected strains were subjected to submerged fermentation and Solid State fermentation for an incubation period of 3 days to determine the influence of fermentation condition indicating that increasing the quantity of cellulase produced by the organisms. Cellulase production in SsF was much higher than that in submerged fermentation SmF (Malarvizhi *et al*., 2003). Similarly in the present study all the 3 isolates showed better enzyme activity in SsF all two substrate wheat bran and rice bran when compared to SmF **(Table 3 and Fig 3)**. The *Bacillus subtilis* (strain 2) produce highest cellulase activity (5.02IU/ml) in solid state fermentation wheat bran as substrate compared with submerged fermentation to increase the 2.81IU/ml enzyme activity. These result showed SsF medium especially wheat bran as substrate is suitable for the cellulase enzyme activity when compared to SmF.

Table 3: Cellulase production during Solid state and submerged fermentation

Name of the Bacteria	Submerged fermentation (IU/ml)	Solid state fermentation (IU/ml)	
		Wheat bran	Rice bran
Bacillus pumilus	1.32±0.056	1.94±0.127	1.54±0.042
Bacillus subtilis (strain 1)	1.72±0.028	4.32±0.141	1.83±0.049
Bacillus subtilis (strain 2)	2.21±0.205	5.02±0.226	2.52±0.106

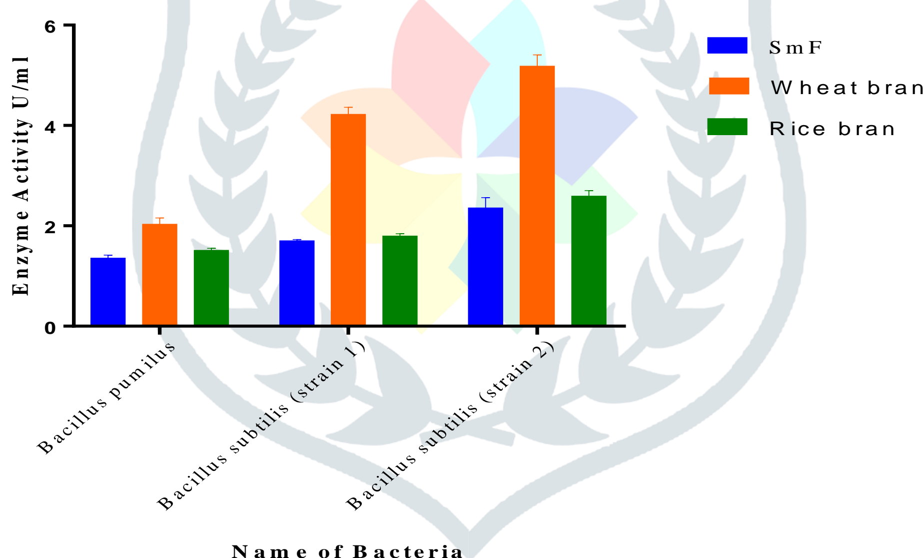


Figure 3: Cellulase production in SmF and SsF (Wheat bran and Rice bran)

In general, it was observed that the enzyme productivity via solid-state fermentation (SSF) is normally much higher than that of submerged fermentation (Agnihotri *et al*., 2010). Several strains of *Bacillus* including *B. brevis, B. firmus, B. polymyxa, B. pumilus, B. subtilis, B. circulans* were reported as cellulase producing bacteria (Priest, 1997; Hakamada *et al*., 2002; Sa-Pereira *et al*., 2002).

IV. CONCLUSION

In the present study the cellulase producing bacterial strains was successfully isolated from soil samples collected from Nilgiri district area. The cellulase producing bacterial strains could be identified as *Bacillus subtilis* (strain 1), *Bacillus subtilis* (strain 2) and *Bacillus pumilus*. Since our present study maximum production of cellulase was obtained from *Bacillus subtilis* (strain 2) selected bacterial stain under solid state fermentation gave 5.02 IU/ml compared with submerged fermentation. The solid state fermentation

condition is better to the cellulase production especially wheat bran as substrate. In future, the cellulase obtained from these strains could be used in pulp bleaching process.

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