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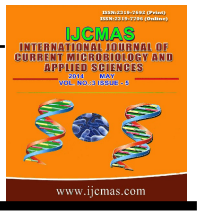

Original Research Article

Isolation and Characterization of Cellulase producing bacteria from Soil

Basavaraj I. Patagundi, C.T.Shivasharan and B. B. Kaliwal*

Department of Studies in Biotechnology and Microbiology,
Karnatak University, Dharwad – 580 003, India
*****Corresponding author*

A B S T R A C T

The present investigation was undertaken to isolate and Screen the Cellulase Producing Bacteria from Soil. Bacterial cultures were isolated from the soil sample collected from Botanical garden, Karnatak University Campus, Karnataka, India. Four different substrates like *Acacia arabica* pod, *Bauhinia forficata* pod, Cassia *surattensis* pod and *Peltophorum pterocarpum* pods (as cellulose substrate) were used in the submerged production medium. A Total of 57 bacterial cultures were isolated based on Morphology and Biochemical characterization. Among all isolated strains, the three cellulolytic bacterial strains, maximum enzyme activity were showed in *Bacillus cereus* (0.440 IU/ml/min and 0.410 IU/ml/min), followed by *Bacillus subtilius* (0.357 IU/ml/min) and *Bacillus thuringiensis* (0.334 IU/ml/min) to the *Acacia arabica* pod. *Acacia arabica* pod showed maximum enzyme activity comparatively other pods.

Keywords

Bacillus Species, CMC-Agar, Submerged fermentation, Substrates.

Introduction

Enzymes are delicate protein molecules necessary for life. Cellulose is the most abundant biomass on the earth (Venkata *et al.*, 2013) Plant biomass contains cellulose as the major component. Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes (Haruta *et al.*, 2003). Presently huge amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Cellulose has attracted worldwide attention as a renewable resource that can be converted into bio-based products and bioenergy (Xing-hua *et al.*, 2009). Celluloses are observed as the most important renewable resource for bioconversion. It has been become the economic interest to develop an effective method to hydrolyze the cellulosic biomass (Saraswati *et al.*, 2012).

Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Immanuel *et al.*, 2006). Cellulase is an important and essential kind of enzyme for carrying out the depolymerization of

cellulose into fermentable sugar (Xing-hua *et al.,* 2009). Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters (Lee and Koo, 2001). Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere (Lynd *et al.,* 2002). Increasing knowledge of mode of action of Cellulase; they were used in enzymatic hydrolysis of cellulosic substances (Kubicek *et al.,* 1993). Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently (Sonia *et al.,* 2013). Cellulases are used in the textile industry for cotton softening and denim finishing, in laundry detergents for colour care, cleaning, in the food industry for mashing, in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications. Over all the cellulose enzymes will be commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes will also grow rapidly (Cherry *et al.,* 2003) Cellulases form bacteria are also more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed. The greatest potential importance is the ease with which bacteria can be genetically engineered (Arifin *et al.,* 2006). Bacteria has high growth rate as compared to fungi has good potential to be used in cellulose production. Some bacterial species viz., Cellulomonas species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* have cellulolytic property (Nakamura and Kappamura, 1982). A large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro*. Numerous investigations have reported the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement (Lee and Koo, 2001). Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production (Sonia *et al.,* 2013). Among bacteria, Bacillus species produce a number of extracellular enzyme including amylases, proteinases, and polysaccharide hydrolases (Mawadza *et al.,* 2000).

For understanding the mechanism of cellulose degradation by cellulase, it is necessary to isolate, purify and characterize this enzyme. Therefore, the present investigation was designed to isolate and Screen the Cellulase Producing Bacteria from Soil.

Materials and Methods

Isolation of Bacteria

Bacteria were isolated from the soil sample collected from Botanical garden, Karnatak University Campus, Karnataka, India. Traditional serial dilution agar plating method was used for the isolation of cellulolytic bacteria. The medium used for cellulolytic bacteria contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin at pH 7. The Plates were incubated for 48 hours at 30˚C.

Screening of Bacteria

The incubated CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. 1M NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having clear zone were selected for identification and cellulase production. Further bacterial strains were purified by repeated streaking. The purified colonies were preserved at 4^{o}C.

Screening for cellulase enzyme

Development of Inoculum

The selected bacterial cultures were individually maintained on CMC agar slants at 4˚C. The selected bacterial cultures were inoculated in broth medium containing 0.03 % $MgSO_4$, 0.2 % K_2HPO_4, 1 % glucose, 0.25 % $(NH_4)_2SO_4$ and 1 % peptone at pH 7 for 24 Hrs of incubation period. After the incubation period these bacterial cells were used as inoculum.

Cellulase enzyme production by Submerged Fermentation Process

The isolated Bacterial strains were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium was prepared by using powders of 1% *Acacia arabica* pod, *Bauhinia forficata* pod, Cassia *surattensis* pod and *Peltophorum pterocarpum* pod (as cellulose substrate), 0.2 % K_2HPO_4, 0.03 % $MgSO_4$, 1 % peptone, 0.25 % $(NH_4)_2SO_4$ and autoclaved at 121^{o}C for 15min. After autoclave, the medium was inoculated with 1 ml of bacterial isolates and incubated in a rotary shaker at 35˚C for 24 hrs of fermentation period with agitation speed of 140 rpm. After fermentation the broth was centrifuged at 14000 × g for 10 min at 4˚C. The supernatant obtained after centrifugation served as crude enzyme source.

Estimation of Cellulase enzyme

Estimation of Cellulase enzyme activity was assayed using Dinitrosalisic acid (DNS) reagent (Miller, 1959) by estimation of reducing sugars released from CMC. Crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M phosphate buffer and incubated at 50˚C for 30 min. After incubation, the reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100˚C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve (Shoham *et al.,* 1999). One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1μmol of glucose per minute under standard assay conditions (Muhammad *et al.,* 2012)

Morphological and biochemical characterization

The bacterial strains which produce cellulose enzyme were further subjected to morphological and MR VP test, Citrate utilization test, Starch hydrolysis test, Gelatin hydrolysis test, Nitrate reduction test, Catalase test, Oxidase test, Glucose fermentation test, Lactose fermentation test, Indole test, Urea hydrolysis test, H_2S production test.

Molecular identification of cellulolytic bacteria

The strain which show maximum cellulase activity was further subjected to molecular identification by analysing 16S r RNA sequence.

Isolation of genomic DNA

2 ml of overnight grown Nutrient broth culture was centrifuged at 10,000 rpm at 4^0C for 10 minutes. The pellet was re suspended in 10 min 10mM Tris, 100 mM Sodium chloride solution and centrifuged at 10,000 rpm 4^0C for 10 minutes. After discarding the supernatant, the pellet was re suspended in 100 µl of $T_{50}E_{20}$ buffer containing 20µl of lysozyme (50mg/ml) and incubated at 37^0C for 20 min, in that solution 1µl of RNase (10 mg/ml) was added and incubated at room temperature for 20 minutes. To this mixture 100µl of SDS (2% in $T_{50}E_{20}$) was added and incubated at 50^0C for 45 min with proper mixing. 2µl of Proteinase K (20mg/ml) was added and incubated at 55^0C for 30 min. The sample was extracted in same volume phenol, Chloroform and Iso-amyl alcohol (25:24:1) and DNA was precipitated with one volume of isopropanol and 0.1 volume of 3M of Sodium acetate. The pellet was washed with 70% Ethanol, dried and dissolved in 100 µl of $T_{10}E_1$ buffer and stored at -20^0C for further use. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at -20ºC for further use (Modified method of Sadashiv and Kaliwal, 2013)

Identification of bacteria by sequencing of the 16s rRNA

PCR amplification was performed using Applied Biosystem verti thermal cycler. The primers for PCR amplification were obtained from Sigma-Aldrich.

Universal Primer (Lane, 1991)

27 forward – 5' AGAGTTTCCTGGCTCAG 3'
1492 reverse – 5' ACGGCTACCTTGTTACGATT 3'

The PCR was performed in 20µl reaction mixture containing 2µl of 10X assay buffer, 1µl dNTP mix of 2.5 mM, 0.5µl of mgcl2, 1µl each of forward and reverse primer (5pmol), 0.5µl of Taq polymerase, 1µl of template DNA and 13.5µl of HPLC grade water with the following amplification for 16s rRNA initial denaturation at 95ºC for 4 min followed by 38 cycles of denaturation, annealing and extension (94ºC for 1 min, 59.9ºC for 2 min and 72ºC for 2 min) and final extension at 72ºC for 20 min followed by hold for infinity at 4ºC. The presence of PCR products was determined by 2.5% agarose gel electrophoresis and to analyse the size of amplified PCR product DNA markers of 100bp was used which was provided by the Puregene. The amplified product was sent for sequencing to SciGenom Labs Pvt Ltd, Cochin, Kerala.

Construction of phylogenetic tree

By using the sequence the bacteria were identified and constructed phylogenetic tree by using NCBI(http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome) and MEGA 5 Software.

Results and Discussion

A Total of 57 bacterial cultures were isolated based on Morphology and

Biochemical characterization. The strains were subjected to Cellulase enzyme production by Submerged Fermentation Process by providing different powders of *Acacia arabica* pod, *Bauhinia forficata* pod, *Cassia surattensis* pod and *Peltophorum pterocarpum* pods (as cellulose substrate). Among all 57 tested bacterial strains B7 (0.440 IU/ml/min) showed maximum enzyme activity, followed by B20 (0. 0.357 IU/ml/min), B37 (0.410 IU/ml/min) and B49 (0.334IU/ml/min) to the *Acacia arabica* pod comparatively other pods (Table 1)

All the 57 strains (B1 to B57) were Gram +ve and showed positive for Methyl red test, Voges Proskauer test, Citrate utilization test, Starch hydrolysis test, Gelatin hydrolysis test, Nitrate reduction test, Catalase test, Oxidase test, Glucose fermentation test, Lactose fermentation test and Negative to Indole test, Urea hydrolysis test, H_2S production test.

The highest cellulatic enzyme production strains (B7, B20, B37 and B49) were further subjected to 16S rRNA. The partial amplification of 16S rRNA confirmed on the agarose gel electrophoresis. (Fig.1). By using NCBI and neighbour joining method in MEGA5 the strains were identified as *Bacillus cereus* (B7, B37) (Fig. 2), *Bacillus subtilis* (B20) (Fig. 3) and *Bacillus thuringiensis* (B49) (Fig. 4).

Cellulose is converted into fermentable sugars by the enzyme cellulase, and cellulase based bio-refinery technologies are versatile and flexible because they utilize cheaper substrates for enzyme synthesis (Mane *et al.*, 2007). The ability to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria. Efforts are going on throughout the world to enhance the production and purity of bacterial cellulases (Sreeja *et al.*, 2013). Studying on cellulolytic activity has isolated various bacteria from different environmental sources. (Hatami *et al.*, 2008).

Different Substrates are used in the present study as a carbon source to produce good yield of cellulase enzyme. *Acacia arabica* pod shows maximum enzyme activity comparatively other pods. Similar attempts have been done by many researchers. Ashish Vyas *et al.,* (2005) used groundnut shell, Shuchi Singh *et al.,* (2013) used Rhinoceros Dung, Atchara Sudto *et al.,* (2008) used Agricultural waste for the production of cellulase enzyme. It has been reported that, physico - chemical factors influence the growth of the organisms and also the Cellulase agro - residues by microorganisms depend on many factors, chemical Composition of the agro-residues (cellulose, hemicellulose, lignin, nitrogen, and minerals), presence of an activator or an inhibitor in the agro-residues, diffusion of the catabolite, and type of organisms for fermentation (Chinn *et al.,* 2006). Several microorganisms have been discovered fordecades which have capacity to convert cellulose into simple sugars (Perez *et al.,* 2002).

Many efforts were taken to generate microorganisms with high ability to produce cellulase that can degrade native cellulose (Aristidou and Penttila, 2000). From the present study among all isolated strains, the three cellulolytic bacterial strains the maximum enzyme activity were showed in *Bacillus cereus* (0.440 IU/ml and 0.410 IU/ml), followed by *Bacillus subtilius* (0.357 IU/ml), and *Bacillus thuringiensis* (0.334 IU/ml) to the *Acacia arabica* pod. Similarly Afza *et al.,* (2012) reported 45.42 U/mg cellulase production, Mukesh Kumar *et al.,* (2012) reported

cellulase activity 66 U/ml from *Bacillus* cereus which showed more activity when compared to our study and in both studies the strain was confirmed by 16s rDNA method. Venkata *et al.,* (2013) also concluded the *Bacillus cereus* is the promising bacteria to produce cellulase. Bacillus cereus was found to produce the endoglucanase type cellulase (Afza *et al.,* (2012) and most of the isolated *B. cereus* / *B. thuringiensis* strains were found to produce extracellular enzymes (Celenk *et al.,* 2009).

In the present study *Bacillus subtilis* also has been isolated and showed cellulase activity. Similarly Yu-Kyoung Kim, *et al.,* (2012), Ramalingam and Ramasamy, 2013 also reported the cellulase activity of 0.9 unit/mL and 0.140 U/ml respectively, which have high growth rate as compared to fungi, good potential to be used in cellulose production. However, the application of bacteria in producing cellulase is not widely used. (Sonia *et al.,* 2013).

Molecular methods being highly sensitive and selective currently used to identify microorganisms. Environmental conditions may have intense impact on morphological and physiological characteristics, hence the accurate identification of isolates turned out to be more difficult (Bakri *et al.,* 2010). The molecular techniques are more significant for the characterization of the new isolates, allowing grouping the strains. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation (Rahna *et al.,* 2013). Species-specific DNA sequences can be used for the identification of bacterial species. The 16s-23s rRNA has proven useful for identification of strains and species (Gurtler & Stanisich, 1996). In the present study the selected three different cellulolytic bacteria such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis* have been identified based on biochemical and 16s rRNA sequencing. The 16s rRNA sequencing makes it possible to identify and distinguish closely related bacterial species. 16s rRNA method was also used by Shuchi *et al.,* (2013) Where they isolated cellulatic bacteria *Bacillus amyloliquefaciens* from Rhinoceros Dung. Rahna *et al.,* (2013) isolated *Bacillus subtilis* using cellulosic waste as carbon source. Therefore present molecular identification work suggest that, the 16s rRNA sequencing is more accurate for the species identification.

Enzyme production is closely controlled in microorganisms and for improving its productivity, these controls can be improved. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth (Immanuel *et al.,* 2006). In enzyme fermentation process, the crude extracts contain different mixtures of proteins and undesirable products as organic acids and other metabolites. So that purification of the required favourable product must be take place by different purification methods. (Mukesh Kumar *et al.,* 2012). Optimization of different physico-chemical parameter of the production medium is required to get the maximum yield of the enzyme. Further studies were in progress to get high yield production, purification and application of cellulase.

Table.1 Enzyme activity by different strains to different substrates

Sl. No	Strain No	Enzyme activity (IU/ml/minute)			
		Acacia arabica	***Bauhinia forficate***	***Cassia surattensis***	***Peltophorum pterocarpum***
1	B7 (*Bacillus cereus*)	**0.440**	0.213	0.187	0.190
2	B20 (*Bacillus subtilis*)	**0.357**	0.201	0.189	0.178
3	B37 (*Bacillus cereus*)	**0.410**	0.217	0.203	0.187
4	B49 (*Bacillus thuringiensis*)	**0.334**	0.219	0.203	0.193

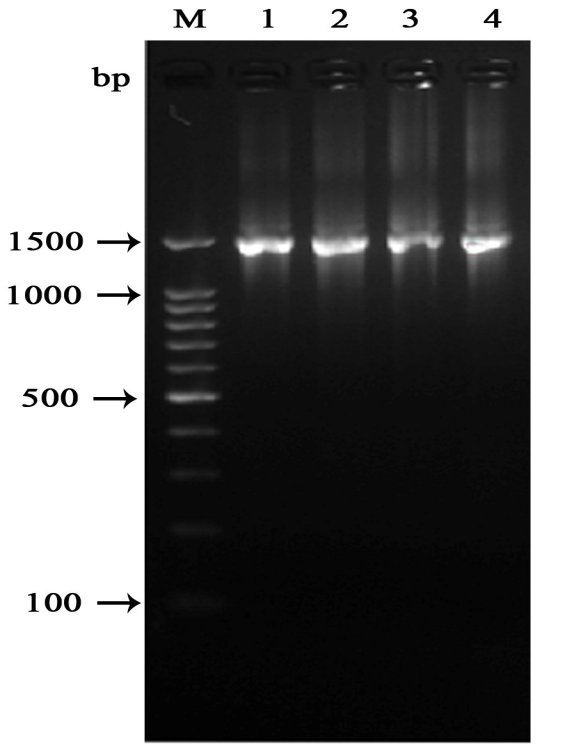

Fig.1 Agarose gel electrophoresis to PCR amplified DNA.

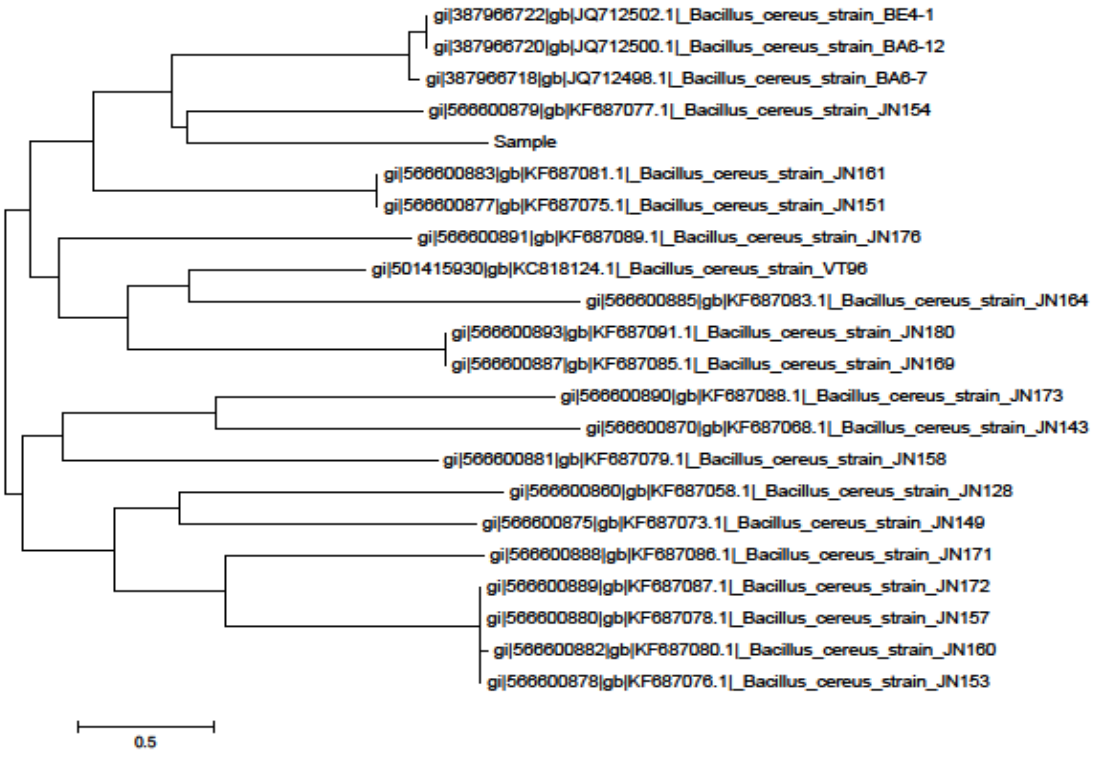

Fig.2 Phylogenetic tree of *Bacillus cereus*

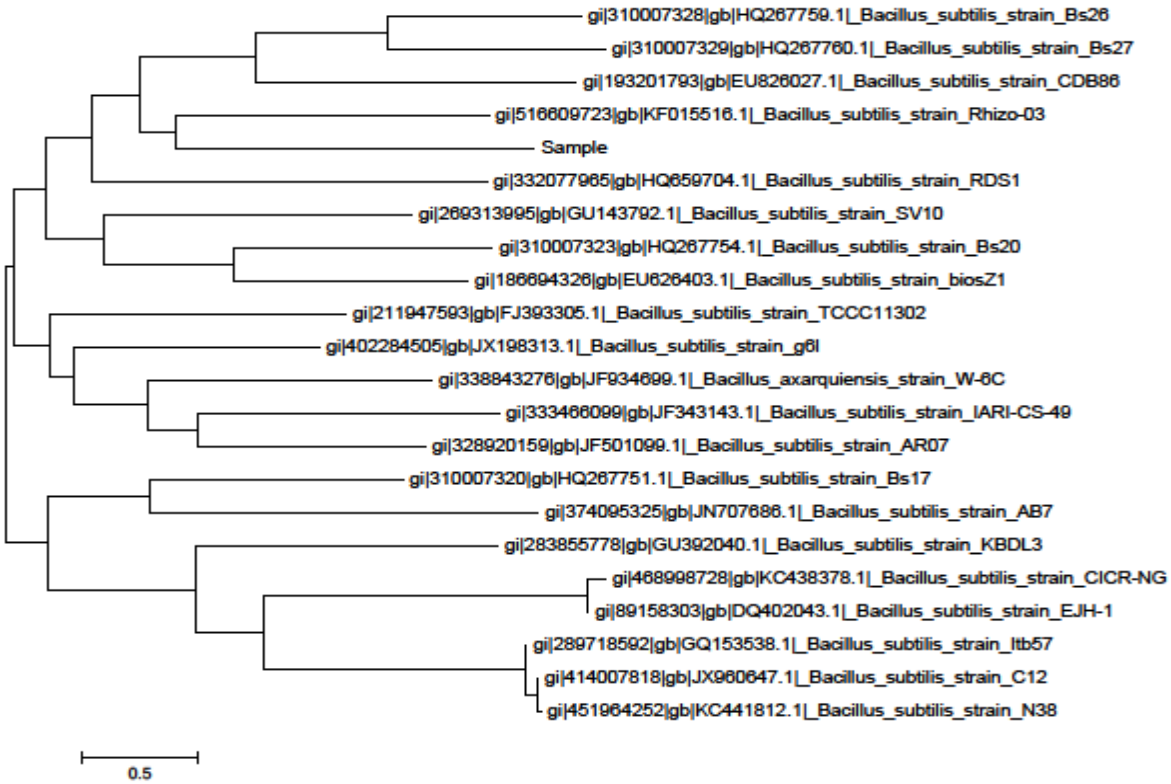

Fig.3 Phylogenetic tree of *Bacillus subtilis*

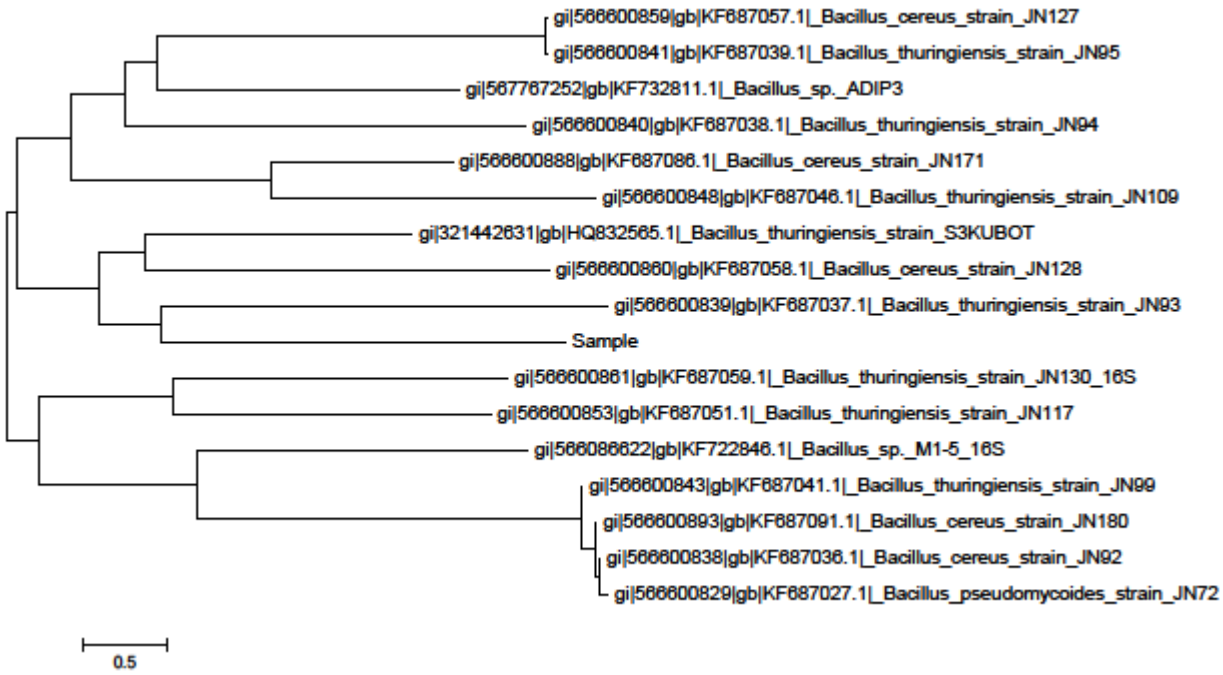

Fig.4 Phylogenetic tree of *Bacillus thuringiensis*

The purified cellulase can be used for various purposes in detergent industries, food industries, and pharmaceutical industries.

In conclusion the three different cellulolytic bacteria such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis* have been isolated. *Bacillus cereus* showed maximum cellulolytic activity compared to other two isolated bacteria. *Acacia arabica* pod shows maximum enzyme activity comparatively other pods. Optimization of different physico-chemical parameter of the production medium is required to get the maximum yield of the enzyme. Further studies were in progress to get high yield production, purification and application of cellulase.

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