

Bacterial strain isolated from the Mango field and identified on the basis of 16S rDNA sequence information and optimization of the alkaline cellulase production

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Abstract

A bacterial strain was isolated from the soil of mango (*Mangifera indica*) orchards. The isolate was identified as *Paenibacillus alvei* [MTCC 7809] through phylogenetic analysis based on the 16S rDNA gene sequencing and also by conventional biochemical processes. The scanning electron microscopic analysis revealed the morphological details of the strain. The isolate is Gram positive, motile, rod shaped, spore forming bacterium with extreme salt tolerance. It can hydrolyze starch and gelatin, and catalase and oxidase were produced. The cellulase activity of the culture broth was determined by measuring the reducing sugar released from carboxymethyl cellulose (CMC). Different cultural conditions were tested to reach the optimum growth conditions in order to obtain large-scale production of alkaline cellulase for commercial importance. Crude cellulase from *Paenibacillus alvei* (MTCC 7809) showed activity and stability at moderately high temperature and pH and the activity remained stable at 40°C and pH 8 after 2h of incubation making the strain a good choice for industrial applications. The rDNA sequence of 1509bp is submitted into the NCBI GenBank database (Accession no. EF429201).

Keywords: Cellulase, *Paenibacillus alvei*, 16S rDNA gene sequencing.

Introduction

Cellulose is the largest renewable carbon source that is frequently found in close association with other compounds such as hemicellulose, lignin and other polysaccharides (Person *et al.*, 1990). In nature microorganisms synthesize different enzymes to degrade cellulosic plant cell wall. Cellulase is one of the most demanded industrial enzymes and also used in plant protoplast isolation, plant virus studies, metabolic investigations and genetic modification experiments (Ray *et al.*, 2007). However, among all cellulases the industrial applications of alkaline cellulases as laundry detergent additive (Horikoshi, 1999) led many microbiologists to carry extensive research on alkaline cellulase producers. Fungi were considered as the chief cellulase producers but recently there is an increasing interest in cellulase production by bacteria (Li and Gao, 1996) because of the high growth rate of bacteria compared to fungi (Ariffin *et al.*, 2006).

We isolated many bacteria from the soil of different mango (*Mangifera indica*) orchards of Malda district, W.B., India (longitude 24°40'20" N to 25°32'08" N and latitude 87°45'50" E to 88°28'10" E) and some of these isolates are really important from industrial perspectives (Roy and Chattopadhyay, 2008). Among these microbes *Paenibacillus alvei* (MTCC 7809) was the potent cellulase producer. The isolate was identified on the basis of 16s rRNA gene sequencing as this process has 90% accuracy for speciation (Moor *et al.*, 2006) and also by other conventional biochemical processes. The size and morphology of the strain were studied by using scanning electron microscope (SEM).

The present investigation is aimed at the isolation of the cellulolytic bacteria *Paenibacillus alvei* (MTCC 7809) from the soil of mango orchards of the Malda district of West Bengal, India and determining the optimal conditions for alkaline cellulase enzyme production and identification on the basis of 16S rDNA gene sequencing.

Materials and Methods

Isolation and Screening of Cellulase Producer

Soil samples from different mango orchards were collected for the isolation of bacterial colonies. The soil dilutions were prepared according to the methods described in Benson's Microbiological Applications (Brown, 2005) and 1ml of the soil suspension was transferred to nutrient agar medium. The preliminary qualitative analysis for cellulolytic activity of the isolates was conducted by using Congo red dye. The isolates were screened for cellulase production on a medium containing (g/L) KH_2PO_4 - 1.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.5, NaCl - 0.5, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.01, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ - 0.01, NH_4NO_3 - 0.3, CMC - 10.0, Agar - 15.0, (pH8.0) (Ariffin *et al.*, 2006), for 3 days at 37°C. After the end of incubation period the CMC agar plates were flooded with aqueous Congo red (1%w/v) solution for 15 min followed by destaining with 1M NaCl for 15 min after pouring off previous stain. The colonies that produce clear zones of hydrolysis around it indicate cellulose degradation (Ariffin *et al.*, 2006) and were picked and maintained in nutrient agar. The biochemical, physiological and morphological tests were performed to determine the strain identity.

Enzyme Assay

For quantitative assay of cellulase production by the strain liquid media was used. Cellulase activity was determined by the release of reducing sugar from carboxymethyl cellulose (CMC) using 0.05M sodium citrate buffer (Ekperigin, 2007 and Ariffin, 2006). 0.5 ml of culture supernatant fluid was incubated with 0.5 ml 1% CMC in 0.05M sodium acetate buffer, pH7.5 at 40°C for 1 h. The reducing sugar released was assayed by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit (U) of cellulase was defined as the amount of enzyme liberating 1mg of glucose equivalent to per min under the assay conditions.

Optimization of Fermentation Conditions

A variety of factors like inoculums size (carbon source and cellulose quality), pH value, temperature, growth time etc. appears to control the cellulase yields (Immanuel *et al.*, 2006). To determine the optimum pH and temperature for the fermentation process media was adjusted at different levels of pH ranging from 6 to 8.5 and for temperature fermentation was carried out at an intervals of 5°C ranging from 25°C to 45°C. Another important parameter for enzyme production is fermentation period and for it the process was carried out up to 120h (Ray *et al.*, 2007). The fermentation medium was seeded with 1.0%, 2.0%, 3.0%, 4.0%, and 5.0% seed culture (tryptone soya broth) for optimizing the inoculum volume and incubated at 37°C (Ray *et al.*, 2007).

Scanning Electron Microscopy of the Strain

The morphological details of the strain *Paenibacillus alvei* (MTCC 7809) was analyzed by scanning electron microscopy (SEM). The specimen was prepared according to the methods of Felgenhauer (1987); fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) at room

temperature for 3h and then dehydration was done with ethanol followed by post fixation in 1.0% osmium tetroxide (OsO_4) in buffer for 2h. The sample was then coated with gold and the micrograph was taken with a JEOL 840 scanning electron microscope (Figure 2).

DNA Extraction for 16s rRNA Gene Sequencing

The strain was grown on nutrient agar medium for 48 h at 37°C for extraction of DNA. DNA was extracted using 250 ml lysis buffer (10 mM/l Tris HCl, 1 mM/l EDTA pH 8.0 with 3 mg/ml lysozyme) and incubated at 37°C for 15 min. 36 ml of 10% (w/v) sarkosyl were added and was further incubated at 37°C for another 30 min. NaCl was added (46.5 ml of a 5 M/l stock) to give a final concentration of 0.7 M/l; 332 ml of a 10% CTAB (prewarmed) solution in 0.7 M/l NaCl were added and the mixture was incubated for 20 min at 60°C. Solvent extraction by 664 ml chloroform was followed by centrifugation at 13,000 g for 5 min. DNA was precipitated after the addition of equal volume of isopropanol and chilling to -20°C and was pelleted by centrifugation at 13,000 g for 5 min. The DNA pellets were then dissolved in 50 ml TE buffer (pH 8.0) (Bell *et al.*, 1999).

PCR Amplification

PCR amplification was carried out in a reaction mixture containing 50 ng template DNA, PCR buffer (10 mM Tris HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl_2 0.001% gelatin), two universal primers each with 0.2 mM concentration, 2.5 U *Taq* DNA polymerase (Genie, Bangalore), and four deoxynucleoside triphosphate each in 0.2 mM concentration. The primer sequence selection was done from the regions of 16s rDNA and the 16s rDNA was amplified by using universal forward primer 5'-TGGAGAGTTTGATCCTGGCTCAG-3' and universal reverse primer 5'-TACCGCGGCTGCTGGCAC-3' (Hamasaki *et al.*, 2005; Hall *et al.*, 2003). After an initial denaturation for 2 min at 95°C, 25 cycles were completed each consisting of 1 min at 94°C, 50 sec at 60°C annealing temperature, and 1.5 min at 72°C. A final extension of 7 min at 72°C was applied. 10 ml of PCR product was loaded onto 2% agarose gel with ethidium bromide staining to determine the size (Lopez *et al.*, 2003). The PCR product was cloned in pGEMT Easy Vector (Roy and Chattopadhyay, 2008) and vector DNA was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologs for the microbe.

Results

Soil dilutions on nutrient agar plates after 7 days of incubation at 37°C were found to contain 36 different bacterial colonies indicating the bacterial flora richness of soil of mango orchards. After the initial screening for cellulase producing bacteria by Congo red method 3 isolates shown positive results but it was the *Paenibacillus alvei* (MTCC 7809) that produces the largest diameter of clear zone of CMC hydrolysis [Fig. 1] and was the strain of interest. The biochemical, morphological and physiological test analysis for the identification of the strain was performed and the results are summarized in Table 1. The rod shaped of the bacterium was confirmed based on the analysis of scanning electron microscopy (SEM) [Fig. 2] and the size ranging from 1.5 to 2 μm .

Cellulase yield was gradually increased with the increase of pH up to 8.0 and then declined beyond pH 8 [Fig. 3]. The temperature profile for fermentation condition is depicted in Figure 4. The maximum enzyme yield was observed at 40°C. Cellulase production was

found increase with the gradual increase in incubation period and was at the peak after 96 h incubation but decreased thereafter [Fig. 5]. The result of the effect of inoculum size reflects that 3% inoculum size was optimum for highest enzyme production though not significantly different from that in 2% inoculum size [Fig. 6]. PCR product of 16S rDNA was fractionated on 1% agarose gel electrophoresis [Fig. 8].

The sequence analysis of 1509 bp of the 16s rRNA gene of the strain was determined and compared using Ribosomal Database project and GenBank database. Based on nucleotides homology and phylogenetic analysis the microbe was identified to be *Paenibacillus alvei* and its nearest homologue species found to be *Paenibacillus apiarius* (ABO73201). The phylogenetic position of the strain was determined by constructing the phylogenetic tree based on comparison of the 16s rRNA gene sequences of the reference *Paenibacillus* sp. [Fig. 7]. Information about other close homologues for the microbe can be found from the alignment view in Table 2.

Table 1. Biochemical, morphological and physiological profiles of the cellulase producing strain *Paenibacillus alvei* (MTCC 7809)

Tests	Results	Tests	Results
Gram's reaction	+ ve	H ₂ S production	-
Endospore	+	Cytochrome Oxidase	+
Motility	+	Catalase Test	+
Fluorescence (UV)	-	Gelatin hydrolysis	+
Growth under anaerobic condition	+	Arginine dihydrolase	+
Indole Test	-	Lysine decarboxylase	-
Methyl Red Test	-	<u>Acid production from carbohydrates</u>	
VP Test	-	Arabinose	+
Starch hydrolysis	+	Dextrose	+
Citrate utilization	-	Dulcitol	-
Casein hydrolysis	+	Galactose	+
Urea hydrolysis	-	Lactose	-
Nitrate reduction	+	Mannitol	+
Growth on MacConkey Agar	-	Maltose	+
		Sucrose	-



Fig 1. Production of the halo zones around the colonies of *Paenibacillus alvei* (MTCC 7809) after staining with Congo red that indicates the cellulase hydrolysis

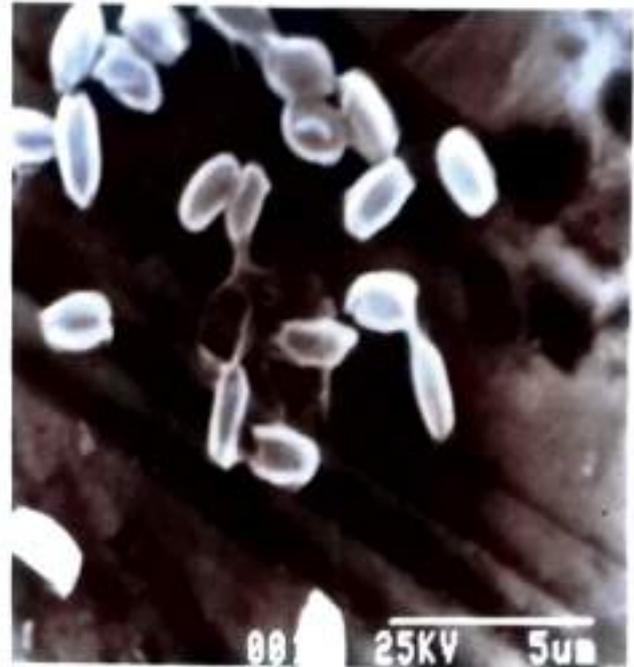


Fig 2. Scanning electron micrograph of *Paenibacillus alvei* (MTCC 7809) after dehydration and fixation indicating the morphological details

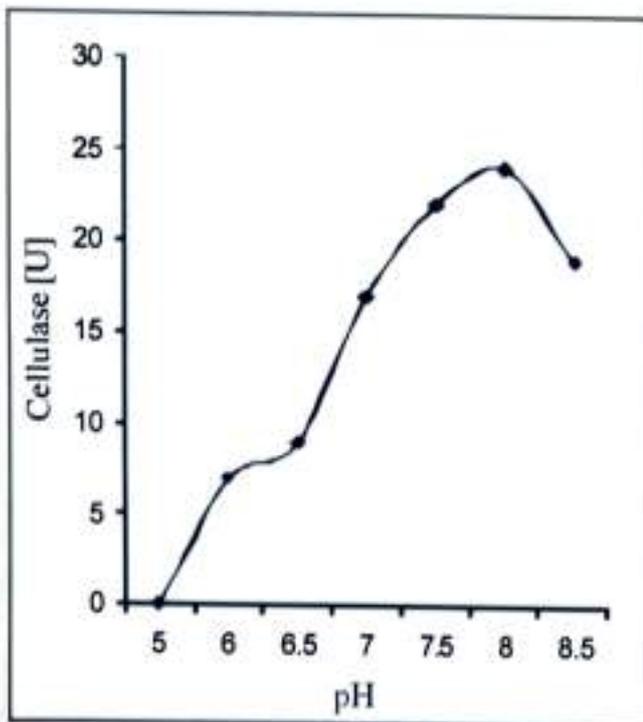


Fig 3. Effect of pH on cellulase production by *Paenibacillus alvei*

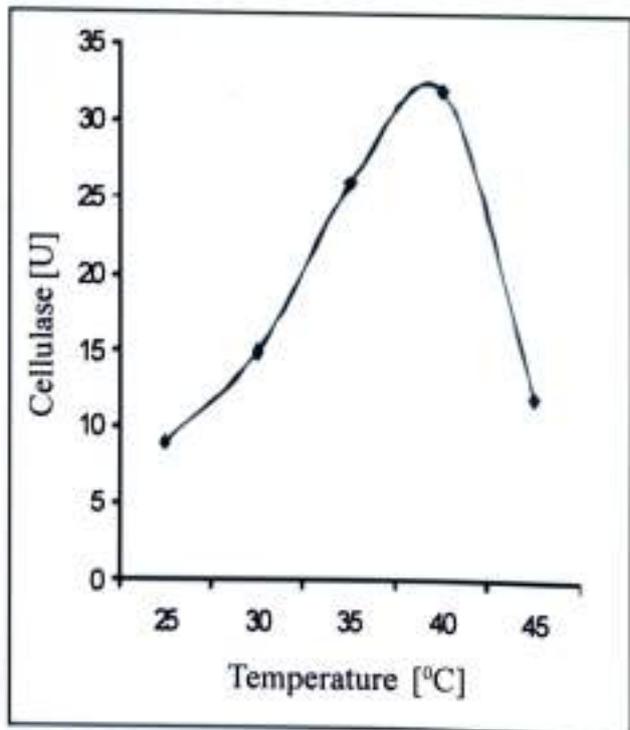


Fig 4. Effect of temperature on cellulase production by *Paenibacillus alvei*

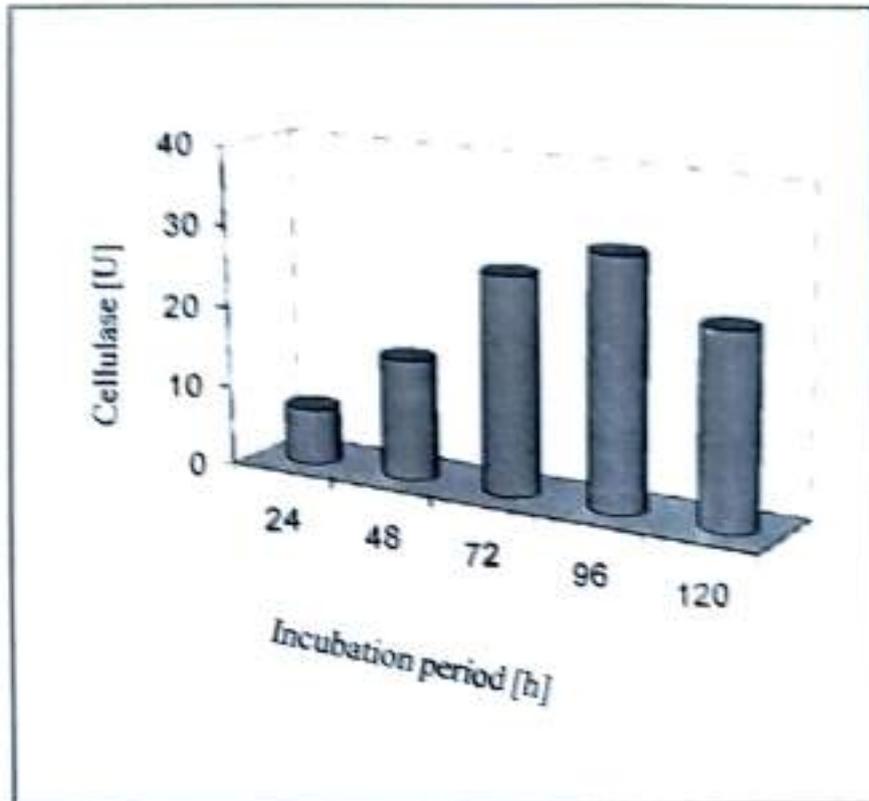


Fig. 5. Effect of incubation period on cellulase production by *Paenibacillus alvei*

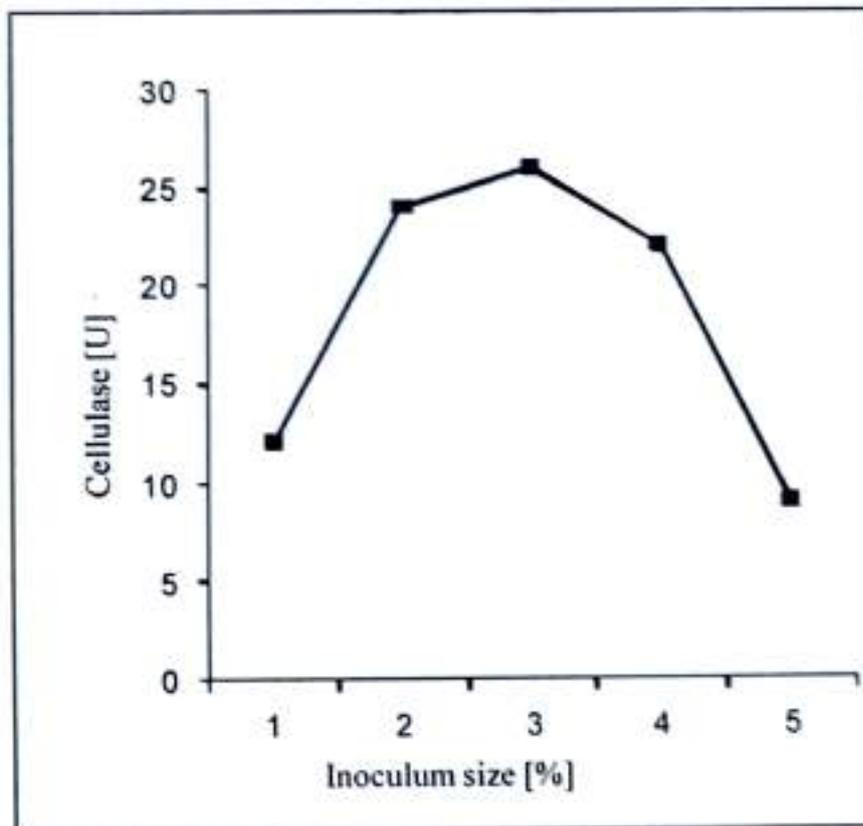


Fig. 6. Effect of percentage of inoculum size on cellulase production by *Paenibacillus alvei*

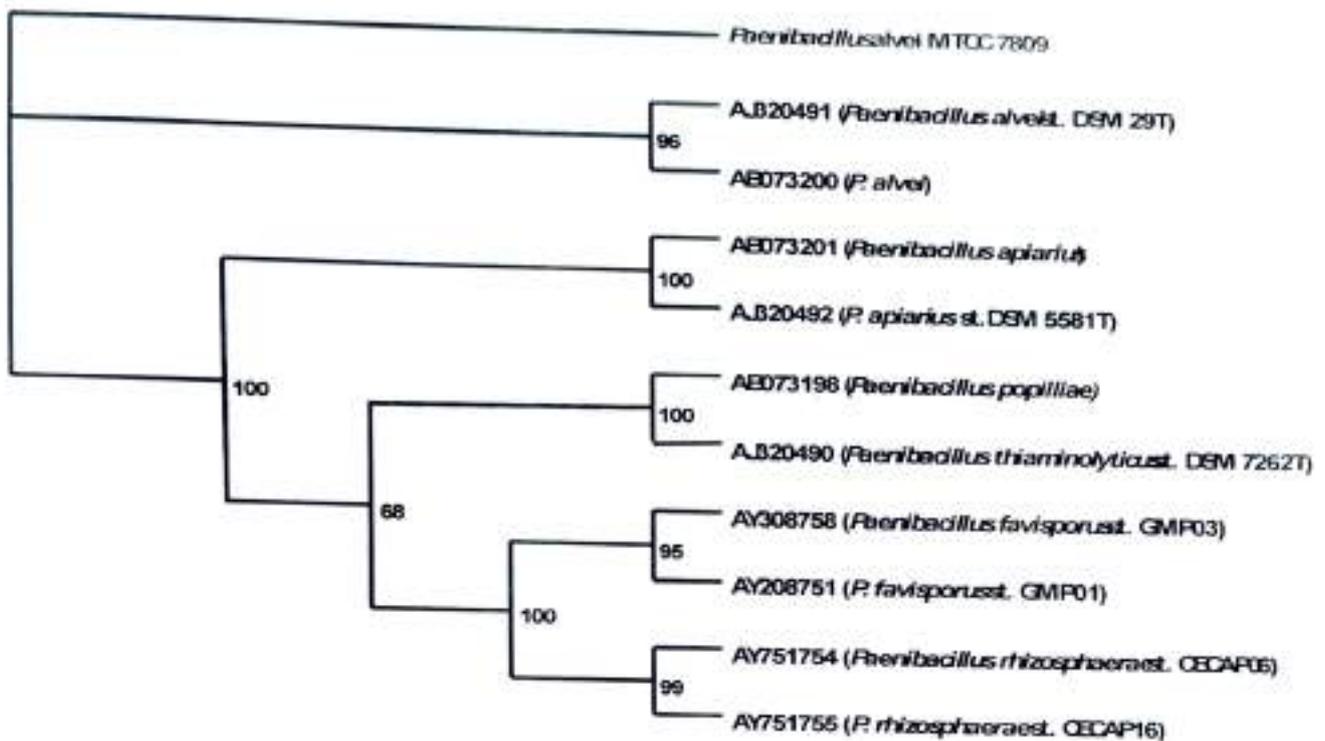


Fig 7. Phylogenetic dendrogram based on 16s rDNA sequence data indicating position of the isolate among the other representatives of the genus *Paenibacillus*. The sequences used in the analysis were obtained from Ribosomal Database project and GenBank. The scale bar indicates evolutionary distance



Fig 8. PCR product of 16rDNA amplification was fractionated on 1% agarose gel electrophoresis after staining with ethidium bromide Lane1, 500 bp ladder DNA marker and lanes 2 &3 showing the 1.5 kb band of 16S rDNA of *Paenibacillus alvei* MTCC 7809 in duplicate

Table 2. Percentage homology based on nucleotide sequence to determine the close homologues of the strain

Sl. No.	Isolates	Percent Homology										
		1	2	3	4	5	6	7	8	9	10	11
1	<i>Paenibacillus alvei</i> MTCC 7809	*	98	98	95	93	94	93	93	93	92	92
2	AJ320491		*	99	96	94	95	94	94	94	93	93
3	AB073200			*	96	94	95	94	94	94	94	94
4	AB073201				*	97	95	95	94	94	94	94
5	AJ320492					*	93	93	92	92	92	92
6	AB073198						*	99	93	93	93	93
7	AJ320490							*	93	93	93	92
8	AY308758								*	99	99	99
9	AY208751									*	99	99
10	AY751754										*	99
11	AY751755											*

Discussions

Cellulase productions depend on a complex relationship involving a variety of factors, which are illustrated. Established fermentation techniques allow microbial enzymes to be produced in large quantities. The enzyme production is controlled in microorganisms and therefore, to improve the productivity the parameters can be exploited and modified (Ray *et al.*, 2007). Commercially available cellulases have optimum activity at pH ranges from 4.0 to 6.0 (Horikoshi, 1999). However, the use of alkaliphiles, which produces alkaline cellulases for laundry purpose in industrial scale has an advantage for their high pH range. Alkaline cellulase from *Bacillus* sp. KSM635 was found to have detergent effects on cotton cloth with reduced washing time and allow washing at lower temperature under European washing conditions (Hoshino *et al.*, 2000). Optimum cellulase activity of *Penicillium* sp. CR-316 (Picart *et al.*, 2007) was recorded at 65°C, that is higher than *Paenibacillus alvei* (MTCC 7809) and far lower pH at 4.5. The thermostable alkaline cellulase strictly alkaliphilic strain of *Bacillus* sp. KSM-S237 (Hakamada *et al.*, 1997) was reported to have optimum pH and temperature of 8.6 to 9.0 and 45°C respectively which is nearly similar to that of our strain. Dasilva *et al.*, (1993) reported *Bacillus* sp. strain B38-2 and *Streptomyces* sp. strain S36-2 with an optimum pH and temperature for the crude enzyme activity ranging from 7.0 to 8.0 at 60°C and 6.0 to 7.0 at 55°C respectively.

The above discussion indicates that the thermostability with high pH tolerance of the enzyme from *Paenibacillus alvei* (MTCC 7809) supports its potential use in detergent industry. However, more research is suggested before its commercial exploitation.

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