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Characterization and cell immobilization of a potent amylase producing mesophilic soil bacteria *Bacillus cereus* strain BRSC-S-A26MB

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Modern biotechnological approach utilizes microbial enzymes having a wide variety of industrial application. Among these enzymes, α-amylase has a high demand in industries such as pharmaceuticals, textiles, food processing etc. Considering the importance of microbial amylase, this study has been focused on isolating, characterizing and identifying potent amylase producing organisms from a natural source like soil. Soil samples were used for isolation of bacteria on nutrient agar which were then screened for their amylase producing ability. Among 71 isolates, 35 isolates were able to produce α-amylase enzyme. Four of them showed high to moderate responses which were further checked for amylase production quantitatively. However, only one of the strains was found to be the best producer of amylase. Hence it was chosen for further characterization. The microorganism was characterized by biochemical tests and 16s rRNA gene sequencing. It was an aerobic, mesophilic, endospore forming, Gram-positive, short rod, capable of fermenting glucose, hydrolyzing gelatin and was positive for simmon citrate, methyl red, catalase and urease tests. It showed 99% homology with *Bacillus cereus.* The organism was hence designated as *B. cereus* strain BRSC-S-A26MB (KC460310). Optimum pH and temperature for the growth of the organism was found to be 7.0 and 37°C respectively. *B. cereus* strain BRSC-S-A26MB showed varying response to heavy metal tolerance and antibiotic sensitivity. It was found that the organism attained late stationary phase of growth in the 72 h, where the enzyme production was maximum (3197.15 U/ml). Enhancement of the enzyme production was observed with immobilized cells when compared to free cells.

Key words: Amylase, *Bacillus cereus*, immobilization, industrial soil, mesophilic,

INTRODUCTION

The increasing demand for novel biocatalysts stimulates exploration of resources from the soil, which is a natural habitat of uncountable species of microorganism. High yielding microbial strains have specific genetic and physiological properties and hence often used for maximum productivity in biotechnological processes. Nowadays, enzymes, chiefly of microbial origin are popularly used by industries due to their higher yield and thermo stability. The chemical hydrolysis of starch have been largely replaced by use of microbial amylases due to their specificity of reaction, mild conditions required for reaction, and less energy consumption than the conventional chemical methods (Shaw *et al.*, 1995; Reddy *et al.*, 2003; Thippeswamy *et al.*, 2006). Among these amylases have gained enormous importance because they are used commercially in the biotechnological process such as starch degradation, detergent, foodstuff, pharmaceutical, textile and paper manufacturing (Burhan *et al.*, 2003). Additionally they play an important role in the biogeochemical cycle of carbon (Pandey *et al.*,

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2000;). Bacterial extra cellular enzymes constitute approximately 20% of the market enzyme (Niu *et al.*, 2009). Amylolytic enzymes are widely distributed in bacteria and fungi. They have been categorized as exo-acting (α-amylase), endo-acting (α-amylase) and de-branching enzyme. (Mishra and Behera, 2008; Dheeran *et al.*, 2010; Poddar *et al.*, 2011).

Acidophilic, alkalophilic and thermo acidophilic bacteria have been reported to produce unusual bacterial amylases (Boyer and Ingel, 1972). Amylase producing microorganisms have been successfully isolated from soil samples, cultivated fields or industrial and domestic waste deposits and have been characterized (Mishra and Behera, 2008; Alamri, 2010; Al-Za Zaee *et al.*, 2011; Bakri *et al.*, 2012; Devi *et al.*, 2012; Kaur *et al.*, 2012). Amongst the microorganisms known to produce amylase *Bacillus sp.* has been found to be widely known for the enzyme production (Thippeswamy *et al.*, 2006; Alamri, 2010). However different strains of *Pseudomonas stutzeri* have also been shown to produce amylases (Zhang and Zeng, 2011).

Since bacterial cells have been found to be a rich source of amylase production attempts have been made to immobilize them on solid matrix as a popular and promising approach for higher productivity by protecting the cells from shear forces and reuse the cells several times. Several matrices have been used for such immobilization studies (Adinarayana *et al.*, 2005; Konsoula and Liakopoulou-Kyriakides, 2006; Poddar *et al.*, 2011). The aim of the present study has been to isolate and screen for amylase producing bacteria from the soil samples collected from different industrial belts of West Bengal. The most potent amylase producer are to be further characterized, identified and explored for usage in starch degradation both as free and immobilized cells.

MATERIALS AND METHODS

Chemicals

Iodine solution, Crystal violet, Glucose, Fructose and Sucrose, 3, 5-dinitrosalicylic acid reagent (DNS), Calcium chloride ($CaCl_2$), Copper chloride ($CuCl_2$), Manganese chloride ($Mncl_2\ 4H_2O$), Lead nitrate [$Pb(NO_3)_2$]. Glucose-Peptone (GP) broth, Ammonium Sulphate (AS) broth, Minimal media (MM), Nutrient Broth (NB), Luria Broth (LB), Fermentation Broth (FM), Luria agar (LA), Starch agar (SA) medium and other chemicals used in this study were of analytical grade and were purchased from SRL (Mumbai) and Himedia (Mumbai).

Isolation and screening of amylase producing microorganism

Soil samples were collected aseptically in sterile containers from different industrial regions of West Bengal, India. Organisms were isolated from soil samples collected from different locations. 1 g of each soil sample was serially diluted in sterile water, mixed thoroughly and carefully spread on Luria agar plates. All the plates were incubated at 37°C and after 24 h colonies were observed. Colonies were henceforth maintained on Luria Agar slants. The stock cultures were preserved in 80% glycerol at -20°C. Individual bacterial colonies were streaked on starch agar plates, incubated at 37°C for 24-48 h and observed for zone of clearance surrounding the growth of the organisms upon flooding with Gram's iodine solution. Organisms with high amylolytic response on starch agar plates were further screened in Luria Broth for extracellular amylase production.

Characterization and identification of microorganism

The isolate was characterized both morphologically, microscopically (both Light and Transmission electron microscope) and biochemically (standard methods). Heavy metal tolerance of the isolate was scored by measuring the relative growth in metal supplemented Luria Broth with respect to control. Antibiotic sensitivity of the organism was determined by using antibiotic impregnated discs on nutrient agar incorporated plates (Octadiscs G VIII plus and G I minus, Himedia). Optimum pH and temperature was also determined by growing the isolate in Luria broth with varying pH (2-10) and temperature (20^0C-50^0C). The isolate was grown in different media to determine the most suitable medium for its growth and enzyme production such as Glucose-Peptone (GP) broth, Ammonium Sulphate (AS) broth, Minimal media (MM), Nutrient Broth (NB), Luria Broth (LB) (Cappuccino and Sherman, 2008) and Fermentation Broth (FM) (Konsoula and Liakopoulou-Kyriakides, 2006). 16s rRNA sequence homology was determined for tentative identification of the organism. (SciGenome).

Growth characteristics and enzyme production

Freshly grown bacterial culture (at 1% level) was

inoculated in 50 ml Fermentation Medium in nephelometric flask and incubated at constant shaking (150 rpm) at 37°C for 96 h. Growth was scored by measuring the optical density (O.D.) at 540 nm using the colorimeter (Elico C-157) at regular intervals of time. Extracellular enzyme production was estimated in the culture supernatant by removal of cells by centrifugation at 10000 rpm for 10 min at 4°C at different time intervals.

Enzyme assay

One ml of crude supernatant was added to 1 ml starch solution (1 mg/ml) and incubated for 3 min at 37°C. After incubation the reaction was stopped by the addition of 2 ml of 3, 5-dinitrosalicylic acid reagent (DNS) and kept in boiling water bath for 5 min. The reaction mixture was then cooled under running tap water and 10 ml of distilled water was added to it. Absorbance was measured using a double beam UV-VIS spectrophotometer (Thermoscientific UV 10) at 540 nm against an enzyme blank (Bernfeld, 1955).

Immobilization

The organism was cultivated at 37°C on a rotary shaker for 24 h in Luria Bertani medium. Cells were harvested by centrifugation (5000 rpm for 20 min at 4°C) and were used both for immobilization and in experiments with free cells. Wet cells (20 mg/ml) were resuspended in 12.5 ml of 2% sodium alginate (w/v). The mixture was extruded drop wise in 25 ml 3.5% $CaCl_2$ solution which on solidification forms capsules entrapping the bacterial cells. After hardening, excess $CaCl_2$ was washed with saline solution. Both immobilized and free cells were then transferred to fermentation medium to estimate the extracellular enzyme production at varying time point (Konsoula and Liakopoulou-Kyriakides, 2006).

RESULTS AND DISCUSSION

Isolation and screening of amylase producing microorganisms

The soil isolates were screened for extracellular amylase production on starch agar plates. A total of 35 test organisms capable of producing clear zones on starch agar plates were obtained from soil samples collected from three different regions. The amylolytic response of the organisms is shown in Fig. 1. It was observed that 2.81% of the organisms were highly amylolytic (A26 and A19), 2.8 % were moderately amylolytic (A7 amd A28), whereas 43.66 % showed poor response, and 50.70% showed negative. Although 35 isolates were able to produce α-amylase enzyme four of them showed high and moderate response. So they were checked for amylase production quantatively. Among them only A26 was found to be the best producer of amylase (Figs. 1a & 1b). Hence A26 was further characterized.

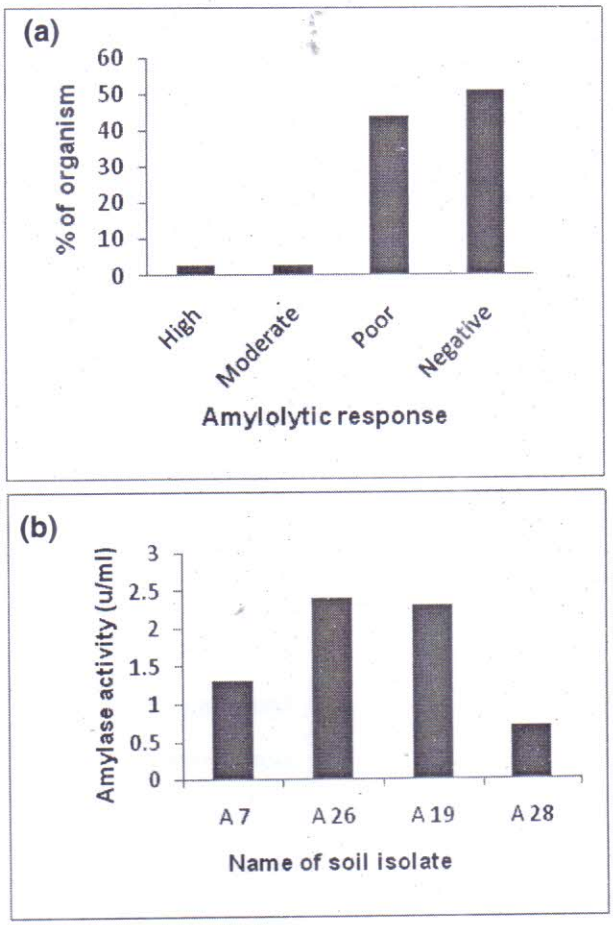


Fig. 1 : (a) Response of the soil isolates in starch agar plates flooded with Gram's iodine. Starch-agar plates streaked with bacterial isolates were flooded with iodine and response of the organisms were scored as high, moderate, poor and negative. **(b)** Amylolytic activity in the cell supernatant of the soil isolates. Organisms showing high amylolytic response on starch-agar plates were screened for amylase production in the cell supernatant.

Characterization and identification of microorganism A26

Cultural characteristics and Microscopic observation

Cultural characteristics of the A26 when streaked

Table1 : Characteristics of A 26

Cultural characteristics	Colony morphology on nutrient agar plate	Circular
	Growth on agar slant	Filiform
	Growth in nutrient broth	Flocculent
Staining characteristics	Positive staining	Short Rod
	Negative staining	Short Rod
	Gram staining	Gram-positive, Short Rod
	Endospore staining	Positive
Oxygen tolerance		Aerobe
Biochemical tests	Catalase test	Feebly +ve
	Urease test	+ve
	Fermentation of Glucose	+ve
	Fermentation of Fructose	Feebly +ve
	Fermentation of Sucrose	-ve
	Gelatin hydrolysis	+ve
	Indole test	-ve
	H_2S production	-ve
	Casein hydrolysis	-ve
	Simmon citrate	+ve
	Methyl Red test	+ve
	VP test	-ve
	Deaminase test	-ve

on nutrient agar plates, nutrient agar slants and grown in nutrient broth is shown in Table 1. On nutrient agar plates the colonies was circular and on agar slant they were filiform in nature. Growth in nutrient broth was flocculent in nature. Simple (positive and negative staining) and differential staining (Gram staining) showed that the organism was a short Gram-positive rod. Transmission electron microscopy of A26 is shown in Fig 2. A26 was found to be an endospore former (Table 1). Oxygen tolerance of the bacterium A26 was also determined. A26 when grown in Luria Agar stab as shown in Table 1 was found to be aerobic in nature.

Biochemical characterization

As shown in Table 1, A26 was urease positive, Simmon citrate and methyl red positive, could ferment glucose, hydrolyze gelatin, and was feebly catalase positive, and ferment fructose feebly but was unable to ferment sucrose.

Phylogeny

Based on nucleotide homology and phylogenetic analysis A26 showed significant similarity with *Bacillus cereus* (Fig. 2). It was submitted in the gene bank, NCBI and was assigned as *Bacillus cereus*

strain BRSC-S-A26MB (KC460310) (SciGenome) (Fig. 3a & 3b).

Heavy metal resistance and antibiotic sensitivity

The growth of the organism was almost inhibited at the highest concentration of the metals tested. Gradual decrease in relative growth was observed

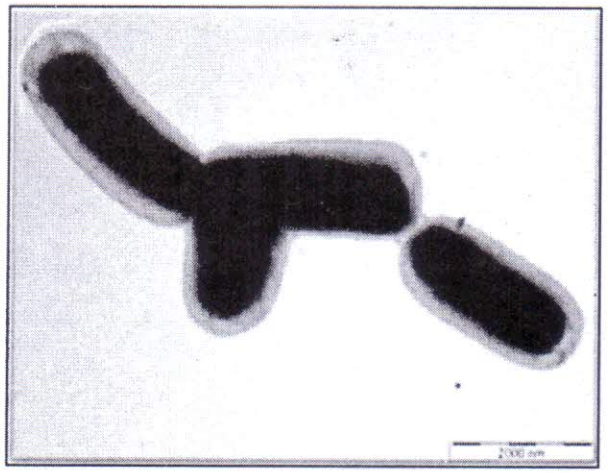


Fig. 2 : TEM (Transmission Electron Microscope) Micrographs of *B. cereus* strain BRSC-S-A26MB (9.9 k magnification)

with increasing concentration of cadmium, copper, lead and manganese (Fig. 4a). Response to antibiotic impregnated discs (Octodisc GVIII-plus: Octodisc G-I minus) showed that *B. cereus* strain BRSC-S-A26MB was resistant to Co-Trimoxazole, Penicillin-B, Ciprofloxacin, Ampicilin, Colistin, Nitrofurantoin. It showed intermediate response to Bacitracin and was sensitive to Gentamicin, Neomycin, Tetracycline, Chloramphenicol, Polymyxin B, and Streptomycin (Fig. 4b).

Growth and enzyme production by B. cereus strain BRSC-S-A26MB

Growth of *B. cereus* strain BRSC-S-A26MB in different media scored as O.D values clearly showed that fermentation media proved to be the best for growth although growth in Luria broth was fairly comparable (Fig. 5a). Hence growth and enzyme production by the organism was further determined in fermentation media (Fig. 5b). As shown in Fig. 5b, the growth pattern of *B. cereus* strain BRSC-S-A26MB revealed that after 3 h of lag phase, it entered log phase which continued for a period of

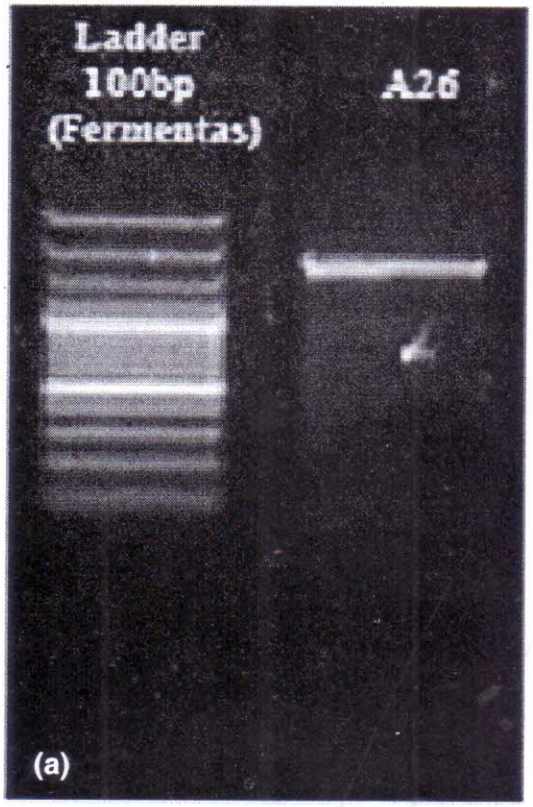
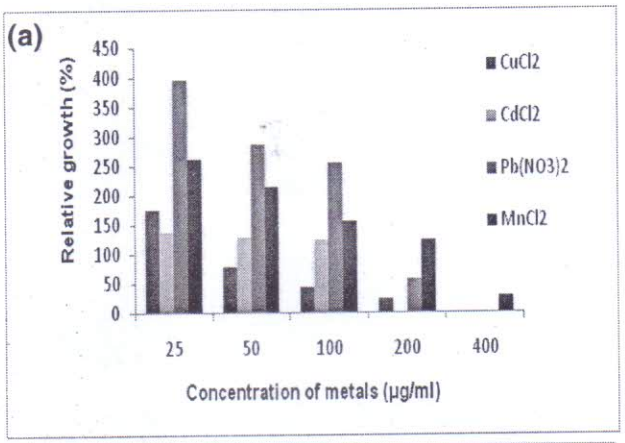


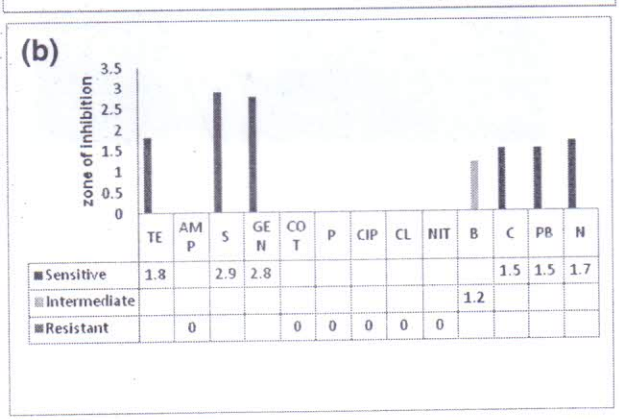
Fig. 3 : Phylogenetic analysis of A26 (Scigenom Labs Pvt. Ltd). **(a)** 16S r DNA gene amplified with F & R primers; **(b)** Phylogram.DNA was isolated, electrophoresed in a 1% agarose gel for obtaining the 16s rRNA genes which was then PCR amplified with F (5'-AGAGTTTGATCCTGGCTCAG-3') and R(5'-ACGGCTACCTTGTTACACTT-3') primers. Amplicon was electrophoresed in a 1% agarose gel and sequence analysis was done by using BLAST search of NCBI. Finally based on the maximum identity scored Dendogram was constructed (SciGenome).

3-4 h. The stationary phase was attained after 24 h of incubation at 37°C. Growth and enzyme production by *B. cereus* strain BRSC-S-A26MB was determined in fermentation media. It was found that although the organism attained late stationary

phase of growth in the 72 h enzyme production was maximum in the late stationary phase of growth (Fig. 5b).

pH and temperature for growth and immobilization of bacterial cells



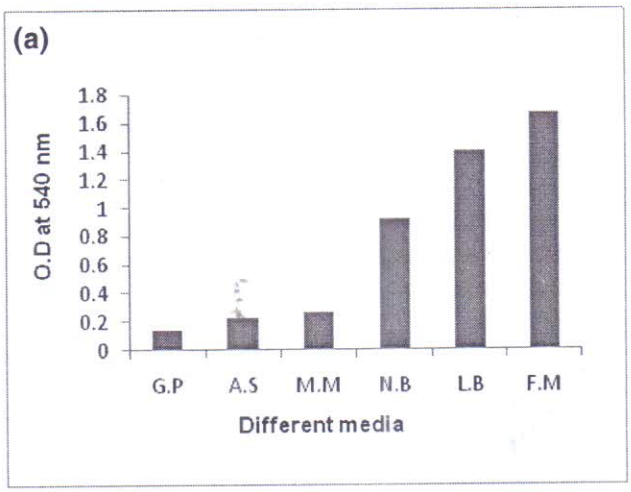


	TE	AMP	S	GEN	COT	P	CIP	CL	NIT	B	C	PB	N
Sensitive	1.8		2.9	2.8							1.5	1.5	1.7
Intermediate										1.2			
Resistant		0			0	0	0	0	0				

Fig. 4 : Heavy metal resistance and antibiotic sensitivity. (a) Heavy metal tolerance pattern of *B. cereus* strain BRSC-S-A26MB. Tolerance to heavy metals by *B. cereus* strain BRSC-S-A26MB was scored by measuring the relative growth in nutrient broth supplemented with varying concentration of metals. (b) Antibiotic sensitivity profile of *B. cereus* strain BRSC-S-A26MB. Antibiotic sensitivity profile of the bacterial strain was tested using Octodisc GVIII-plus: Octodisc G-I minus, Himedia. TE 30 = Tetracycline (30 mcg). AMP 10 = Ampicillin (10 mcg), S 10 = Streptomycin (10 mcg), GEN 10 = Gentamicin (10 mcg), COT 25 = Co-Trimoxazole (25 mcg), P 10 = Penicillin-B (10 units), CIP 10 = Ciprofloxacin (10 mcg), CL 10 = Colistin (10 mcg), NIT 300 = Nitrofurantoin (300 mcg). B 10 = Bacitracin (10 units). C 30 = Chloramphenicol (30 mcg). PB 300 = Polymyxin-B (300 units), N 30 = Neomycin (30 mcg).

The optimum pH and temperature required for the growth of *B. cereus* strain BRSC-S-A26MB was determined. It was found that the optimum pH and temperature for growth of organism was 7 (Fig. 6a) and 37°C (Fig. 6b) respectively.

Immobilization of bacterial cells in 2% sodium alginate showed higher (~ 2.0 fold increase) amylolytic



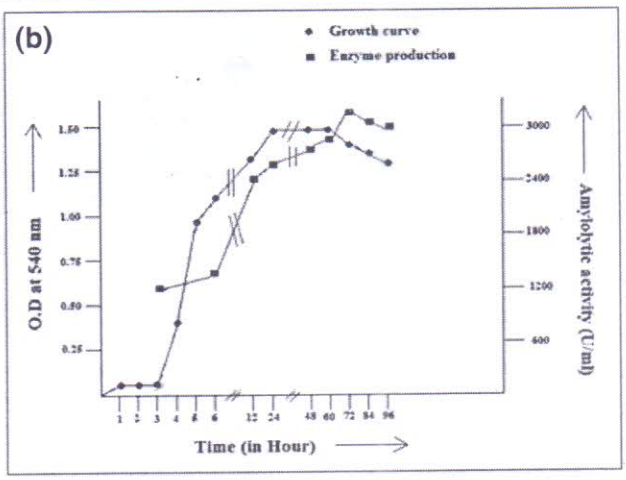


Fig. 5 : Effect of growth of *B. cereus* strain BRSC-S-A26MB in different media and enzyme production. **(a)** *B. cereus* strain BRSC-S-A26MB was grown in Glucose-Peptone (G.P) Broth, Ammonium Sulphate (A.S) Broth, Minimal media (M.M), Nutrient Broth (N.B), Luria Broth (L.B), Fermentation media (F.M) to determine the best medium for amylase production. **(b)** Correlation of growth and enzyme production by *B. cereus* strain BRSC-S-A26MB in Fermentation medium. *B. cereus* strain BRSC-S-A26MB was grown in fermentation medium and monitored for its growth and enzyme production.

activity as compared to the free cells (Fig. 7) when grown in fermentation medium. Experiments were performed in triplicates and average of three results indicated here.

Soils from different industrial locations of West Bengal were used to isolate potent amylase producing microorganisms. Among the 71 bacterial strains isolated from the soil samples 35 were found to be amylase positive. Two organisms were highly amylolytic, two were moderate; rest was either poor or negative in amylase production (Fig. 1a &1b). *B. cereus* strain BRSC-S-A26MB was found to have the ability to produce maximum amount of amylase among all the potent isolates and hence was

selected for further study.

Potent amylase producing strain(s) have been isolated from different environmental conditions encompassing domestic & industrial soil sediments (Mishra and Behera, 2008; Alamri, 2010; Kumar *et al.*, 2012). Among these *Bacillus* group of organisms predominate as amylase producers al-

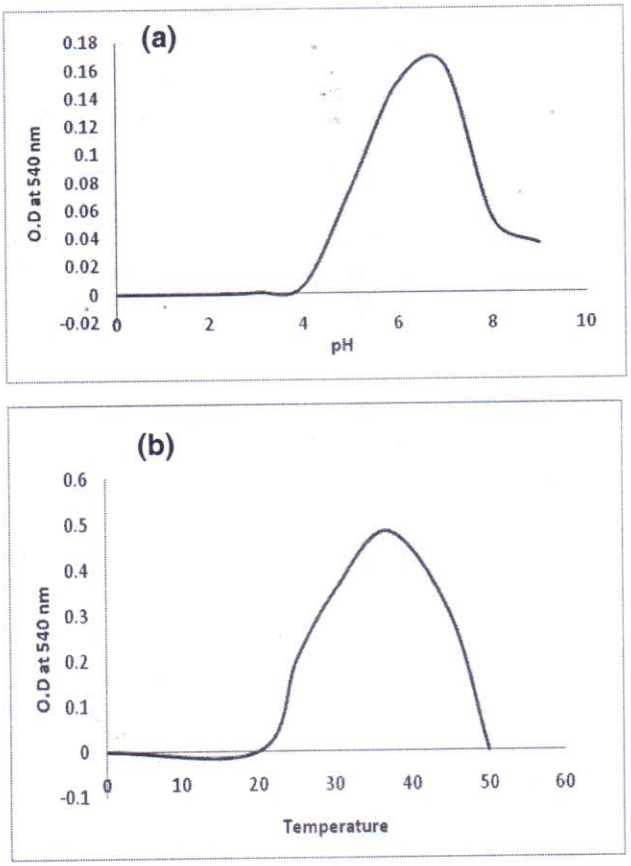


Fig. 6 : Optimum pH and temperature for growth of *B. cereus* strain BRSC-S-A26MB. **(a)** *B. cereus* strain BRSC-S-A26MB was grown in LB medium with varying pH and the optimum pH for growth was determined. **(b)** *B. cereus* strain BRSC-S-A26MB was grown in LB medium with varying temperature and the optimum temperature for growth was determined. Graph showing the optimum temperature for *B. cereus* strain BRSC-S-A26MB.

though *Pseudomonas* species have also being known to produce and secret a large number of useful enzymes (Alamri, 2010; Zhang and Zeng 2011). Kumar *et al.*, 2012; Devi *et al.*, 2012; Tambekar and Dhundale, 2013; Biochemical characterization of the soil bacteria *B. cereus* strain BRSC-S-A26MB as shown in Table 1 revealed that the strain was catalase and urease positive, whereas indole negative & have the ability to ferment glucose & sucrose. It was a Gram-positive, short rod, producing endospores and was aerobic in nature. Showing close homology with the *Bacillus* genus, the organism exhibits similar nature as reported by several group of workers (Behal *et al.*, 2006; Alamri, 2010; Al-ZaZaee *et al.*, 2011). However, variation has been found between the optimum temperature for growth and amylase production as reported earlier (Burhan *et al.*, 2003; Konsoula and Liakopoulou-Kyriakides, 2006). The optimum temperature and pH for growth of *B. cereus* strain BRSC-S-A26MB was found to be 37°C (Fig. 5a) & 7 (Fig. 5b) respectively. This may

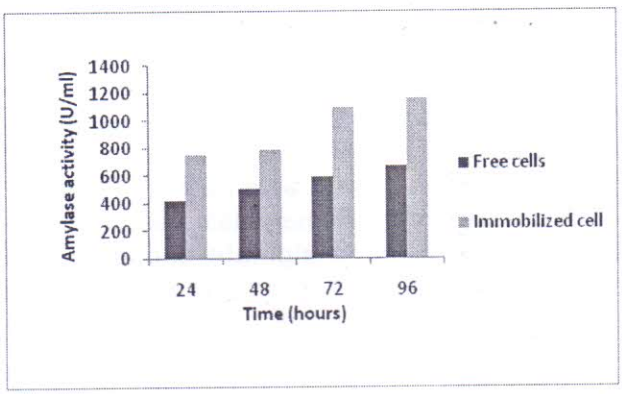


Fig. 7 : Amylase production by immobilized & free cells of *B. cereus* strain BRSC-S-A26MB. Bacterial cells were entrapped in calcium alginate beads & enzyme production was measured compared to free cells.

be correlated with the fact that the organism was originally isolated from a mesophilic environment unlike those strains reported to be thermophilic, acido or basophilic (Dheeran *et al.*, 2010; Alamri, 2010).

Optimizations of growth and enzyme production by bacterial isolates in different media have been determined (Santos and Martins, 2003). Enzyme production was growth associated as reported in *B. subtilis* (Stephenson *et al.*, 1998), *B. licheniformis* TCRDC- B13 (Bajpai and Bajpai, 1989) and *B. subtilis* JS-2004 (Asgher *et al.*, 2007). Correlation of growth characteristics with enzyme production by *B. cereus* strain BRSC-S-A26MB revealed that it is only in the late stationary phase of growth the organism produced maximum amount of enzyme. This is coherent with the fact that although the cells may not be exponentially dividing they were metabolically active.

Cell immobilization offers several advantages over free cells, such as relative ease of product separation, re-use of biocatalysts, prevention of washout, reduced risk of contamination and operational stability (Das *et al.*, 2007). Productivity of α-amy-

lase by immobilized cell was found to be significantly greater than that by free cells without alteration of the property of the enzyme (Konsoula and Liake Poulou-Kyriakides., 2004; Konsoula., 2006). In this study too as shown in Fig. 7 whole cell immobilization of *B. cereus* strain BRSC-S-A26MB have yielded greater enzyme production as compared to free cells. However some workers have shown contradictory results suggesting that immobilized biocatalyst produced fewer enzymes in comparison to free cells due to diffusional barrier and reduced oxygen availability to immobilized aerobic cells (Mamo and Gessesse, 1997); Kanasawud *et al.,* 1989). Although several procedure of immobilization have been used entrapment in calcium alginate gel offers many advantages due to its simplicity and non-toxic character (Gombotz and Wee., 1998; Kanasawud *et al.,* 2001; Poddar *et al.,* 2011). Hence preliminary data has been generated using calcium alginate capsules only whereas use of different immobilizing matrices and optimization of conditions favouring maximal enzyme production needs to be worked out.

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