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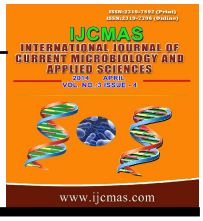

Original Research Article

Amylase production by *Bacillus cereus* strain BRSC-S-A26MB under optimal laboratory condition

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Keywords

Bacillus cereus strain BRSC-S-A26MB (KC460310), Fermentation medium, Conditions for optimization, Amylolytic activity.

A B S T R A C T

Amylases are one of the main enzymes used in food, fermentation and pharmaceutical industries. Due to the enormous importance of this enzyme, this study has focused on optimization of the conditions favoring the maximum enzyme production by *Bacillus cereus* strain BRSC-S-A26MB (KC460310), a potent amylase producing strain. Optimization of culture components and growth parameters is a prime step in using microorganisms in fermentation technology. Sucrose and casein were found to be the best carbon and organic nitrogen sources respectively that enhanced enzyme production. Starch supplemented at 0.5% level in the medium also was found to have a positive effect on enzyme production. Among the heavy metals tested copper and cobalt at a concentration of 25µg/ml favourably supported both growth and enzyme production by the organism. Among physiological parameters, maximum amylase production by isolate A26MB was attained at an initial temperature of 37°C and pH 6.5.

Introduction

Amylases are one of the main enzymes used in industry. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. However, with the advances in biotechnology, the amylase application has expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries.

Amylases have been purified earlier from various *Bacillus sp.* such as *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus licheniformis* SPT 27 [Aiyer, 2004]. Out of different factors affecting microbial environment, temperature is one of the most important as it influences most biochemical reactions [Chi et al., 2007]. Thereby psychrotolerant microbes have developed various adaptive strategies to live in harsh environmental conditions. Bacterial amylases are produced at a much wider range of temperature (30-50) (Ashwini et al., 2011). Certain hyper

thermophiles such as *Thermococcus profundus* and *Thermatogamaritima* have been reported to produce amylase at 80 °C (Vieille et al., 2001). pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. pH is known to affect the synthesis and secretion of a-amylase just like its stability (Fogarty et al., 1983). Bacterial cultures such as *B.subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* required an initial pH of 7.0.

Rhodothermus marinus was reported to yield good enzyme levels at initial pH range 7.5 to 8. In general, amylase activity is connected with the substrate utilization. The inducibility nature of amylase has been assured in different microorganisms (Aiyer, 2004; Abou Dobara et al., 2011). Amylase production is also appeared to be subjected to catabolite repression by maltose and glucose, like most other inducible enzymes that are affected by substrate hydrolytic products (Bhella & Altosaar, 1988; Morkeberg et al., 1995). However, amylase synthesis by *Bacillus* strains was reported to not subject to catabolite repression by monosaccharides (Kalishwaralal et al., 2010). Authors have classified xylose and fructose as strongly repressive to amylase synthesis (Gupta et al., 2003). Addition of starch to the medium has normally been employed for the production of amylase from various microorganisms as reported in the literature.

Nitrogen source as a basal component of the medium is a major factor affecting amylase production. Its effect was not only as a nitrogen source but also as a metal ion source and a pH controller as well. Many investigators had recorded that organic nitrogen sources supported maximum amylase production by various bacteria (Aqeel & Umar, 2010; Abou Dobara et al., 2011). The increased α-amylase production by organic nitrogen sources could be attributed to the high nutritional amino acids and vitamins content. However, various inorganic salts have been reported to support better production in fungi (Gupta et al., 2003). As a metal ion source, ammonium chloride was found to enhance the production of amylase by *T. vulgaris*, where chloride is a stabilizer, over that of other ammonium salts (Abou Dobara et al., 2011).

Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production (as most α-amylases are known to be metallo enzymes). Ca^{2+} ions are reported to be present in majority of these enzymes. Addition of $CaCl_2$ to the fermentation media increased the enzyme production (Francis et al., 2003). Positive results of the influence of $CaCl_2$ (0.1%) and NaCl (0.1 %) on α-amylase production in SSF using *Amaranthus* grains as substrate were recorded. $LiSO_4$ (20 mM) and $MgSO_4$ (1 mM) increased α-amylase production by *Bacillus* sp. I-3 (Sodhi et al., 2005), but $FeCl_3$ and$MgSO_4$ exhibited negative influence on α-amylase production.

Materials and Methods

Microorganism: Isolate A26MB (KC460310) that was originally isolated from soil samples from the industrial regions of West Bengal was used in this study. Freshly grown bacteria were used as inoculum at 1% level.

Chemicals: Strach agar, Iodine solution,

Ammonium sulphate, NaCl, $CaCl_2$, $MgSO_4$, Sucrose, Glycerol, Dextrose, Mannitol, Tryptone, Casein, Urea, Peptone, Yeast extract, Starch, $MnCl_2$, $NiCl_2$, $CuCl_2$, $CoCl_2$, Sodium di-hydrogen phosphate, Disodium hydrogen phosphate, 3,5-dinitrosalicylic acid reagent (DNS). Chemicals used were from SRL and Himedia.

Media and Buffers: Fermentation medium (Konsoula and Liakopoulou-Kyriakides, 2006), Phosphate buffer, Glycine-HCl (pH 3.0-4.0), Acetate buffer (pH 5.0-6.0), Phosphate buffer (6.0-8.0) Glycine NaoH (9.0-10.0).

Qualitative and quantitative screening for optimization of time for maximum amylase production by A26MB

Amylase production by A26MB was determined qualitatively by cup plate method and also quantitative method. A26 was inoculated in fermentation medium (at 1% level) and incubated at 37˚C for varying time period (24, 48, 72, 96 h) under constant shaking. The culture was centrifuged at 6000 rpm for 20 min at 4˚C and cell free culture supernatant was harvested. Wells were bored in starch agar plates and filled with the supernatant. The plates were incubated for 24 hours at 37˚C. Later on the plates were flooded with Gram's iodine solution to observe the halo zone. Also the amylolytic activity in the culture supernatant was determined quantitatively.

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 minutes. 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)**.** One unit of amylase (U) was defined as the quantity of enzyme extract releasing 1μmole of reducing sugar from soluble starch in a minute.

Partial purification of enzyme

The enzyme amylase was partially purified by ammonium sulphate precipitation followed by dialysis (Bollag et al., 1996). 50 ml of cell free extract was saturated with ammonium sulphate upto 80% saturation at 4°C in an ice bath. The precipitated protein was collected by centrifugation at 6000 rpm for 15 min at 4˚C and dissolved in a minimum volume of phosphate buffer (pH 6.5). The enzyme mixture was dialyzed at 4˚C against the same buffer with continuous stirring for three hours while changing the buffer after every hour. The final concentrated enzyme solution was assayed by DNSA method.

Molecular weight determination of Amylase produced by A26

The protein samples in crude culture supernatant of (24 h, 48 h, 72 h & 96 h) and 80% ammonium sulphate cut were run on 10% SDS PAGE following standard protocol at 40 mA constant current. The gel was stained with Coomassie Blue. Equivalent amount of sample were loaded in the wells. Molecular weight of the protein bands were determined using Page ruler prestained protein ladder (Fermentus).

Optimization of conditions for best enzyme production by A26

Effect of various parameters like pH, temperature, carbon, nitrogen, heavy metals and substrate on enzyme production were determined. A26MB was inoculated in the fermentation medium and incubated under varying conditions for a period of 72 h with continuous shaking. Amylolytic activity in the cell free culture supernatant was measured by DNSA method.

pH

Optimum pH for enzyme production by A26MB was established by using production media maintained at different pH (4-10).

Temperature

The optimum temperature for amylase production by A26MB was determined by incubating the fermentation medium inoculated with the bacterial strain at varying temperatures ranging from 20-50˚C.

Effect of substrate concentration on amylase production

Fermentation medium was supplemented with 0.5-2% (w/v) of starch to evaluate the effect of substrate concentration on amylase production by A26MB.

Effect of carbon, nitrogen and heavy metals on amylase production

Effect of carbon and nitrogen sources and heavy metals on enzyme production by A26MB was studied by supplementing the production media with different carbon sources (sucrose, fructose, mannitol and dextrose), nitrogen sources (tryptone, casein, urea, peptone, yeast extract) and heavy metals ($CuCl_2$, $CoCl_2$, $MnCl_2$, $NiCl_2$.

Results and Discussion

Qualitative and quantitative screening for optimization of time for maximum amylase production by A26MB

The soil isolate A26MB when screened for amylase production for varying time periods (24 h, 48 h, 72 h, 96 h) by cup plate method shows that maximum zone of clearance occurs with respect to the supernatant from the bacterial culture grown for 72 h (Fig. 1a). Amylolytic activity in the culture supernatant was maximum (3197.15 U/ml) in the 72 h old culture as well (Fig. 1b) (1Unit/ml = amount of enzyme which releases 1µ mole glucose under the assay condition).

Partial purification of enzyme

The partial purification of the enzyme was performed by ammonium sulphate precipitation followed by dialysis. It revealed that pellet after ammonium sulphate cut yielded partially purified amylase with a specific activity of 119.21 U/mg protein and fold purification equals 0.91 whereas the dialyzed product yielded partially purified enzyme with a specific activity of 144.92 U/mg protein and fold purification equals 1.21 (Table 1).

Molecular weight determination of amylase produced by A26MB

SDS PAGE of crude culture supernatant of A26MB grown for different time period versus the dialyzed product obtained after ammonium sulphate precipitation is shown in Fig. 2. Stepwise purification of the

crude culture supernatant eventually showed the presence of two distinct bands corresponding to molecular weight ~50 kDa and ~92 kDa (approximately).

pH

pH of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane. In this study the effect of pH on amylase production by *Bacillus cereus* strain BRSC-S-A26MB was investigated with varying pH (4-10).

The optimum pH for enzyme production was found to be 6.5 above and below which there is a significant decrease in the enzyme production (Fig. 3).

Temperature

As shown in Figure 4 the optimum temperature for amylase production by A26MB was found to be 37°C and further increase of temperature resulted in decrease of enzyme production.

Effect of substrate concentration on amylase production

Starch is ubiquitous in nature and is an accessible source of energy for any organism. Although the strain grew well with all the different starch concentrations used, 0.5 % starch was necessary for the maximum amylase production. Figure 5 shows that *Bacillus cereus* strain BRSC-S-A26MB produced maximum amylase (2020.42 U/ml/) at 0.5% starch as substrate.

Increasing the starch concentration in the medium beyond 0.5 % did not lead to an increase in the enzyme activity.

Effect of carbon, nitrogen and heavy metals on amylase production

On supplementation of the various carbon sources (sucrose, glycerol, dextrose, mannitol) and nitrogen sources (tryptone, casein, urea, peptone, yeast extract) in the medium sucrose was found to be the best carbon source to enhance enzyme production (1021.32 U/ml) as shown in Fig. 6 and casein gave comparatively higher yield (3308.16 U/ml) of amylase than other nitrogen sources (Fig. 7). Among the heavy metals tested to affect enzyme production by A26MB $CuCl_2$ and $CoCl_2$ (2000 U/ml and 2331.26 U/ml respectively) enhanced enzyme production while others exhibited a significant reducing effect (Fig. 8). Heavy metals are abundant in soil and thus might have an impact on enzyme production by the organism.

Bacillus cereus strain BRSC-S-A26MB (KC460310) that was originally isolated from soil was found as an effective producer of amylase. Various factors often influence the nature of the metabolic process and the enzyme produced. The optimization of cultural conditions is thus very important for the production of enzymes. In this study effect of variation of pH, temperature, substrate concentration, heavy metal concentration on amylase production was assessed to determine the optimum condition for maximum enzyme yield in the culture supernatant. It showed that amylolytic activity of BRSC-S-A26MB is 3197.15 U/ml (Fig 1b). It has been reported that amylolytic activity in crude culture supernatant from *Bacillus* sp. SI-136 was 407.79 U/ml (Sarethy et al., 2012) where as 5010 U/ml in case of *B. cereus* GA6 (Roohi et al., 2013). The enzyme amylase was partially purified by ammonium

sulphate precipitation followed by dialysis. After ammonium sulfate (80%) precipitation specific activity of 119.21 U/mg and fold purification equals 0.91 whereas the dialyzed product yielded partially purified enzyme with a specific activity of 144.92 U/mg and fold purification equals 1.21(Table 1). However similar protein purification by ammonium sulfate precipitation of the culture supernatant of *B. cereus* GA6 resulted in 2.8 fold purification & 50.84 U/mg specific activity (Roohi et al., 2013).

Purification of the crude culture supernatant eventually showed the presence of two distinct bands corresponding to molecular weight ~50 kDa and ~92 kDa (approximately) (Fig 2). However variation in molecular weight of amylase has been reported by several workers. Purified amylase from *B. cereus* GA6 showed 55kDa product (Roohi et al., 2013). Reports have shown the highest molecular weight of amylases as 210 kDa, from *Chloroflexus aurantiacus* (Ratanakhanokchai et al., 1992); whereas, 10 kDa corresponding to *Bacillus caldolyticus* amylase was reported to be the lowest value (Gupta et al., 2003). The optimum pH for enzyme production in BRSC-S-A26MB was found to be 6.5 (Fig 3). *Bacillus sphericus* also has been found to produce α-amylase if grown at pH 6-9 range (Utong et al., 2006). Hence slightly acidic pH favoured better production of amylase. Temperature is also one of the important parameters that have to be controlled for any enzyme production (Chi et al., 2007). The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic. Bacterial amylases are produced at a much wider range of temperature (30-50°C) (Ashwini et al., 2011). The optimum temperature for enzyme production in BRSC-S-A26MB was found to be 37°C (Fig 4). So, it is mesophilic. *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce amylase at temperatures ranging from 37–60°C (Mielelz et al., 1983; Mishra et al., 2005). Certain hyper thermophiles such as *Thermococcus profundus* and *Thermatogamaritima* have been reported to produce amylase at 80°C (Vieille et al., 2001). However our study reveals that the organism used in this study is mesophilic in nature and the amylase produced by it is not as thermotolerant as reported in literature.

Bacillus cereus strain BRSC-S-A26MB produced maximum amylase (2020.42 U/ml/) at 0.5% starch as substrate (Fig 5). Best amylase production by *B. licheniformis* CUMC 305 was also obtained with a starch concentration of less than 1 % (Krishnan et al., 1983). At higher starch concentrations, the enzyme productivity was comparatively low; and such a behavior may be attributed to the high viscosity of the broth at such concentrations, which interferes with O_2 transfer leading to a limitation of dissolved O_2 for bacterial growth. Amylase production appears to be subjected to catabolite repression by maltose and glucose, like most other inducible enzymes that are affected by substrate hydrolytic products (Bhella & Altosaar 1988; Morkeberg et al., 1995). However, amylase synthesis by *Bacillus* strains was reported not to be subjected to catabolite repression by monosaccharides (Kalishwaralal et al., 2010). Authors have classified xylose and fructose as strongly repressive to amylase synthesis (Gupta et al., 2003).

Fig. 1 (a) **Qualitative demonstration of amylolytic activity by A26MB**
The extracellular supernatant was added in wells bored on starch agar plates flooded with iodine to observe the halo zone. (b) Bacterial cells were inoculated into fermentation media and incubated for 24, 48, 72, 96 h and the amylolytic activity in the supernatant was measured.

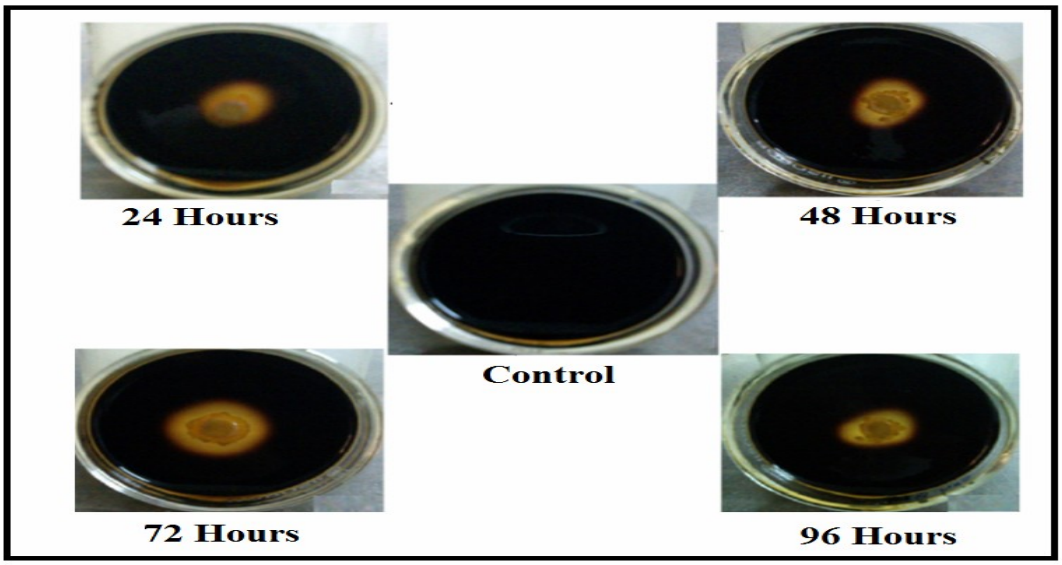


Fig. 1(b)

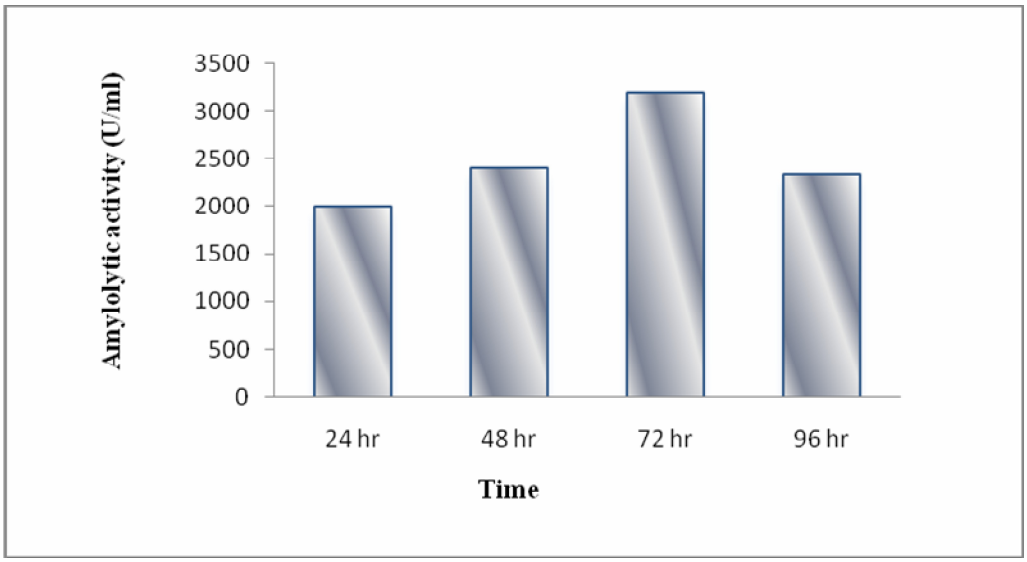


Table.1 Partial purification of amylase from A26MB

Partial purification of amylase was performed by ammonium sulphate precipitation followed by dialysis

Purification steps	Enzyme Activity (U/ml)	Specific activity (U/mg)	Fold purification
Crude sup	**3197.15**	**130.49**	**-**
Pellet after Ammonium sulphate cut	**3308.16**	**119.21**	**0.91**
Dialyzed sup	**3552.39**	**144.99**	**1.21**

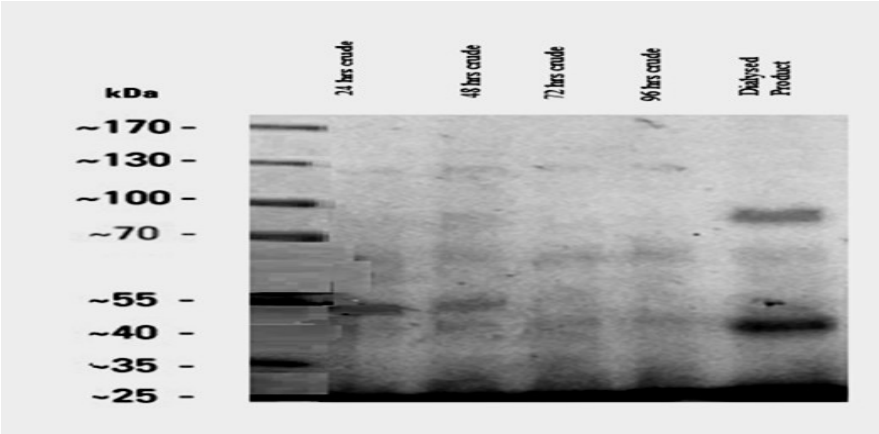


Fig. 2 SDS PAGE gel of crude culture supernatant and partially purified amylase from A26MB.
SDS PAGE of crude culture supernatant of A26 grown for different time period shows many band corresponding to the amylase product; however the dialyzed product obtained after ammonium sulphate precipitation shows the presence of two distinct bands corresponding to molecular weight ~50 kDa and ~92 kDa (approximately) which could be due to two subunits of the enzyme.

Fig.3 Effect of pH on amylase production by A26MB

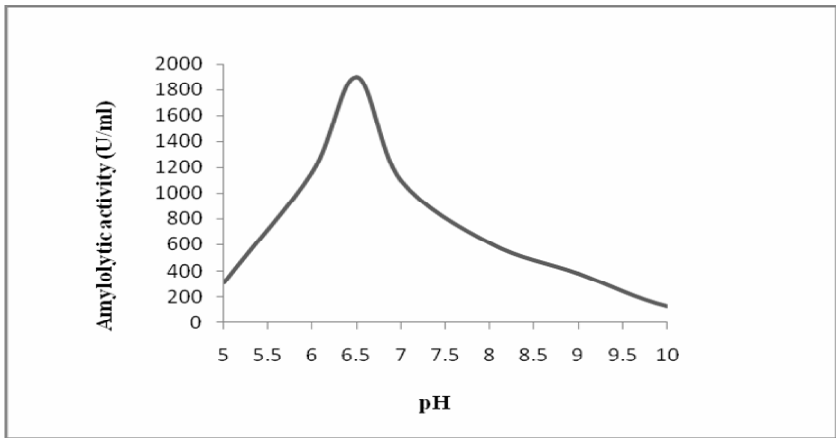


Fig.4 Effect of temperature on amylase production by A26MB

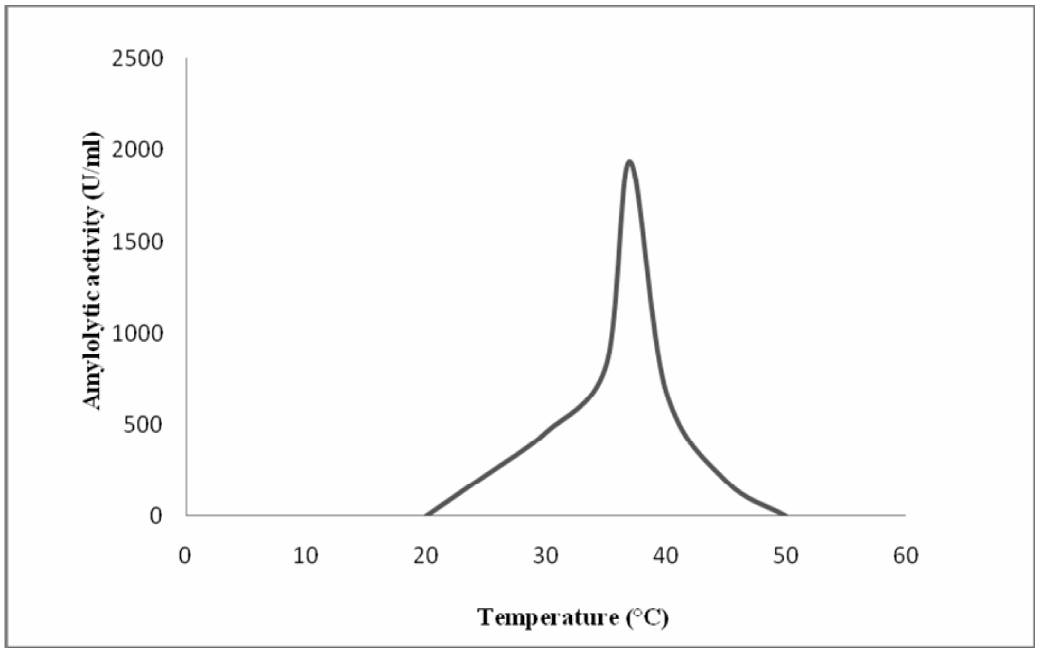


Fig.5 Effect of different concentration of starch on growth and amylolytic activity by A26MB.
Fermentation medium supplemented with different concentration of starch was inoculated with A26, incubated at 37°C for 72 h and amylolytic activity in culture supernatant was measured.

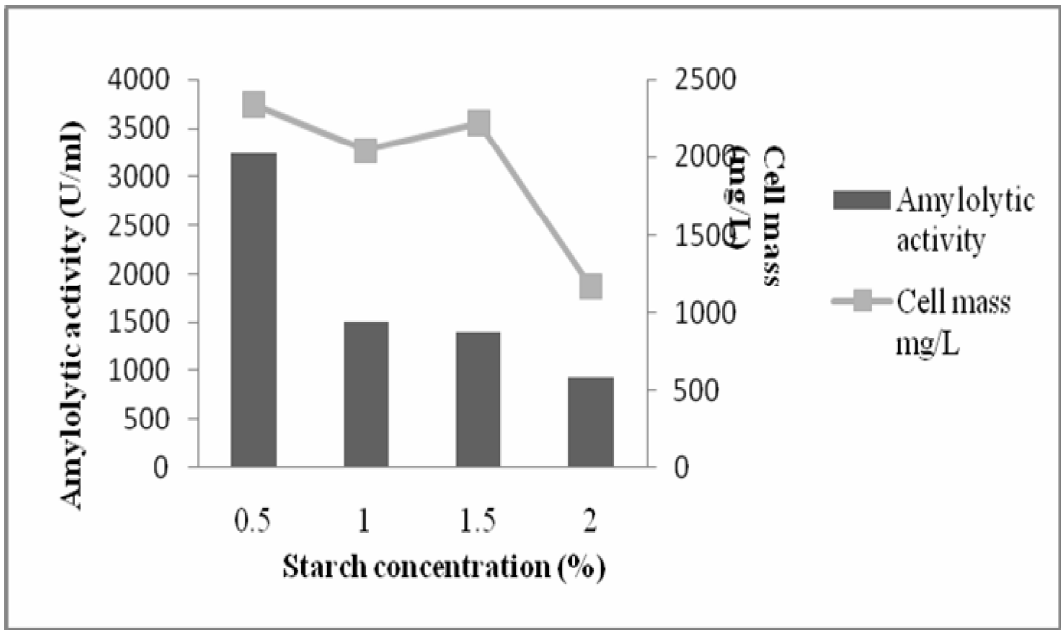


Fig.6 Effect of various carbon sources on amylase production by A26MB
Bacteria was grown in fermentation medium supplemented with various carbon sources, incubated at 37°C for 72 h and amylolytic activity in culture supernatant was measured.

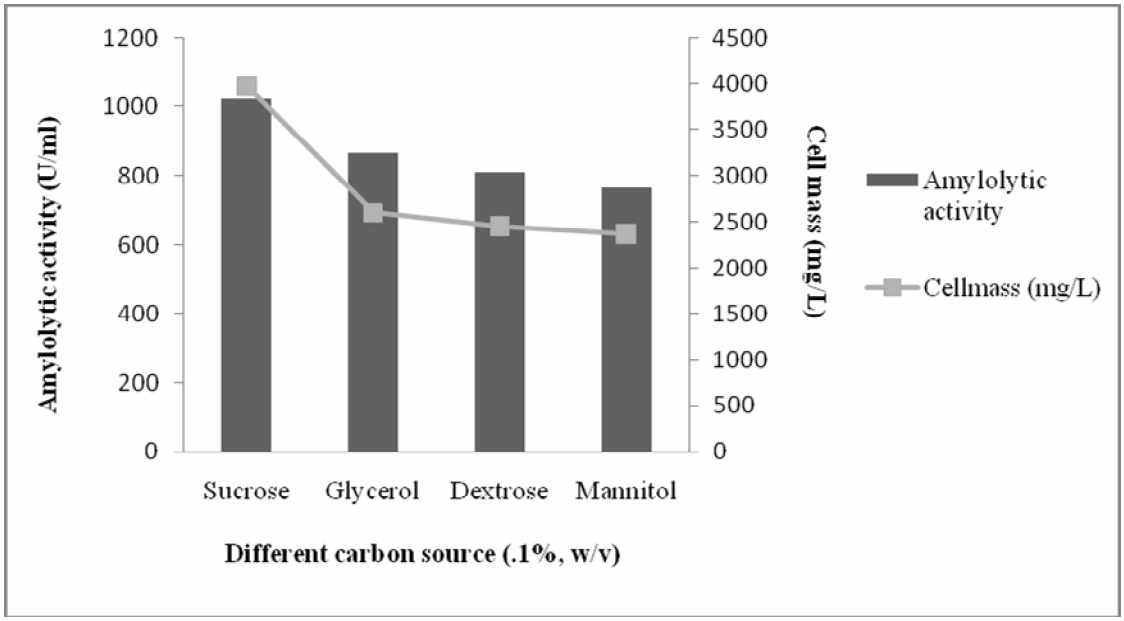


Fig. 7 Effect of nitrogen sources on amylase production by A26MB.
Bacteria was grown in fermentation medium supplemented with various nitrogen sources, incubated at 37°C for 72 h and amylolytic activity in culture supernatant was measured.

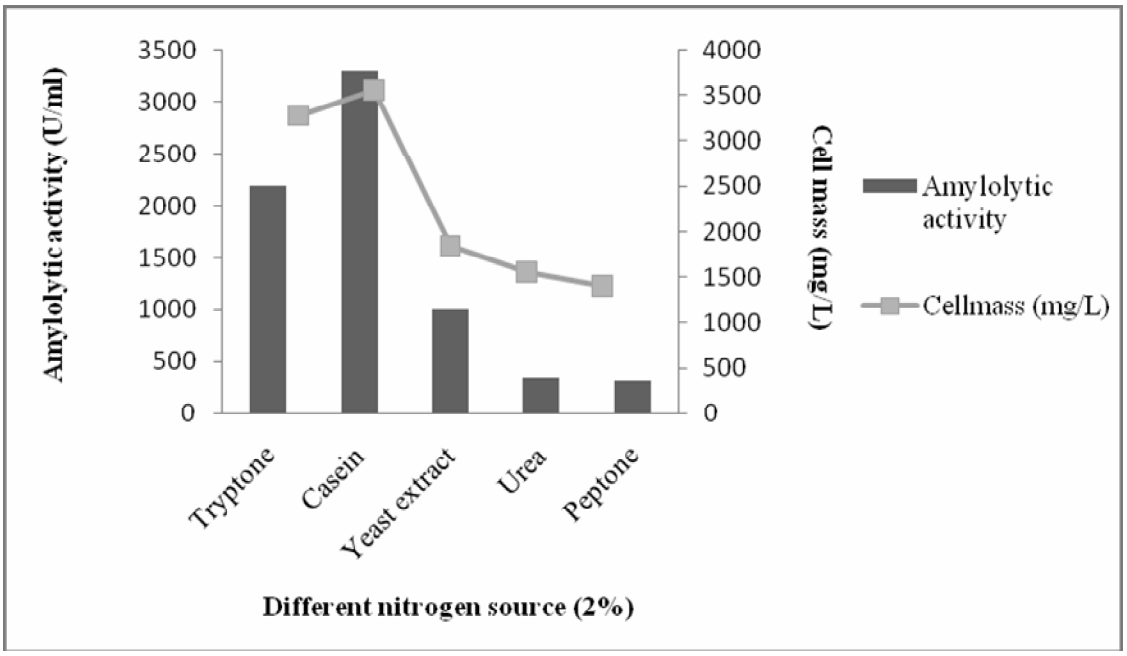
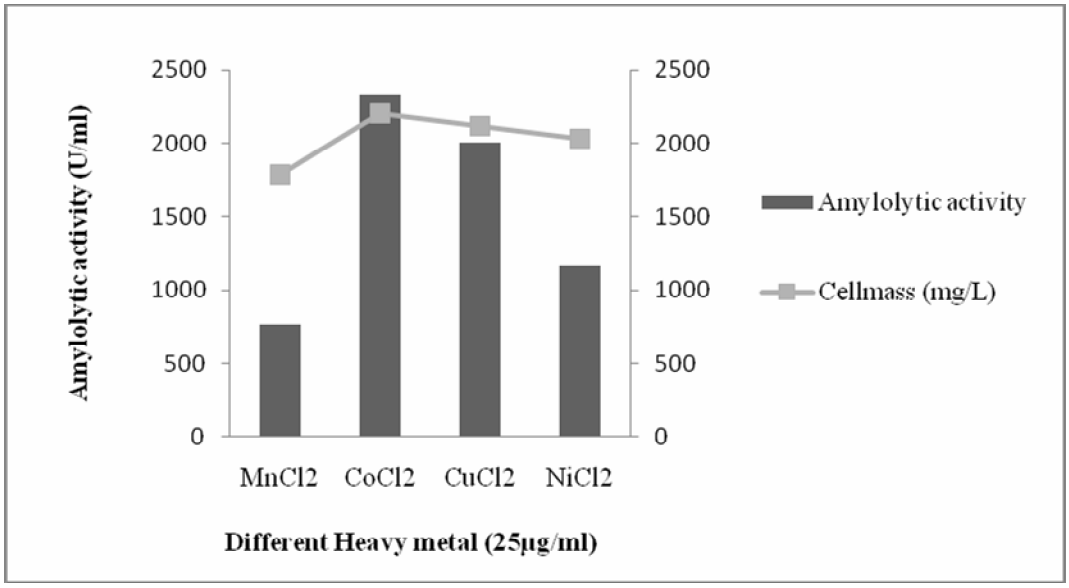


Fig.8 Effect of heavy metals on amylase production by A26MB
$MnCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$ (concentration 25µg/ml) were added to the fermentation medium investigated for their effect on enzyme production.



Lactose was found to be the better carbon source for amylase production (Ashwini et al., 2011). Carbon sources in the form of carbohydrates in the production media influence enzyme production (Kumar et al., 2012). Sucrose was found to be the best carbon source to enhance enzyme production (1021.32 U/ml) as shown in Fig. 6. Nitrogen sources are necessary for the proper growth and metabolism of microorganisms. It was reported that organic nitrogen sources supported maximum amylase production by various bacteria (Aqeel & Umar, 2010; Abou Dobara et al., 2011). It has been reported that ammonium dihydrogen phosphate to be a better nitrogen source for enzyme production by *B. licheniformis* SPT 278 than other tested inorganic nitrogen sources (Aiyer, 2004). Casein was the best among the organic nitrogen sources followed by tryptone, while most others reduced enzyme production than the usual level (Fig. 7). A26MB enhanced enzyme production in the presence of $CuCl_2$ and $CoCl_2$ (2000 U/ml and 2331.26 U/ml respectively) while others exhibited a significant reducing effect (Fig. 8).

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