Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme

S Mrudula, R Kokila

Citation
S Mrudula, R Kokila. Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme. The Internet Journal of Microbiology. 2009 Volume 8 Number 1.

Abstract
Thermostable α-amylase production under solid state fermentation was investigated using isolated thermophilic Bacillus cereus MK. Optimization of process parameters followed by leaching parameters for enhanced enzyme yields were carried out. The optimum temperature, pH, incubation period, inoculation size and substrate to moisture ratio were found to be 55 °C, 7.0, 24 h, 15 % (v/w) and 12.5 (w/v), respectively. Among different carbon, nitrogen and trace elements supplemented, glucose, peptone and calcium chloride, respectively enhanced enzyme production. The optimum leaching parameters such as solvents, solvent volume, physical state, solvent temperature and solvent pH for effective extraction of amylase from the fermented bran were found to be sodium acetate buffer (0.1M, pH 5.6), 1:2.5 (w/v), agitation, 50 °C and 7.0, respectively. An overall 14 fold increase in enzyme production was attained by optimization of process conditions and leaching parameters in SSF. The crude amylase showed maximum activity at pH 7.0 and 90 °C. The enzyme was stable at 90 °C for 1h. However in the presence of 4% (w/v) starch, the stability of enzyme was increased to 100 °C up to 2h.

INTRODUCTION
Alpha amylases (endo-1,4-β-D-glucan glucanohydrolase, E.C. 3.2.1.1) are extracellular endo enzymes that randomly cleave the 1,4 β-linkage between adjacent glucose units in the linear amylase chain and ultimately generate glucose, maltose and maltotriose units. Among various extracellular enzymes, β-amylase ranks first in terms of commercial exploitation (Babu and Satyanarayana, 1993) and accounts 12% of the sales value of the world market (Baysal et al, 2003). Spectrum of applications of β-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in bakery, brewery, detergent, textile, paper and distilling industry (Ramachandran et al, 2004).

Alpha amylases has been derived from several fungi, yeasts, bacteria and actinomycetes. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al, 2000). Almost all microorganisms of the genus Bacillus synthesize β-amylase, thus this genus has the potential to dominate the enzyme industry ( Pretorius et al, 1986). Thermostability is a feature of most of the enzymes sold for bulk industrial usage and thermophilic organisms are therefore of special interest as they could be used for saccharification processes occurring at high temperatures (Peixoto et al, 2003). The advantages of using thermostable amylases in industrial processes include the decreased risk of contamination, cost of external cooling and increased diffusion rate (Lin et al, 1998). Apart from this, they are resistant to denaturing agents, solvents and proteolytic enzymes (Bragger et al, 1989). Amylases with broad pH range have potential applications for starch saccharification in starch and textile industries and also are an ingredient in detergents for automatic dish washers and laundries (Kim et al, 1995). The industrially important Bacillus strains, which are extensively used to produces β-amylase are B. amyloliquefaciens, B. amyloliquefaciens (Fogarty and Kelly, 1980), B. licheniformis (Wind et al, 1994), B. subtilis (Takasaki, 1985), B. subtilis (Takasaki, 1983).

Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, solid-state fermentation (SSF) constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly (Pandey, 1992; Nigam...
Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme

and Singh, 1995; Chadha et al, 1997; Pandey et al, 2000). SSF is preferred to SmF because of simple technique, low capital investment, lower levels of catabolic repression and end product inhibition, low waste water output, better product recovery, and high quality production (Lonsane et al, 1985).

Production of these enzymes using agricultural residues as substrates under SSF conditions provide several advantages in productivity, cost effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc (Pandey et al, 2000). There are several reports describing use of agro industrial residues for the production of amylases, e.g, wheat bran and Bacillus amyloliquefaciens (Gangadharan et al, 2006), wheat bran and Bacillus cereus (Anto et al, 2006), wheat bran and Thermomyces lanuginosus (Adinarayana et al, 2005), wheat bran and rice husk and B. subtilis (Baysal et al, 2003), oil seed cakes and B. licheniformis CUMC305 (Krishnan and Chandra, 1982), banana waste and Aeromonas caviae CBTK185 (Krishna and Chandrasekaran, 1995), raw starch and Bacillus sp. TS-23 (Lin et al, 1998).

In SSF, the products are formed at or near the surfaces of the solid materials with low moisture content (Selvakumar and Pandey, 1999). Therefore, it is necessary to select solvent and leaching of the product from the fermented bran. Leaching of the product from the fermented bran is a difficult process (Lonsane and Krishnaiah, 1992). Attempt has been made by researchers to isolate the desired product from the fermented bran by various techniques due to its implication on process economics (Bjurstrom, 1985 and Calton et al, 1986).

The purpose of the present study was to investigate the production of amylase under SSF conditions by Bacillus cereus MK strain which was isolated and identified. In this work, we report a number of factors that influence amylase production in SSF and also partial purification and characterization of the enzyme.

MATERIALS AND METHODS

ISOLATION, SCREENING AND IDENTIFICATION OF THERMOPHILIC BACTERIUM PRODUCING AMYLASE

The soil samples for the isolation of thermophilic amylolytic organisms were collected from a potato cultivated land, Hosur, Tamil Nadu, India. The soil sample (1.0g) was suspended in saline (100 ml) and serially diluted. The diluted soil suspension (0.1ml) was spread on starch agar plates (Teodoro and Martins, 2000) and incubated at 55 °C for 48h. The plates were screened for amylolytic microorganisms by flooding with Grams iodine solution [2% (w/v) I₂ and 0.2% (w/v) KI]. The colonies that showed largest halo-forming zones were picked up and transferred into starch broth and incubated at 55 °C for 24h.

The isolated microorganism was further characterized according to Bergey’s manual of determinative bacteriology (Holt et al, 1994 and Olajuyigbe et al, 2005).

SUBMERGED FERMENTATION (SMF)

In submerged fermentation (SmF), the organism was routinely grown in 100 ml Erlenmeyer flasks containing 20 ml starch broth medium consisted of (g/l): peptone, 5.0; beef extract, 15.0; yeast extract, 15.0; sodium chloride, 5.0; soluble starch, 2.0; pH, 6.8. The medium was sterilized by autoclaving at 121 °C for 15 min.

SOLID STATE FERMENTATION (SSF)

Solid state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks that contained 10 g of wheat bran and 10 ml of distilled water (moistening agent). The flasks were sterilized at 121 °C for 15 min and cooled to room temperature. About 1.0 ml (v/w) of exponential phase culture was added, mixed well and incubated at 55 °C in a humidified incubator. Flasks were periodically mixed by gentle shaking. At the end of incubation period (24 h), the flasks were taken out and the contents from the each flasks were extracted with 50 ml of sterile distilled water.

PREPARATION OF ENZYME

For preparation of enzyme, the organism was grown in starch broth medium with [0.2% (w/v)] soluble starch. At the end of incubation period, the broth was centrifuged at 6000 rpm for 20 min in cooling centrifuge (REMI). The supernatant was used as extracellular enzyme and the cell pellet which was suspended in distilled water was used as cell bound enzyme.

In solid state fermentation (SSF) the enzyme was extracted from the bacterial bran by mixing homogenously the entire bran with (1:10 w/v) distilled water and agitated on a rotary shaker at 100 rpm with a contact time of 1h at 55 °C. Dampered cheese cloth was used to filter the extract (Ramesh and Lonsane, 1990) and pooled extracts were centrifuged at 6000 rpm for 20 min at 4 °C and the clear
supernatant was used as the source of extracellular enzyme.

**ENZYME ASSAY**

Amylase activity was assayed by measuring the reducing sugars released from the action of amylase.

**AMYLASE ACTIVITY**

The amylase activity was routinely assayed by measuring the reducing sugars liberated in the reaction mixture. The reaction mixture (3ml) consisted of 0.5ml of (1.0% w/v) soluble starch and 0.5ml of appropriately diluted enzyme source in 2ml of 0.1M sodium acetate buffer (pH 5.6). After incubation at 60 °C for 30 min, 1ml of DNS was added to the tubes and the reaction was stopped by boiling the tubes in boiling water bath for 15 min. The reducing sugars released by enzymatic hydrolysis of starch were determined (Miller, 1959). A separate blank was set up to correct the non-enzymatic release of sugars.

One unit of amylase is defined as the amount of enzyme which releases 1μ mole of reducing sugar, per minute with glucose as standard, under the assay conditions described above.

**OPTIMIZATION OF PROCESS PARAMETERS AND LEACHING PARAMETERS FOR INCREASED PRODUCTION OF ENZYME SSF**

The protocol adopted for the optimization of process parameters and leaching parameters influencing α-amylase production was to evaluate the effect of an individual parameter. The parameters optimized were: (1) effect of solid substrates (10 substrates); (2) substrate mixture (6 combinations); (3) incubation temperature (35-75 °C); (4) the initial pH of the medium was varied between 4.0 and 12.0 by adding either 1N H₂SO₄ or 1N NaOH to distilled water before using it to moisten the medium; (5) incubation time (24 - 96 h); (6) moisture content [wheat bran: distilled water ratio (1:0.5 to 1:2 w/v)]; (7) inoculums size [5 to 25% (v/w)]; (8) the effect of carbon, nitrogen and trace mineral source on enzyme production were tested by incorporation at 1% (w/w) in the distilled water; (9) effect of type of solvent on extraction of enzyme from the fermented bran was evaluated by mixing 10 g of fermented bran with 50 ml [10% (v/v)] each of organic solvents followed by shaking at 100 rpm for a contact time of 1 h; (10) effect of solvent volume [fermented bran : sodium acetate buffer (0.1M)] between 1: 1.35 and 1: 7.5 (w/v); (11) effect of physical state; (12) effect of solvent temperature (30-80 °C); (13) effect of solvent pH (4-9) on extraction of enzyme from the fermented bran was carried out by mixing 10 g of fermented bran with (0.1 M) buffers of different pH.

**PARTIAL PURIFICATION AND CHARACTERIZATION OF ENZYME**

Solid ammonium sulphate was added to 1 l of the culture supernatant at 80 % saturation. After incubating overnight at 4 °C, and centrifugation (15,000 x g, 30 min at 4 °C) the re suspended precipitate (in 25 ml of 0.1M sodium acetate buffer pH 5.6) was dialyzed overnight against the same buffer, and re centrifuged. The supernatant was used as partially purified enzyme.

**ESTIMATION OF PROTEIN**

Protein content was determined by the method of Lowry (1951) with bovine serum albumin as standard.

**TLC ANALYSIS OF THE PRODUCTS**

Starch hydrolysates were analyzed by TLC. The reaction mixture containing 0.5 ml of enzyme solution and 0.5 ml of 1% soluble starch in 2 ml of 0.1M sodium acetate buffer were incubated at 60 °C for 30 min and reaction was stopped by heating reaction mixture in boiling water bath for 5 to 10 min. 100 l of reaction mixture was applied along with glucose and maltose as reference standards was applied onto pre coated silica gel plates (Kieselgel; E.Merck; Germany) previously activated at 100 °C for 30 min. The plates were developed with butanol / glacial acetic acid/distilled water (3:1:1, v/v/v) (Kim et al, 1995). After developing hydrolysates, sugar spots were made visible by spraying the chromatograms with mixtures containing aniline (4 ml), diphenylamine (4 gm), 85% orthophosphoric acid (30 ml) and acetone (200 ml) and incubating the plates at 105 °C for 1h. Sugar spots of hydrolysates were identified by comparing their Rf values with those similarly obtained for standard sugar spots.

**EFFECT OF TEMPERATURE AND PH ON ENZYME ACTIVITY**

The effect of temperature on the activity of amylase was determined by performing enzyme assays between 40 and 100 °C in sodium acetate buffer (0.1 M, pH 5.6). The effect of pH on enzyme activity was determined by measuring the activity at 60 °C using sodium acetate (0.1 M, pH 4-6), sodium phosphate buffer (0.1 M, pH 6-8) and glycine-NaOH (0.1 M, pH 9-11) buffers. The temperature stability was determined by incubating the enzyme solution at different temperatures in the absence of starch for 2 h and in the
Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme

presence of starch at 100 °C for 2h, for the determination of pH stability the enzyme solution in sodium acetate buffer (0.1 M, pH 5.0) was pre incubated at various time intervals for 2 h and then residual activities were assayed under standard assay conditions.

RESULTS
SCREENING AND MAINTENANCE OF AMYLASE PRODUCING BACTERIA

The isolates having a higher ratio of clearing zone to colony size were grown in starch broth and the amount of amylase production was determined from the culture filtrate. The isolate found to be a promising producer for thermostable amylase. Subsequently the selected isolate was grown on nutrient agar slants at 55 °C and maintained at 8+2 °C and sub cultured every fifteenth day.

CHARACTERIZATION AND IDENTIFICATION OF THE ISOLATE

The strain was aerobic, rod shaped, often arranged in chains or pairs. Cells were gram positive, non capsulated, spore forming and actively motile. The strain showed catalase positive. Based on the above characteristics of an organism suggests that it belongs to genus Bacillus (Holt et al, 1994).

The strain has the ability to hydrolyse both starch and gelatin. Nitrate was reduced to nitrite. Oxidase, Voges proskauer test and citrate utilization test were found positive where as indole test, methyl red test and urease gave negative result. The strain fermented xylose, glucose, lactose, sucrose, maltose and raffinose. Carbohydrate fermentation of galactose, arabinose and mannitol was not found with the strain. The strain has shown an ability to grow on MacConkey agar. On the basis of above morphological and biochemical characteristics, it was identified as Bacillus cereus following the criteria laid down Bergey’s manual of determinative bacteriology (Holt et al, 1994 and Olajuyigbe et al, 2005).

ENZYME YIELDS IN SUBMERGED FERMENTATION (SMF) AND SOLID STATE FERMENTATION (SSF)

Initially, the strain produced 900 units of extracellular enzyme per litre of culture broth when the strain was grown in starch broth with 0.2% (w/v) soluble starch at 55 °C for 24h.

The same strain when grown on wheat bran moistened with 10 ml distilled water (moistening agent) at 55 °C for 24h, it produced 1096 units of enzyme per kg of dry bacterial bran (DBB).

OPTIMIZATION OF PROCESS PARAMETERS FOR AMYLASE PRODUCTION BY BACILLUS CEREUS MK IN SSF

Screening of solid substrates

Among the 11 substrates screened for SSF, wheat bran fine gave the highest enzyme activity (3700U/kg DBB) followed by groundnut oil cake and coconut oil cake, respectively (Fig. 1). Considerable amount of enzyme production was observed when grown on gingely oil cake, maize bran, corn bran, rice bran fine and wheat bran coarse respectively and the remaining substrates tamarind seed powder, rice husk and gram bran gave low amount of yields.

Figure 1

Figure. 1. Effect of solid substrates on α-amylase production by MK in SSF

Screening of substrate mixture

Among the 6 substrate mixtures screened for SSF, wheat bran + gingely oil cake (WB+GIOC) gave the highest enzyme activity (3120U/kg DBB) respectively followed by wheat bran + coconut oil cake (WB+COC). 50% of enzyme activities were observed with substrate mixtures of wheat bran + groundnut oil cake (WB+GOC) and coconut oil cake + gingely oil cake (COC+GIOC). Considerably, low amount of enzyme yields were observed with the remaining substrates (GIOC+GOC and COC+GOC) (Fig. 2).
**Figure 2**
Figure. 2. Effect of substrate mixture on α-amylase production by MK in SSF

**Effect of temperature**

Bacillus cereus MK was capable of producing amylase in the range of 35 to 70 °C with maximum production at 55 °C (2030U/kg DBB). However, increase in temperature beyond 55 °C led to decline in the production of the enzyme (Fig. 3).

**Figure 3**
Figure. 3. Effect of temperature on α-amylase production by MK in SSF

**Effect of pH**

Maximum enzyme yields were observed between the pH range of 7.0 and 7.5 with an initial optimum pH of 7.0 (2070U/kg DBB). Marginal decrease in enzyme yields were observed when grown at pH below 7.0 and above 7.5, respectively (Fig. 4).

**Figure 4**
Figure. 4. Effect of pH on α-amylase production by MK in SSF

**Effect of incubation time**

From the figure 5, it is clear that maximum enzyme production was observed up to 24 h of incubation (2030U/kg DBB). The enzyme yield showed a gradual decrease on further extension of incubation.

**Figure 5**
Figure. 5. Effect of incubation time on α-amylase production by MK in SSF

**Effect of moisture content**

The data presented in figure 6, shows that amylase production was high in wheat bran fine to distilled water ratio of 10:10 (w/v)(3700U/kg DBB). Any further increase or decrease in the ratio resulted in decreased enzyme activity.
Effect of inoculum size

Significant enzyme yields were observed with 5.0 to 25.0% (v/w) of inoculum with maximum enzyme yield at 15.0% of inoculum (3120U/kg DBB). Higher or lower inoculum size resulted in a significant decrease in enzyme activity (Fig. 7).

Effect of carbon sources

Among the carbon sources tested, glucose (4670U/kg DBB) produced maximum amylase yield followed by maltose, fructose and lactose respectively (Fig. 8). In contrast carbon sources such as galactose, starch soluble and sucrose showed lower enzyme yields.

Effect of nitrogen sources

Among the organic and inorganic nitrogen sources tested, peptone type-I (2070U/kg DBB) showed maximum yields followed by urea and sodium sulphate, beef extract, ammonium chloride and ammonium nitrate respectively. Comparatively, less enzyme yields were observed with casein, yeast extract and ammonium sulphate (Fig. 9).

Effect of trace elements

All the trace elements tested, showed enhanced amylase yield. Calcium chloride (3980U/kg DBB) produced maximum yields followed by sodium chloride, cobalt
Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme

chloride, manganous sulphate, magnesium sulphate and ferric chloride, respectively (Fig.10).

Figure 10
Figure. 10. Effect of trace elements on α-amylase production by MK in SSF

Effect of solvents
Among the various solvents selected for the extraction of amylase from the fermented bran, sodium acetate buffer (0.1M, pH 5.6) (9000U/kg DBB), leached maximum amylase enzyme from the fermented bran. Considerable amount of enzyme yields were obtained with the other solvents (Fig. 11).

Figure 11
Figure. 11. Effect of solvent selection on extraction of α-amylase from the MK in fermented bran

Effect of solvent volume
The optimum solvent volume was found to be 1:2.5 (w/v) (8500U/kg DBB) and was capable of extracting maximum enzyme from the fermented bran (Fig. 12).

Figure 12
Figure. 12. Effect of solvent volume on extraction of α-amylase from the MK in fermented bran

Effect of physical state
From the figure 13, it is observed that the effect of agitation on extraction of amylase from the fermented bran was found to be best condition for the maximum recovery when compared to static position.
Figure 13
Figure. 13. Effect of physical state of leaching on extraction of \( \alpha \)-amylase from the MK in fermented bran

Effect of solvent temperature

To study the effect of temperature on the leaching process, the temperature was varied from 30 to 80 °C each at 10 °C intervals. From the figure 14, it is observed that 50 °C (10000U/kg DBB) was found to be the most effective condition for the leaching of the enzyme. Further increase or decrease in temperature resulted decrease in enzyme yield.

Figure 14
Figure. 14. Effect of solvent temperature on extraction of \( \alpha \)-amylase from the MK in fermented bran

The effect of solvent pH on the extraction of enzyme was studied by incubating the fermented bran with buffers of different pH ranging between 4.0 and 10.0 (10000U/kg DBB). From the figure 15, it is clear that as the pH of the buffer increases from 4.0 to 7.0, the leaching of the enzyme from the fermented bran increased and found maximum at pH 7.0. Further increase in pH showed decreased enzyme leaching from the fermented bran.

Figure 15
Figure. 15. Effect of solvent pH on extraction of \( \alpha \)-amylase from the MK in fermented bran

Evaluation of optimized process parameters on amylase production by Bacillus cereus MK in SSF

Under the optimum conditions described above, the strain produced 15250 units of amylase per kg of dry bacterial bran (Fig. 16).
Figure 16

Figure. 16. Yields of thermostable α-amylase by MK in SmF and SSF (Before optimization and after optimization)

Partial purification of the enzyme

The enzyme produced by Bacillus cereus strain was partially purified by ammonium sulphate precipitation to 80% saturation level. The strain MK produced 900 units extracellular of thermostable α-amylase per litre of culture broth. After centrifugation, the crude culture supernatant contained approximately 418 units of α-amylase per litre. In the present study, the specific activity of α-amylase is 1.105 units per mg of protein. This is an increase of 4 folds of purity of α-amylase, with a recovery of 46% (Table. 1).

TLC ANALYSIS OF THE PRODUCTS

TLC analysis of reaction products of B. cereus MK amylase on starch showed that glucose and maltose as main products. The appearance of maltose and glucose as major hydrolysis product implies that the amylase produced by this organism is of α-type.

Figure 17

Table. 1. Summary of partial purification procedure of MK α-amylase

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Culture supernatant</td>
<td>3567</td>
<td>900</td>
<td>0.252</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Salting out with ammonium sulphate</td>
<td>378</td>
<td>418</td>
<td>1.105</td>
<td>4.38</td>
<td>46</td>
</tr>
</tbody>
</table>

Effect of temperature on enzyme activity and stability

Amylase activity was maximal at 90 °C. Amylase retained 80% and 89% activity at 70 °C and 80 °C respectively (Fig. 17). The thermal stability of amylase was examined by pre incubating the enzymes at 90 °C for 2h. The enzyme was stable up to 1h showing very little loss of activity after 1h (Fig. 18). However the presence of 4% starch increased the thermal stability of enzyme at 90 °C for 2h (Fig. 19).

Figure 18

Figure. 17. Effect of temperature on the activity of α-amylase from the MK
Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme

DISCUSSION

Bacillus sp. are considered to be the most important source of α-amylase and have been used for enzyme production using solid state fermentation (SSF) (Pandey et al., 2000). Production of α-amylase by Bacillus cereus has been studied by growing the isolate on various natural solid substrates. Selection of an ideal agro-residues for enzyme production in a SSF process depends upon several factors, mainly related to cost and availability of the substrate material, and thus may involve screening of several agro-residues (Pandey et al., 2000). The results in the present study indicated that α-amylase production pattern varied with type of agro-residue. Among which wheat bran was found to be most suitable, while minimum amylase production was noticed with gram bran. The suitability of wheat bran may be due to the fact that it contains sufficient nutrients and is able to remain loose even in moist conditions, thereby producing a large surface area (Babu and Satyanarayana, 1995). The mixtures of substrates containing WB gave significant increase in enzyme production, but the highest enzyme activity was found with WB+GIOC. The results are in accordance with α-amylase production by B. amyloliquefaciens (Gangadharan et al., 2006).

The production of enzyme was determined at different temperatures ranging from 35 to 75 °C and optimum enzyme production was obtained at 55 °C. Similar patterns were reported for B. licheniformis (Saito and Yamamoto, 1975), B. stearothermophilus (Wind et al, 1994) and Bacillus sp.
(Mamo and Gessesse, 1999), where as Bajpai and Bajpai (1989) reported that the enzyme synthesis and growth temperature of B. licheniformis TCRDC-B13 strain was 25-50 °C and maximum enzyme production was obtained at 35 °C.

pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during growth of microorganism also effects the product stability in the medium (Rani Gupta et al., 2003). Maximum amylase production was achieved at pH 7.0 by B. cereus MK, although pH 4.0 to 12.0 supported amylase production. Similar findings have been reported by Bajpai and Bajpai (1989) for growth and production of amylase by B. licheniformis TCRDC-B13 and Haq et al. (2005) for production of amylase by B. subtilis. Where as Pyrococcus furiosus, Pyrococcus woesei and Thermococcus profundus yielded optimum α-amylase at pH 5.0 (Veille and Zeikus, 2001).

The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on the growth rate and enzyme production. Maximum amylase production was observed at 24h of incubation time. Further increase in incubation time showed decreased enzyme yields. The decrease in enzyme yield may be because of the decomposition or degeneration of amylase due to interaction with other components in the media (Ramesh and Lonsane, 1990). These results are in accordance with the observations made by Dharani Aiyer (2004).

Among the several factors that are important for microbial growth and enzyme production under solid state fermentation using a particular substrate, moisture level / water activity is one of the most critical factors (Pandey et al, 1994). Because, solid state fermentation processes are different from submerged fermentation culturing since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents (Pandey et al, 2000). Thus, it is crucial to provide optimized water level that controls the water activity (a_w) of the fermenting substrate for achieving maximum product. In the present study, high enzyme titre was obtained when the substrate: moisture ratio was maintained at 1:1 (w/v). Babu and Satyanarayana (1995) reported maximum β-amylase enzyme production by thermophilic Bacillus coagulans at a high level of substrate moisture ratio of 1:2.5 where as Anto et al (2006) reported substrate moisture ratio of 1:2 as optimum for production of β-amylase by Bacillus cereus.

Inoculum level selected for this study ranged from 5 to 25% of 24h. Enzyme production is varied with inoculum level and showed parabolic nature in the present study. Maximum amylase synthesis was noticed with 15% inoculum size.

Supplementation of carbon sources in the form of mono saccharides, di saccharides and poly saccharides to solid medium at 1.0% level showed different impact on enzyme production with different compounds. Glucose supported maximum production followed by maltose. These data suggested that glucose was not a repressor of a amylase enzyme in this bacterial strain unlike the observed catabolic repression by glucose in Bacillus coagulans (Babu and Satyanarayana, 1995). The results of the present study were in accordance with reported β-amylases from B. thermoleovorans (Narang and Satyanarayana, 2001) and B. cereus (Anto et al, 2006).

Addition of nitrogen sources have been reported to have an inducing effect on the production of various enzymes including β-amylase in a SSF medium (Pedersen and Nielsen, 2000). Results revealed that complex nitrogen sources supported better for β-amylase production over inorganic nitrogen sources. Peptone showed maximum influence in enhancement of enzyme production. Similar observations were noticed in case of amylase production by different microbial species (Gangadharan et al, 2006 and Saxena et al, 2007). In contrast, Dharani Aiyer (2004) reported amylase production from a combination with ammonium hydrogen phosphate.

All trace elements tested enhanced amylase yield significantly and calcium chloride was found to be best metal ion for the production of enzyme followed by sodium chloride. Allan et al (1997), reported that in case of B. licheniformis, addition of calcium salt in the medium increased β-amylase production. The stability of β-amylase is calcium dependant (Kennedy and White, 1979), where as addition of Mn²⁺ in the fermentation medium favoured the synthesis of amylase (Kadrekar and Ramasarma, 1990).

In SSF the products are formed at or near the surfaces of solid materials with low moisture content (Selvakumar and Pandey, 1999). So it is necessary to select a solvent for leaching out the product from the fermented bran. Leaching of enzymes from the fermented bran is a different task, and is an important aspect for the development of cost effective
process for enzyme production in SSF. Among the solvents tested, sodium acetate buffer (0.1M, pH 5.6) gave the best result. In contrast, glycerol (10%) in sodium acetate buffer was found to be the best solvent for extraction of α-amylase from the fermented bran of B. circulans GRS313 (Palit and Banerjee, 2001).

In SSF system as the free flowing solvent is limited, therefore sufficient amount of solvent is required to leach out the product from the fermented bran. In the present study, 1:2.5 (w/v) fermented bran to solvent ratio was found optimum. Palit and Banerjee (2001) reported an optimum solid to solvent ratio of 1:3 for extraction of α-amylase from the fermented bran of Bacillus circulans GRS313. Where as solvent to bacterial bran ratio (S/BB) of 4 was found optimum for leaching of thermostable pullulanase by Clostridium thermosulfurogenes SV2 from the fermented bran (Rama Mohan Reddy et al, 2000).

The leaching of α-amylase increased upon agitation. This may be because on agitation, fermented bran gets homogenously distributed in a continuous phase of solvent (Tunga et al, 1999). These results are in agreement with the reports of Rama Mohan Reddy et al (2000) who reported maximum thermostable pullulanase leached from the fermented bran at an agitation of 50 rpm.

The effect of temperature on leaching process was carried out by varying solvent temperature from 30 to 80 °C each at 10 °C intervals, the solvent temperature of 50 °C was found most effective for leaching of enzyme. Whereas temperature did not showed a significant effect on leaching of thermostable pullulanase from the fermented bran (Rama Mohan Reddy et al, 2000).

The solvent pH of 6.0 was found to be optimum for the leaching of α-amylase and any increase or decrease in the pH of the solvent resulted in sharp decrease in the leaching of the amylase. Similar results were reported by Rama Mohan Reddy et al (2000) for pullulanase enzyme.

Fractionation of culture supernatants showed the existence of α-amylase in the type strain of Bacillus cereus. The strain produced maltose and maltotriose with trace amounts of glucose from soluble starch. This indicates that it cleaves β-1,4 linkages in starch. Similar observations were made for Clostridium thermohydrosulfuricum (Melasniemi, 1987). The reactions of amylase and starch were characterized by thin layer chromatography by determining its hydrolysed products Amylase produces maltose (G₂), maltotriose (G₃) and trace amounts of glucose from soluble starch.

The amylase activities at different temperatures were active in a broad temperature range (50 to 100 °C) and displayed optimum activity at 90 °C. This attribute can be exploited in starch processing industries which requires a broad temperature range. Similar findings have been reported by Melasniemi (1987) for Clostridium thermohydrosulfuricum. The optimum temperature of 60 °C was observed for β-amylase for Clostridium thermocellum SS8 (Swamy et al, 1994), Bacillus circulans (Kwan et al, 1993) and Bacillus megaterium (Ray et al, 1995). The amylase activity was maximum at 85 °C for Rhodothermus marinus (Gomes et al, 2003).

The effect of temperature on heat stability on amylase in the absence of substrate showed that the enzyme activities were entirely stable up to 90 °C for 30 min. Similarly, thermostability of pullulanase from Clostridium thermohydrosulfuricum (Saha et al, 1998) was found to be stable upto 90 °C in the absence of substrate for 30 min.

The presence of 4% starch increased in thermostability of amylase at 100 °C to 2h. The heat stability of enzymes reported for thermostable β-amylases (Hyun and Zeikus, 1985 and Rama Mohan Reddy et al, 1998) were at 80 °C.

The pH optima were determined in three buffer systems the enzyme showed good activity at pH 7.0. The optimum activity was around 79% between 7.0 and 8.0 pH ranges. The effect of pH on β-amylase activity indicates that the amylase is active in the wide pH range of 4.0 to 8.0. This suggests that the enzyme would be useful in processes that require a wide pH range from acidic to slightly alkaline and retained activity about 58% and 80% activities at pH 4.0 and 8.0 respectively. β-amylase from Clostridium thermocellum SS8 (Swamy et al, 1994) and amylase from fermented cassava waste water showed an optimal activity at pH 6.0 (Oboh, 2005).

The enzyme was found to be stable at pH 7.0 for 2h. Oboh (2005) reported that the amylase from fermented cassava showed pH stability for 4h at pH 6.0 and 7.0.

CONCLUSIONS

In the present study, initially the strain produced 1096 units of amylase per ml of culture broth. After optimization, the same strain produced 15250 units of amylase per gram of dry bacterial bran in solid state fermentation. The enzyme yields obtained were 14 times more in SSF than before.
Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme

optimization. Therefore these results clearly indicated the scope for utilization of Bacillus cereus MK for extracellular thermostable α-amylase production through SSF. The ability of Bacillus cereus MK up to 100 °C and its activity at a broad pH range showed its potential for usage in industrial starch liquefaction and detergent industries.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the Management, M.G.R. College, Hosur for providing laboratory facilities.

References

Production of Thermostable α-amylase by Bacillus cereus MK in solid state fermentation: Partial purification and characterization of the enzyme


r-57. Vieille C and Zeikus GJ. (2001). Hyperthermophilic enzymes: sources, uses and molecular mechanisms of

Author Information

S. Mrudula, M. Sc., Ph. D
Department of Microbiology, M. G. R. College

R. Kokila, M. Sc., M. Phil
Department of Microbiology, M. G. R. College