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Partial Characterization and Optimization of Production of Extracellular α-amylase from *Bacillus subtilis* Isolated from Culturable Cow Dung Microflora

MANAS R. SWAIN[1], SHAKTIMAY KAR[1], GOURIKUTTI PADMAJA[2] and RAMESH C. RAY[1]*

[1]Regional Center of Central Tuber Crops Research Institute, Bhubaneswar, India
[2]Central Tuber Crops Research Institute, Sreekarium, Trivandrum, India

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A b s t r a c t

Studies of α-amylase production by *Bacillus subtilis* (CM3) isolated earlier from cow dung microflora, were carried out. The optimum temperature, pH and incubation period for amylase production were 50–70°C, 5.0–9.0 and 36 h, respectively. Enzyme secretion was very similar in the presence of any of the carbon sources tested (soluble starch, potato starch, cassava starch, wheat flour, glucose, fructose, *etc*.). Yeast extract and ammonium acetate (1%) as nitrogen sources gave higher yield compared to other nitrogen sources (peptone, malt extract, casein, asparagine, glycine, beef extract), whereas ammonium chloride, ammonium sulphate and urea inhibited the enzyme activity. Addition of Ca^{+2} (10–40 mM) to the culture medium did not result in further improvement of enzyme production, whereas the addition of surfactants (Tween 20, Tween 40, Tween 80, and sodium lauryl sulphate) at 0.02% resulted in 2–15% increase in enzyme production. There were no significant variations in enzyme yield between shaked-flask and laboratory fermentor cultures. The purified enzyme is in two forms with molecular mass of 18.0 ± 1 and 43.0 ± 1 kDa in native SDS-PAGE.

K e y w o r d s: *Bacillus subtilis*; cow dung microflora; extracellular α-amylase

Introduction

Various microorganisms are associated with rumen microflora of cattle, sheep, buffalos and goats, which are largely responsible for digestion in these animals (Ware *et al.*, 1988). The general culturable microflora of the cattle gut involves *Bacillus*, *Bifidobacterium*, *Lactobacillus*, and yeasts (Wallace and Newbold, 1993). According to EEC directive 70/524, several microorganisms have been authorized as additives for feedstuffs (Abe *et al*., 1995; Auclair, E., 2006 http://resources.ciheam.org/om/pdf/c54/01600010.pdf). These microorganisms belong to different species: *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis*, *Enterococcus faecium* and *Saccharomyces cerevisiae*. All these strains have a positive effect on the health of different animal species such as beef cattle, dairy cows, pigs and rabbits (Breul, 1998; Guo *et al*., 2006).

In our previous study it was found that *B. subtilis* strains were one of the predominant groups of bacteria isolated from culturable cow dung microflora (Swain and Ray, 2006). These strains exhibited several beneficial attributes, which include biocontrol of pathogenic fungi, *i.e. Fusarium oxysporum* and *Botryodiplodia theobromae*, plant growth promotion, sulphur oxidation, solubilization of rock phosphorus and production of industrially important enzymes (amylase and cellulase) *in vitro*.

Among the starch hydrolyzing enzymes that are produced on an industrial scale, thermostable α-amylases are of significant commercial interest. α-Amylases (E.C. 3.2.1.1) randomly hydrolyze α-1,4, glycosidic linkages in starch and its partial hydrolysis products. Bacteria belonging mainly to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylase (Tonkova, 2006). The most important characteristic of thermophilic amylase producers is their ability to produce the enzyme with higher operational stability and longer shelf-life. The α-amylases presently used in starch saccharification

* Corresponding author: C. Ray, Cen. Tuber Crops Res. Inst., Bhubaneswar 751019, Orissa, India; e-mail: rc_ray@rediffmail.com

require Ca^{2+} for their activity and/or stability. There is a continuous search for microorganisms producing α-amylase which do not require Ca^{2+} (Tonkova, 2006).

The present study was carried out to explore the possibility of production of thermostable Ca^{2+} independent α-amylase by *B. subtilis* strains isolated from cow dung microflora, to characterize the enzyme, and to investigate conditions for its production.

Experimental

Materials and Methods

***B. subtilis* strain.** The *B. subtilis* strain CM3 isolated from culturable cow dung microflora (Swain and Ray, in press) was used in this study. The culture was maintained on Nutrient Agar slants at 4°C.

Production of α-amylase. The production of α-amylase from *B. subtilis* strain CM3 was carried out in a basal medium with following composition: 1% soluble starch, 0.2% yeast extract 0.5% peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar (pH adjusted to 7.0 before autoclaving). The medium (100 ml taken in 250 ml Erlenmeyer flasks) was inoculated with 2% (1×10^6 CFU/ml) of 24 h seed culture and incubated with shaking (150 rpm) and 50°C for 36 h in an orbital shaker-incubator (Remi Pvt. Ltd, Bombay, India). Different carbon and nitrogen sources (1%) were used for optimization of nutritional factors. The various carbon sources tested were: soluble starch, potato starch, sweet potato starch, cassava starch, wheat flour, glucose, fructose, maltose, lactose and sucrose. Different levels of soluble starch (0.5–3.0%) were also used. The nitrogen sources tested were: yeast extract, peptone, malt extract, casein, asparagine, glycine, beef extract, ammonium chloride, ammonium sulphate, urea and ammonium acetate. Different concentrations of yeast extract (0.25–3.0%) and ammonium acetate (0.25–3%) were also used.

The physical parameters, including pH of the medium (4.0–11.0), temperature of incubation (40–90°C), and incubation period (12–60 h) were also tested. The effect of different surfactants (0.02%): Tween 20, Tween 40, Tween 80 and sodium lauryl sulphate on amylase activity was studied. Similarly, the effect of Ca^{2+} ions (10–40 mM) was also tested. All the experiments were carried out in triplicate and mean data with standard deviations (±) were calculated. At the end of the incubation period (36 h for all experiments except the experiment in which incubation period was studied), the cell-free enzyme supernatant was obtained by centrifugation at 8000 × g for 15 min at 4°C.

Enzyme stability at various temperatures and pH was also studied by incubating cell-free supernatants at different temperatures (40–90°C) and assay buffer pH (4.0–10.0). The pH of 4.0–5.0 was maintained with acetate buffer (0.2 M) while pH of 6.0–8.0 and 9.0–10.0 was achieved with phosphate (0.1 M) buffer and borax-NaOH (0.05 M) buffers, respectively. *B. subtilis* CM3 was also cultivated in a 2-liter fermentor (Model Biostat B, B. Braun, Germany) with a working volume of 1l (Glass Jar Vessel) containing α-amylase production medium (pH 7.0) at 60°C and 150 rpm. The aeration level was maintained at 1vvm (volume of air/unit volume of the medium/min). The enzyme production and growth of the organism were compared with shaked-flask cultures incubated at 60°C and 150 rpm in an orbital shaker incubator.

***B. subtilis* growth.** The growth of *B. subtilis* strain CM3 was determined by measuring the optical density of the growth medium at 600 nm in a UV-Vis spectrophotometer (Cecil Instruments, UK).

Thin-layer chromatographic analysis. The products liberated by the action of amylase on starch were identified by spotting the starch digest and standard sugars (glucose and maltose) on a silica gel plate activated at 80°C for 30 min. The plates were developed in butanol:ethanol:water (50:30:20) and dried overnight at 32 ± 2°C. The individual sugar(s) were visualized with acetone-silver nitrate solution (0.1 ml saturated solution of $AgNO_3$ in 20 ml of acetone).

Amylase assay. The amylase assay was based on the reduction in blue colour intensity resulting from enzyme hydrolysis of starch (Palanivelu, 2001). The reaction mixture consisted of 0.2 ml enzyme (cell free supernatant), 0.25 ml of 0.1% soluble starch solution and 0.5 ml of phosphate buffer (0.1 M, pH 6.8) incubated at 50°C for 10 min. The reaction was stopped by adding 0.25 ml of 0.1 N HCl and colour was developed by adding 0.25 ml of I/KI solution (2% KI in 0.2% I). The optical density (OD) of the blue colour solution was determined using a UV-Vis spectrophotometer (Cecil Instruments, UK) at 690 nm. One unit of enzyme activity is defined as the quantity of enzyme that caused 0.01% reduction of blue colour intensity of starch iodine solution at 50°C in one min per ml (Palanivelu, 2001). The optimal activity and stability of the partially purified enzyme (described in the following section) at various pH values (5.0–10.0) and temperature (40–80°C) were studied.

Partial purification of the enzyme. α-Amylase was partially purified by ammonium sulphate fractionation followed by dialysis and gel filtration chromatography. A total of 100 ml of bacterial culture filtrate was centrifuged at 8000 × g for 20 min at 4°C to remove the cells. The supernatant was brought to 50% ammonium sulphate saturation at 4°C in an ice bath. The precipitated protein was collected by centrifugation at 8000 × g at 4°C and dissolved in a minimum volume of phosphate buffer (0.1 M; pH, 6.0). The enzyme solution was dialyzed at 4°C against the same buffer for 24 h at 4°C with continuous stirring and three changes of the same buffer. The DEAE cellulose ion exchange column was pre-equilibrated with the same buffer. The dialysate was concentrated with a rotary evaporator at 50°C and applied to the DEAE cellulose column at a flow rate of 0.6 ml/min with 200 ml linear NaCl gradient (0 to 1.0 M). Fractions of 10 ml were collected and each fraction was analyzed for protein concentration and α-amylase activity. The active fractions were pooled and concentrated with a rotary evaporator at 50°C. The final enzyme solution was taken for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Electrophoresis and molecular mass determination. SDS-PAGE was performed with 12% polyacrylamide gel using a Mini GEL electrophoresis system (Model No 0502, Bangalore Genei Pvt. Ltd., Bangalore, India) as described by Laemmli (1970). The bacterial proteins were stained with 0.2% Coomassie Brilliant Blue. The molecular mass of the partially purified amylase was estimated using standard 'protein markers' (PMW-M) of known molecular mass (14.3–97.4 kDa) (Bangalore Genei Pvt. Ltd., Bangalore, India).

Results

Amylase production by *B. subtilis* strain CM3 started in the log phase of growth and maximum enzyme production was achieved during the stationary phase (36 h) of the growth of the organism (Fig. 1). Further, this strain produced amylase optimally at growth temperature of 50–70°C (Fig. 2 A). To examine the thermostability of the enzyme, the enzyme solution buffered at pH 6.8 was incubated at various temperature (40–90°C) for 30 min. The maximum activity was 4900–4960 units at temperature of 60–70°C (Fig. 2 A).

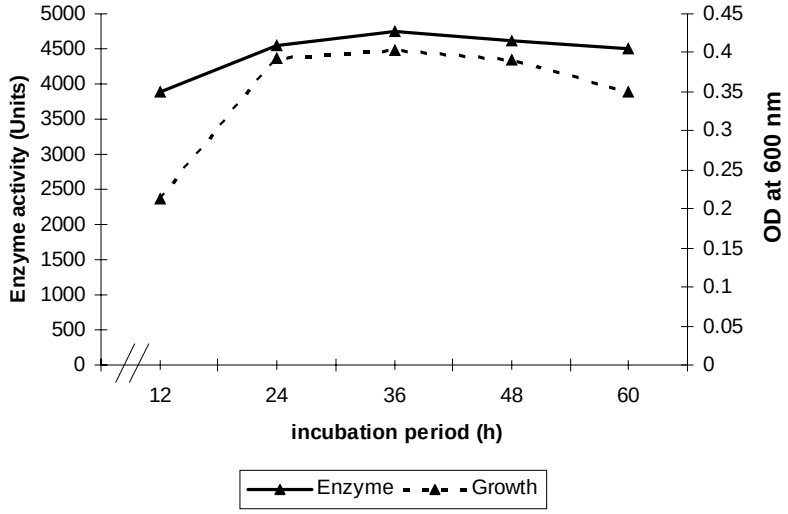

Fig. 1. Effect of incubation period on growth and α-amylase production by *B. subtilis* strain CM3

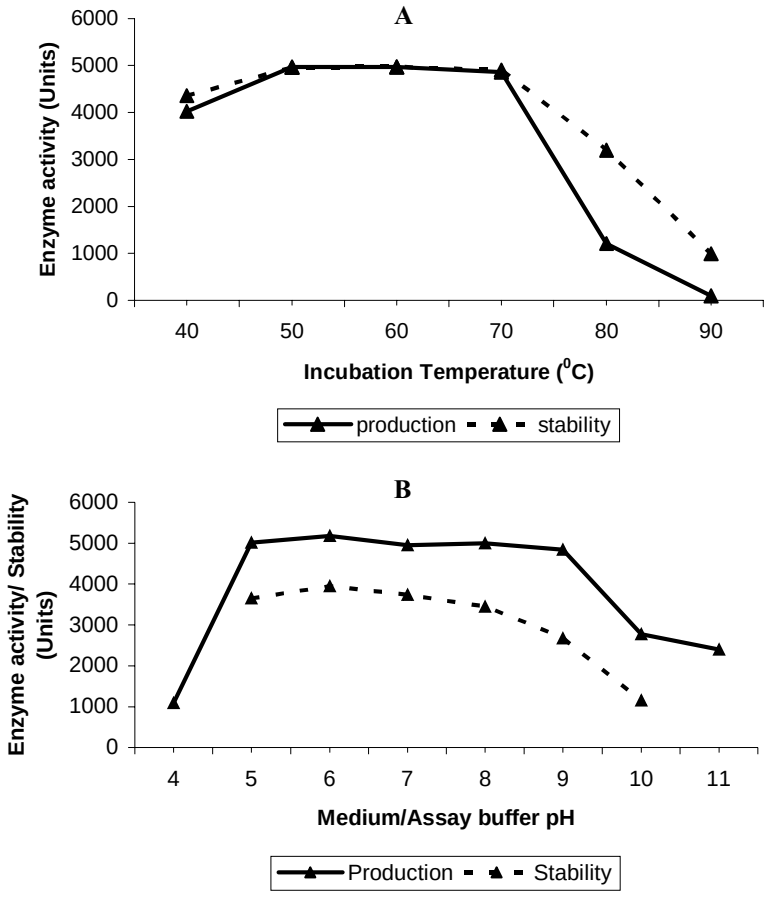

Fig. 2. Effect of temperature (A) and pH (B) on α-amylase production and stability by *B. subtilis* strain CM3

Table I
Effect of different carbon sources (starch and sugar) and nitrogen sources (inorganic and organic) on α-amylase production by *B. subtilis* strain CM3

Carbon sources	Enzyme production (Units)	Nitrogen sources	Enzyme production (Units)
Soluble starch	4870.50 ±103.4	Peptone	4865.23 ± 106.2
Potato starch	4970.50 ± 098.0	Casein	4462.00 ± 093.23
Sweet potato starch	4923.50 ± 121.1	Malt extract	1088.23 ± 065.2
Cassava starch	4923.50 ± 110.4	Yeast extract	4918.56 ± 121.0
Wheat flour	4970.50 ± 103.4	Beef extract	4491.13 ± 095.2
Glucose	4970.50 ± 087.2	Asparagine	4751.56 ± 103.6
Fructose	4978.54 ± 056.6	Glycine	4311.00 ± 056.3
Maltose	4976.20 ± 00.0	Ammonium chloride	857.00 ± 103.2
Lactose	4967.81 ± 121.3	Ammonium sulphate	1065.15 ± 069.6
Sucrose	4957.08 ± 103.2	Urea	107.50 ± 106.5
		Ammonium acetate	4938.32 ± 103.2

± Standard deviations

When the crude enzyme was heated at 90°C for 30 min, 80% of the original enzyme activity was lost. Using thin-layer chromatographic analysis, the end products of starch hydrolysis detected were glucose and maltose which suggested an endo-mode of action for the amylase, *i.e.* α-amylase (data not shown).

Following optimization of parameters, the optimum pH for enzyme production was in the range of 5.0–9.0 (4950–5180 units/ml culture medium) and stability was in the range of 5.0–8.0 (3455–3947 units)

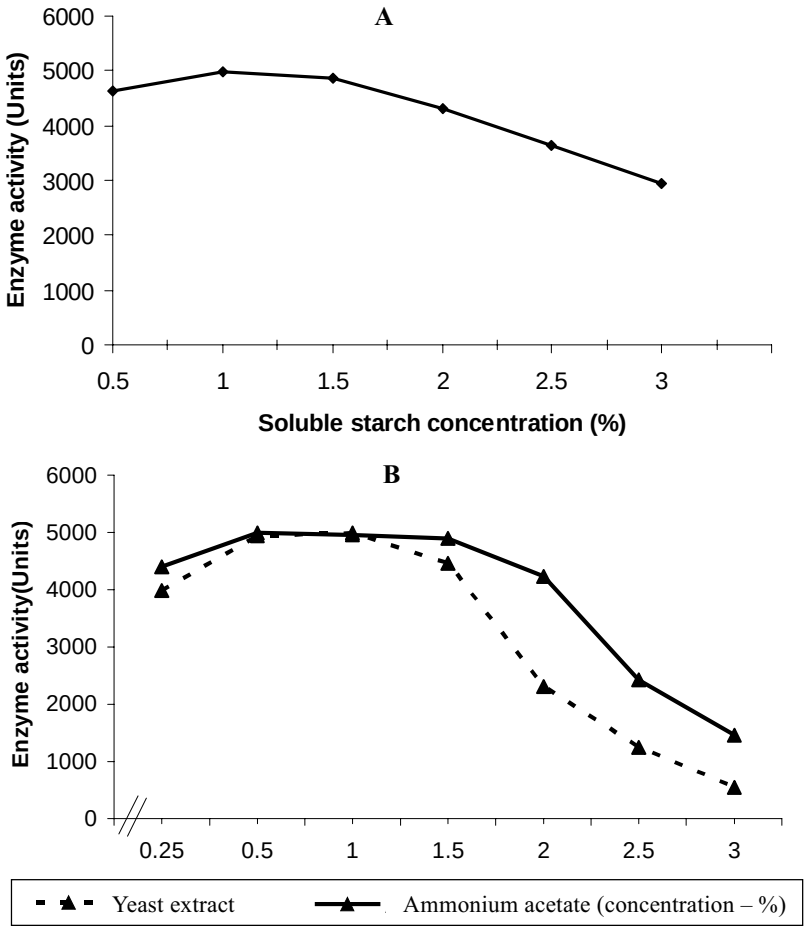

Fig 3. Effect of different concentrations (%) of soluble starch (A), yeast extract and ammonium acetate (B) on α-amylase production by *B. subtilis* strain CM3

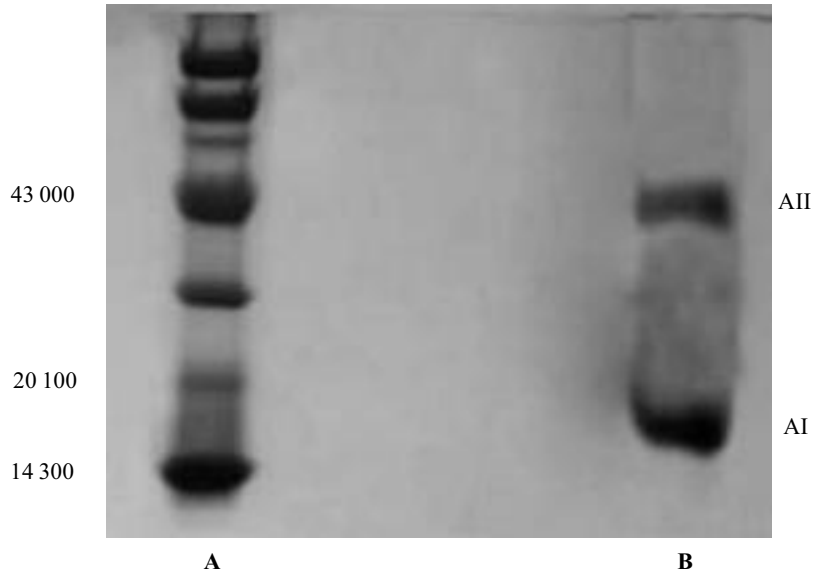

Fig. 4. Determination of molecular weight by SDS-PAGE. (A) molecular mass markers: (97.0–14.3 kDa) (B) α-amylase(s) from *B. subtilis* strain CM3. Isozymes AI and AII

(Fig. 2B). The organism could grow and produce almost equal amount of amylase in medium containing different carbon sources (1% w/v), *i.e.* soluble starch, glucose, fructose, wheat flour, *etc.* (Table I). Moreover, among the various starch concentrations used, 1% soluble starch gave maximum enzyme yield (Fig. 3A). Similarly, enzyme production was more efficient in medium containing organic nitrogen sources, *i.e*. peptone, yeast extract, beef extract, etc., as compared with inorganic nitrogen sources (ammonium acetate was the exception) (Table I). The enzyme production increased concomitantly with increase in concentrations of yeast extract and ammonium acetate up to 1% level, beyond that there was a gradual decline (Fig. 3B).

Amylase production increased in culture medium due to addition of surfactants such as Tween 20, Tween 40, Tween 80 and sodium lauryl sulphate. While the medium without surfactant (control) yielded 4756 units of amylase, 5290, 5416, 5104 and 5020 units of enzyme were produced in medium containing 0.02% of Tween 20, Tween 40, Tween 80 and sodium lauryl sulphate, respectively. Addition of Ca^{2+} ion (10–40 mM) had no significant difference (increase or decrease) on α-amylase activity. The enzyme production varied in the range of 4965–5011 units in medium with or without Ca^{2+} ion.

α-Amylase was partially purified using ammonium sulphate fractionation. The crude extract contained 327.23 mg/ml protein and showed a specific activity of 14.53 units/mg protein. After partial purification, the specific activity increased to 39.61 units/mg protein with a yield of 19% and three fold purification.

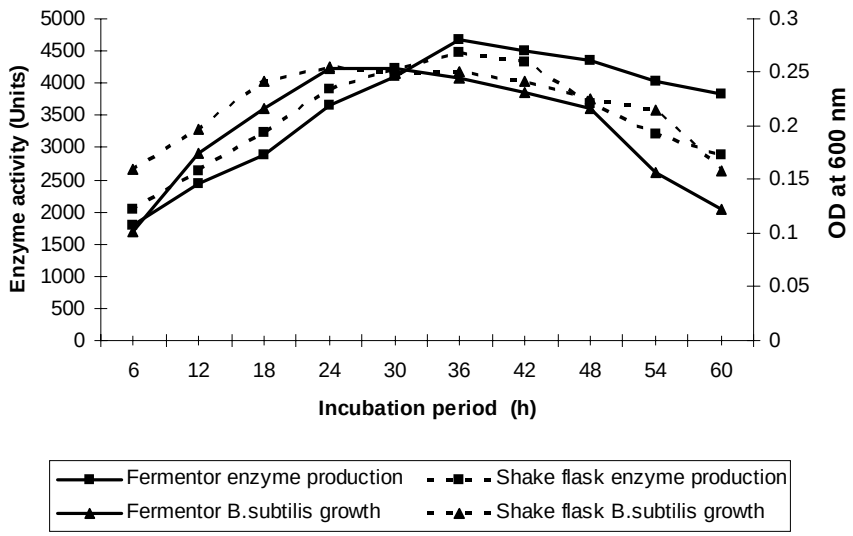

Fig. 5. Enzyme production and growth of *B. subtilis* strain CM3 in laboratory fermentor and shake flask cultures

Electrophoretic studies showed that there were two forms of the α-amylase (AI and AII) and the molecular mass of partially purified enzymes were approximately 18 000 ± 1000 (AI) and 43 000 ± 1000 (AII) Da, respectively (Fig. 4). The partially purified enzyme showed similar pH (7.0) and temperature (60°C) optima and stability as the crude enzyme preparation (data not shown)

When *B. subtilis* strain CM3 was cultivated in a laboratory fermentor, peak activity (4685 units) was obtained at 36 h which was similar to the shake-flask cultures (4487 units) (Fig. 5) while the cell density attained in the fermentor (0.253 at 600 nm) was similar to that in the shake flasks (0.249 at 600 nm).

Discussion

Members of the genus *Bacillus* produce a large variety of extracellular enzymes, of which amylases are of particularly significant industrial importance. Similar to other *Bacillus* species, *i.e. B. coagulans*, *B. thermooleovorans*, *B. licheniformis*, *etc.*, *B. subtilis* strain CM3 was found to produce α-amylase maximally at the optimal growth temperature of 50–60°C (Babu and Satyanarayana, 1993; Malhotra *et al*., 2000; Das *et al*., 2004; Najafi *et al*., 2005). The pH range (5.0–9.0) was found to be optimal for amylase production by *B. subtilis* CM3 as also reported for *B. brevis* (Tsvetkov and Emanuilova, 1989), *B. coagulans*, *B. licheniformis* (Krishnan and Chandra, 1983), *B. thermooleovorans* (Malhotra *et al*., 2000) and *B. subtilis* (Das *et al*., 2004).

Cultural conditions have a profound influence on amylase production. Enzyme production was maximal when the cell population entered into stationary phase of growth. Similar findings have been recorded for several other *Bacillus* species *i.e. Bacillus amyloliquefaciens* (Roychoudhary *et al*., 1989), *B. thermooleovorans* (Malhotra *et al*., 2000), and *B. subtilis* (Baig *et al*., 1984; Najafi *et al*., 2005).

Amylase production by this strain was constitutive since biosynthesis of the enzyme took place not only in the presence of starch but also with other carbon sources. Moreover, the amylase yield was similar in all types of carbon sources such as soluble starch, potato starch, glucose, maltose, sucrose, etc. and therefore, this was not considered to reflect inducibility (Tonkova, 2006). Starch at a concentration of 1% (w/v) supported optimal enzyme production, followed by a decline at higher concentrations (Fig. 2A). This can be attributed to the high viscosity of culture broth at such concentrations, which interferes with O_2 transfer leading to limitation of dissolved O_2 for the growth of bacteria (Rukhaiyar and Srivastava, 1995).

Among nitrogen sources, organic nitrogen supported higher amylase secretion in comparison with inorganic nitrogen sources, with ammonium acetate as the only exception. Moreover, urea, ammonium chloride and ammonium sulphate at 1% level inhibited (78.1–97.5%) enzyme activity. However, there was no difference in the growth of *B. subtilis* grown with any of these nitrogen sources. Similar results were obtained for other *Bacillus* spp. *i.e.*, *B. licheniformis* (Aiyer, 2004), *B. subtilis* (Haq *et al*., 2002), *B. thermooleovorans* (Narang and Satyanrayan, 2001) and *B. coagulans* (Babu and Satyanrayan, 2001). In contrast, Das *et al.* (2004) reported maximum α-amylase production by *B. subtilis* DM-03 obtained by using ammonium chloride as the nitrogen source. Further, there was no significant variations in the enzyme yield among organic nitrogen sources (beef extract, peptone, yeast extract, *etc.*) incorporated at 1% level in the basal medium. Similar results were reported for *B. thermooleovorans* (Malhotra *et al*., 2000), *B. stearothermophilus* (Davies *et al*., 1980) and *B. amyloliquefaciens* (Babu and Satyanarayana, 1993). The concentration of yeast extract or ammonium acetate was also critical for obtaining maximum enzyme yield. The enzyme levels were high at 0.5–1.0% levels and declined sharply thereafter as in the case of *B. coagulans* (Malohotra *et al.*, 2000). The decline in amylase production at increased nitrogen concentration could be due to the lowering of pH of the production medium or the induction of protease, which suppresses the amylolytic activity (Tonkova, 2006). It is generally known that surfactants often increase enzyme secretion and production (Ray *et al*., 1990) but the explanation how they act to increase enzyme yield is largely conjectural (Reese and Maguire, 1969). In this study, the various surfactants (0.02%) applied enhanced amylase activity (2–15%) over control (no surfactant). The increase enzyme accumulation might be due to increase in cell membrane permeability (Rao and Satyanarayana, 2003) and/or modification (swelling) of starch (Moorthy, 2002). Similar results were found in the case of other microorganisms, *i.e. Thermomyces lanuginosus* (Arnesen *et al*., 1998), *Bacillus circulans* (Palit and Banerjee, 2001), *etc.*

For production and stability of amylase of many *Bacillus* spp., addition of Ca^{2+} ion is often necessary (Tonkova, 1991; 2000). In this study, addition of Ca^{2+} had no effect at all on enzyme activity. Ca^{2+} independent α-amylase from *Bacillus* spp. have been reported by several authors (Malhotra *et al*., 2000; Kumar *et al*., 1990; Mamo *et al*., 1999). Ca^{2+} independent amylase merits consideration for starch liquefaction, especially in the manufacture of fructose syrup, where Ca^{2+} is a known inhibitor of glucose isomerase (Tonkova, 2006).

Control environmental conditions are critical in achieving higher concentration or yield of any microbial product. Since *B. subtilis* strain CM3 could grow and produced α-amylase over a wide range of pH (5.0–9.0) and temperature (40–60°C), this might be the reason for not obtaining a higher yield in the fermentor in comparison with shaked flask culture.

Several bacteria, *i.e. Lactobacillus plantarum, L. casei*, *L. acidophilus*, *B. subtilis*, *Enterococcus diacetylactis*, *etc.* were isolated from the lower part of the gut of cow (Ware *et al.*, 1988). Other than these, the cow rumen contains various species of *Bacillus* and *Bifidobacterium* as well as yeasts (*S. cerevisiae*) for better rumen fermentation (Kung, L. Jr., www.das.psu.edu/dairynutrition/documents/kung.pdf.), which form the initial culturable microflora of cow dung (Swain and Ray, in press). Among the culturable cow dung microflora, *B. subtilis* strains were found to be the most common bacteria (Swain and Ray, 2006). The starch-digesting characteristics of these organisms, *i.e.* pH, 5.0–9.0; temperature, 40–60°C and capable of growing in various carbon and organic nitrogen sources may be useful parameters for the bacteria to digest starch as a component of feed under a wide-range of gut environmental conditions. Preliminary studies have also shown that this organism possesses endo- and exo- cellulase activities (Swain and Ray, 2006). The multi-complex enzyme (amylase and cellulase) activities of *B. subtilis* will be useful to the cattle in digesting a wide range of feed rich in starch and cellulose.

There were wide variations in molecular mass of amylases of different *Bacillus* spp., *i.e. B. coagulans* and *B. subtilis* (42 800–67 000 Da.) (Babu and Satyanarayan, 1993; Ozcan *et al.*, 2001; Das *et al.*, 2004), *Bacillus* spp.(97000 Da) (Kim *et al*., 1995). In this study, the two forms of amylase (AI and AII) of *B. subtilis* strain CM3 had molecular masses of 18 ± 1 and 43 ± 1 kDa, respectively. In a recent study, Najafi *et al.* (2005) reported molecular mass of α-amylase by a strain of *B. subtilis* AX 20 was 14.9 kDa.

In the present study, the α-amylase of *B. subtilis* strain CM3 isolated from cow dung is Ca^{2+} independent and thermostable and can therefore be of importance for the starch-processing industries. Further work is in progress in our laboratory on the use of this enzyme in cassava (*Manihot esculanta* L.) and sweetpotato (*Ipomoea batata* L.) starch saccharification for production of ethanol, organic acid and other bioproducts.

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