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Optimization of process parameters for α-amylase production under solid-state fermentation by *Bacillus Cereus* MTCC 10202

Kaliappan Kalaiarasi[1]* and Ramasamy Parvatham[2]

[1]Department of Textiles and Clothing, Avinashilingam University for Women, Coimbatore, Tamil Nadu, India.
[2]Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam University for Women, Coimbatore, Tamil Nadu, India.

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An α-amylase producing bacterial strain was isolated from sago factory effluent discharged soil and identified as *Bacillus cereus*. Production of extracellular α-amylase by *B. cereus* was studied in solid-state fermentation. Different substrates like wheat bran, maize bran, corn bran, millet bran, rice bran, greengram bran, blackgram bran, cassava peel powder, cotton seed oil cake, coconut oil cake, sesame oil cake and groundnut oil cake were screened to select the suitable substrate for the production of α-amylase. Among the agro-wastes, wheat bran was found to be the best substrate. Various physical and chemical parameters were optimized. Maximum α-amylase yield was achieved at pH 7 with inoculum level of 10% at 50°C and an incubation period of 72 h. The optimum ratio of substrate to moisture level was found to be 1:2 and substrate weight to flask volume was 1:50. Supplementation of starch at 1% (w/w) concentration and yeast extract at 1.5% (w/w) concentration resulted in maximum production of α-amylase. Calcium chloride at a concentration of 1% (w/w) was found to stimulate α-amylase production. Thus, *B. cereus* produced a high titre of α-amylase in solid state fermentation using inexpensive agro-residue under optimized conditions.

Key words: α-Amylase, *Bacillus cereus*, solid-state fermentation, optimization.

INTRODUCTION

α-Amylases (α-1,4-glucanohydrolases,E.C.3.2.1.1) are endo-amylases that catalyze the hydrolysis of α-D-(1,4) glycosidic linkages in starch components or related carbohydrates, releasing malto-oligosaccharides and glucose in the α-anomeric form (Nazmi et al., 2006). The potential for commercial application of α-amylases is enormous. It is a key enzyme in the conversion of starch to sugar syrups, production of cyclodextrins, preparation of digestive aids, production of chocolates, cakes and fruit juices. They are very extensively used in beverages, baby foods and pharmaceutical industries. Besides their use in starch saccharification, they also find application in brewing, paper and distillery industries (Ramachandran et al., 2004).

α-Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes. However, enzymes from bacterial sources have dominated applications in Industrial sectors. The major advantages of using microorganism for the production of α-amylase are in economical bulk production capacity and they are easy to manipulate to obtain enzymes of desired characteristics. In addition, they allow an economic technology with low resource

*Corresponding author. E-mail: kalaiarasik@rediffmail.com.

consumption and low emission involving no social or political issues as in the case of animal and plant sources (Venkat, 2007). Microorganisms utilize various substrates as nutrient source for their growth and metabolic activities and subsequently produce metabolism related products. However, fine tuning of nutrient concentrations regulate the microbial metabolism and associated metabolic product formation. Balancing of nutrient concentrations with minimum experimentation and other cultural parameters is an art in microbial metabolism to optimize enzyme production (Prakasham et al., 2007).

α-Amylase is produced either by submerged (Oyeleke and Oduwole, 2009) or solid-state fermentation (Varalakshmi et al., 2009). Solid-state fermentation dominates over submerged fermentation in aspects such as better yield, simple technique, low capital investment, lower levels of catabolite repression, high stability and better product recovery (Babu and Satyanarayana, 1995). Agro-residues are generally considered as the best substrate for the solid-state fermentation processes (Ellaiah et al., 2002). Many agro-industrial by-products such as wheat bran, rice bran, molasses, barley bran, maize meal, soybean meal, potato peel and coconut oil cake have been screened as low cost solid substrates for microbial production of α-amylase in solid-state fermentation (Shukla and Kar, 2006). The major factors that affect microbial synthesis of enzymes in a solid-state fermentation system include the selection of a suitable substrate, microorganism, inoculum concentration, particle size and moisture level of the substrate. The aim of the present study is to investigate the production of α-amylase from *Bacillus cereus* under solid-state fermentation using inexpensive and abundantly available agro-residues as substrate thereby reducing the cost of enzyme production.

In this paper we have reported the factors that influence maximization of α-amylase production by *B. cereus.*

MATERIALS AND METHODS

Screening and isolation of α-amylase producing bacteria

A total of 433 bacterial isolates were isolated from different sources including field soils, waste water discharged soils, effluents and spoiled food sources by serial dilution technique. The bacterial isolates were screened for α-amylase production on starch agar plates. The hydrolysis zone formed on starch agar plates were visualized by flooding the plates with Gram's iodine solution. The amylolytic potential was estimated using the amylolytic Ratio (R/r) defined as the diameter of the hydrolyzation zone (R) divided by the diameter of the producing colony (r) (Bernhardsdotter et al., 2005). Isolates having a higher amylolytic ratio (above 3) of clearing zone to colony size were grown in liquid broth and the amount of amylase production was determined from cell free supernatant. The bacterial isolate that produced maximum amylase was selected and identified in the Institute of Microbial Technology (IMTECH) at Chandigarh, India. The selected bacterial strain was maintained in nutrient agar slants. The bacterial strain was sub-cultured periodically after every 30 days and stored at 4°C.

Preparation of bacterial inoculum

A volume of 50 ml nutrient broth supplemented with 1% soluble starch was taken in a 250 ml Erlenmeyer flask. The flask was sterilized in an autoclave at 15 lb pressure (121ºC) for 15 min. After cooling the medium, a loopfull of bacteria from a 24 h old slant was aseptically transferred to the flask and kept at 37°C in a rotary shaker (150 rpm). After 24 h of incubation, 1 ml of this nutrient broth culture was used as the inoculum for 100 ml of production medium.

Production of bacterial α-amylase in solid-state fermentation

Solid-state fermentation (SSF) was carried out in 250 ml Erlenmeyer flasks containing 5 g of wheat bran. The substrate was moistened with 10 ml of distilled water, autoclaved (15 lb) at 121°C for 15 min and cooled. The flasks were inoculated with 1% (v/w) bacterial inoculum and incubated at 37°C for 72 h.

Enzyme extraction

The enzyme from the fermented bacterial bran was extracted twice with 50 ml of 10 mM phosphate buffer (pH 7.0). Extraction was done by soaking the fermented solids with phosphate buffer for 30 min at 30°C on a rotary shaker (150 rpm). The slurry was squeezed through a damp cheese cloth. The extracts were pooled and centrifuged at 4°C for 15 min at 5000 rpm to separate small wheat bran particles, cells and spores. The brown clear supernatant was used as the source of α-amylase (Ellaiah et al., 2002).

α-Amylase activity

α-Amylase activity was determined as per the method described by Bernfeld (1955). One unit of α-amylase activity is defined as the number of µmol of maltose liberated by 1 ml of enzyme solution per minute. Amylase production was expressed as units per gram of dry substrate.

Optimization of process parameters for α- amylase production

Various process parameters such as inoculum level, incubation period, moistening agents, moisture level, pH, temperature, carbon sources, nitrogen sources and metal salts were optimized. The strategy followed was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments.

Effect of different agro-residues as solid substrates

The bacterial strain was inoculated (1%v/w) in 250 ml Erlenmeyer flasks each containing 5 g of various substrates (wheat bran, maize bran, corn bran, millet bran, rice bran, greengram bran, blackgram bran, cassava peel powder, cotton seed oil cake, coconut oil cake, sesame oil cake and groundnut oil cake. The flasks were moistened with 10 ml of distilled water and incubated at 37°C for 72 h. The enzyme was then extracted and assayed for α-amylase activity. The best solid substrate achieved by this step was used for subsequent experiments.

Effect of inoculum level

Culture flasks (250 ml) each containing wheat bran (5 g) moistened with 10 ml distilled water were autoclaved and inoculated with dif-

ferent amounts (2, 4, 6, 8, 10 and 12% v/w) of bacterial inoculum. All the flasks were incubated at 37°C for 72 h. The contents of the flasks were harvested and assayed for α-amylase activity. The optimum inoculum level achieved was used for further optimization studies.

Effect of incubation period

The inoculated flasks were incubated at different time intervals such as 24, 48, 72, 96 and 120 h. After each incubation period, the enzyme was extracted and assayed for α-amylase activity. The optimum incubation period found was followed for further experiments.

Effect of moistening agents

The effect of moistening agents (MA) on the production of α-amylase was studied using the following five mineral salt solutions prepared in distilled water as follows:

MA I (g/L) - $MgSO_4$ $7H_2O$ - 0.5; K_2HPO_4 -1.5 - pH 7.2
MA II (g/L) - KH_2PO_4 - 11.0; NaH_2PO_4 - 6.1; KCl - 3.0; $MgSO_4$ $7H_2O$ - 0.1-pH 7.2
MA III (g/L) - K_2HPO_4 - 0.1; $(NH_4)H_2PO_4$ -1.0; $MgSO_4$ $7H_2O$ - 0.5; $CaCl_2$ -0.1; $FeSO_4$,- 0.1; $MnSO_4$ - 0.1- pH 7.2
MA IV (g/L) - $(NH_4)NO_3$ - 2.0; K_2HPO_4 - 5.0; $MgSO_4$ $7H_2O$ - 0.5; KCl - 0.5 - pH 7.2
MA V (g/L) - KH_2PO_4 - 11.0; NaH_2PO_4 - 6.1; KCl- 3.0; $MgSO_4$ $7H_2O$ - 1.0 - pH 7.2

Besides the aforementioned moistening agents, distilled water and tap water were also used as moistening agents. The enzyme was extracted and assayed from each set. The selected moistening agent was used to moisten the substrates in the subsequent experiments.

Effect of moisture level

The effect of different moisture level on α-amylase production was studied by varying the ratio of weight of the substrate to the volume of moistening agents (1:1.0, 1:1.5, 1:2.0, 1:2.5 and 1:3.0 w/v). The fermentation was carried out at 37°C for 72 h having other experimental conditions at their optimum levels. The optimum ratio of weight of solid substrate to volume of moistening agent achieved by this step was fixed for subsequent studies.

Effect of initial pH

To determine the effect of initial pH on α-amylase production, the pH of the moistening agent was varied from 4 to 10 with one unit interval using 0.1 N HCl and 0.1 N NaOH. An inoculum level of 10% and moisture ratio of 1:2 was employed. The fermentation was carried out at 37°C for 72 h. After the incubation period, the enzyme was extracted and assayed. The optimum initial pH of the solid substrate determined by this step was fixed for subsequent experiments.

Effect of incubation temperature

The fermentation was carried out at various temperatures such as 30 to 90°C with an interval of 10°C, keeping all other parameters at their optimum levels. After incubation, the enzyme was extracted and assayed for α-amylase activity.

Effect of selected supplementary carbon sources

The effect of various carbon supplements on α-amylase production was evaluated by adding carbon sources (1% w/w) such as starch, maltose, lactose, sucrose, glucose and fructose. The crude enzyme extract was analyzed for α-amylase activity. The most suitable carbon supplement was found to be starch.

Effect of different concentrations of starch

Wheat bran was supplemented with starch at different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5% w/w) individually. All the other experimental conditions were kept at their optimum. The optimum starch concentration determined was used for further studies.

Effect of selected supplementary nitrogen sources

Various nitrogen sources such as yeast extract, peptone, tryptone, casein, beef extract, urea, ammonium sulphate, ammonium chloride and sodium nitrate at 1% (w/w) concentration was incorporated separately into the fermentation medium to select the best nitrogen supplements for α-amylase production. α-Amylase activity was determined in the cell free supernatant. The most suitable nitrogen supplement was found to be yeast extract.

Effect of different concentrations of yeast extract

Different yeast extract concentrations such as 0.5, 1.0, 1.5, 2.0 and 2.5% (w/w) were supplemented in the fermentation medium separately. All the other experimental conditions were kept at their optimum.

Effect of the amount of substrate to flask volume

The effect of the amount of substrate to flask volume was studied by changing the ratio of weight of the substrate to flask volume. Different amounts (5, 10, 15, 20 and 25 g) of wheat bran were taken individually in 250 ml conical flasks. The experiment was conducted at 50°C for 72 h keeping all other conditions at their optimum levels. The optimum ratio of the amount of substrate to flask volume achieved by this step was fixed for subsequent experiments.

Effect of different metal salts on α-amylase production

Influence of different metal salts on α-amylase production was studied by incubating the culture medium with various metal salts, namely, calcium chloride, magnesium sulphate, ferric chloride, manganese sulphate, copper sulphate, mercuric chloride, zinc sulphate, silver chloride, sodium chloride, potassium chloride, lead nitrate and lithium sulphate each at a concentration of 1 mM was mixed with the moistening agent individually (Sharma et al., 2007). After incubation, α-amylase was extracted and assayed for its activity.

Effect of different concentrations of calcium chloride

Wheat bran was incorporated with various concentrations of calcium chloride separately (0.2, 0.4, 0.6, 0.8 and 1.0% w/w) and fermentation was carried out under optimized conditions. After incubation period, the enzyme was extracted and assayed.

Table 1. α-Amylase production on selected agro-residues as substrates.

Agro-residue	α-Amylase activity (U/gds)
Wheat bran	24.85±0.98
Maize bran	17.42±1.20
Corn bran	9.35±1.33
Millet bran	14.56±1.17
Rice bran	18.55±0.58
Greengram bran	12.92±1.00
Blackgram bran	16.57±1.02
Cassava peel powder	20.62±0.90
Cotton seed oil cake	13.87±0.80
Coconut oil cake	20.03±0.52
Sesame oil cake	7.47±1.01
Groundnut oil cake	15.95±0.73

U/gds, Units per gram of dry substrate

RESULTS AND DISCUSSION

Isolation and identification of the selected amylolytic bacterial strain

Among the 433 bacterial isolates, 42 showed amylolytic ratio greater than 3 (good), 209 showed between 2 to 3 (moderate) and 182 showed less than 2 (poor). Bernhardsdotter et al. (2005) and Oyeleke and Oduwole (2009) have recorded an amylolytic ratio of 3 for *Bacillus* sp. isolate L 1711 and 3.1 for *Bacillus subtilis* respectively. The bacterial isolates were grown in liquid broth and the amount of α-amylase production was determined in cell free supernatant. Bacterial strain isolated from sago factory effluent discharged soil showed maximum α-amylase activity (36.85 U/ml) and was selected as the best potent producer of α-amylase. The selected bacterial isolate was identified as Gram positive, rod shaped and non-motile with the size ranging from 2 to 3 µm. Off-white colonies were produced on nutrient agar medium. The biochemical characterization of the isolate showed positive results for methyl red, voges proskauer, nitrate reduction, catalase, oxidase, gelatin and starch hydrolysis; negative results for indole test, citrate utilization and urea hydrolysis. Regarding carbohydrate fermentation test, the selected bacterial isolate produced acid with glucose and maltose, whereas there was no acid production with lactose, sucrose, mannitol and xylose as substrates. Based on these morphological, physiological and biochemical tests, the selected bacterial strain was identified as *B. cereus* at the Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited in their culture collection with the accession number MTCC 10202.

Many workers have reported *Bacillus* sp. as a potent producer of α-amylase (Sodhi et al., 2005; Anto et al.., 2006; Thippeswamy et al., 2006; Asgher et al., 2007; Ahmed, 2008).

Optimization of various physical and chemical parameters for α- amylase production

Effect of different agro-residues as solid substrate

In SSF process, the selection of a suitable solid substrate for fermentation process is a critical factor and thus involves the screening of a number of agro-wastes for microbial growth and product formation. Various agro-residues as substrates were screened for amylase production and the results are presented in Table 1. All the substrates supported growth and enzyme production. A high titre of α-amylase activity was obtained with wheat bran followed by cassava peel powder and coconut oil cake. The results of the present study are in concurrence with Baysal et al. (2003), Anto et al. (2006) and Gangadharan et al. (2006), who have reported wheat bran as the best substrate for optimal production of amylase by *B. subtilis*, *B. cereus* MTCC 1305 and *Bacillus amyloliquefaciens*, respectively. Wheat bran was found to be the suitable substrate for α-amylase production when compared to all the other agro-residues. This might be due to the presence of sufficient nutrients and ability to remain loose even under moist conditions, thereby providing a large surface area (Babu and Satyanarayana, 1995). In the subsequent experiments, wheat bran was used as the substrate for the production of α-amylase.

Effect of inoculum level

The fermentation profile of an organism is usually affected by the initial inoculum concentration. An increase in inoculum level was found to improve the growth and production of α-amylase and reached maximum at 10% v/w (29.74 U/gds). Increase in inoculum level above 10% was found to decline the enzyme production (Figure 1a). This might be due to higher concentration of inoculum resulting in increased competition for carbon source and nutrients, which might lead to exhaustion of nutrients. This imbalance would have resulted in reduced enzyme production and also the free excess liquid present in an unabsorbed form would have given rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate might lead to decrease growth and enzyme production (Baysal et al., 2003). Lower inoculum level would have resulted in a lesser number of cells in the production medium. These require a longer time to grow to optimum number to utilize the substrate and form the desired product (Kashyap et al., 2002). The results of the present study is in concurrence with Anto et al. (2006) who have reported 10% (v/w) as optimum inoculum concentration for the production of amylase with *B. cereus* MTCC 1305.

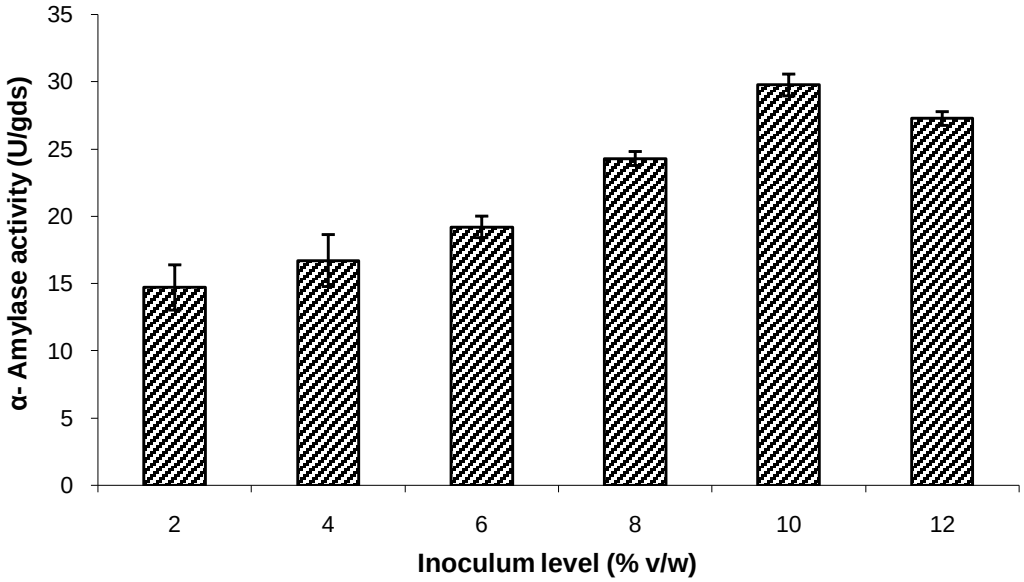

Figure 1a. Effect of inoculum level on α- amylase production.

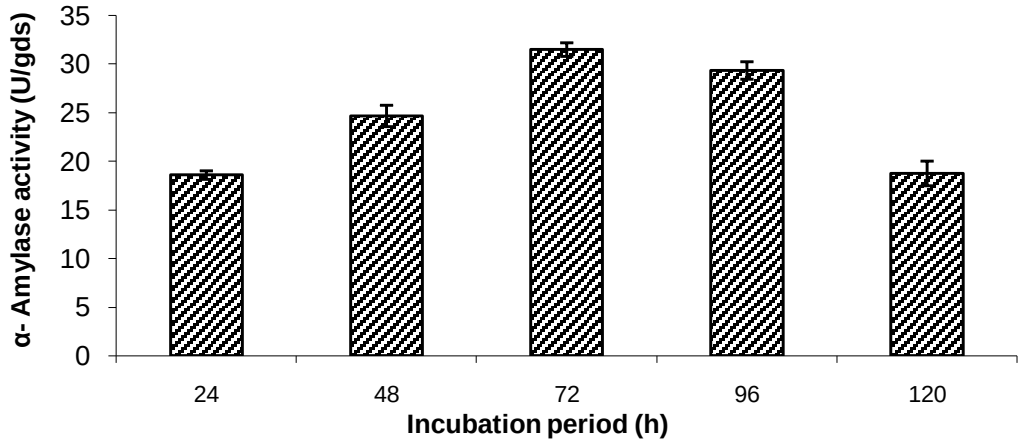

Figure 1b. Effect of incubation period on α- amylase production.

In contrast, Baysal et al. (2003) have reported that 20% inoculum led to good enzyme production with *B. subtilis*. An inoculum concentration of 10% (v/w) was fixed for further optimization experiments.

Effect of incubation period

The optimum incubation time for achieving the maximum enzyme production is governed by the characteristics of the culture and is based on the growth rate of the microorganisms and its enzyme production pattern. α-amylase production reached the peak (31.50 U/gds) at 72 h of incubation (Figure 1b). The enzyme production showed a gradual decrease on further extension of fermentation period. This could be due to loss of moisture or denaturation or decomposition of the enzyme resulting in variation in pH during fermentation or due to interaction with other components in the culture medium (Ahmed, 2008). The observed results are in agreement with Babu and Satyanarayana (1995) and Anto et al. (2006) who have reported maximum amylase production at 72 h of incubation with *Bacillus coagulans* and *B. cereus* MTCC 1305, respectively; whereas, Baysal et al. (2003) have reported 48 h as optimum incubation time for *B. subtilis*. In most cases, the optimum incubation period for α-amylase production in SSF using *Bacillus* sp. varied from 48 to 72 h, depending on the environmental conditions (Sivaramakrishnan et al., 2006). An incubation period of 72 h was followed for further experiments.

Table 2. Effect of moistening agents on α-amylase production.

Moistening agent	α- Amylase activity (U/gds)
MA I	34.93±0.39
MA II	30.76±0.15
MA III	15.51±0.95
MA IV	25.67±0.62
MA V	14.94±1.05
Tap water	21.40±0.55
Distilled water	22.01±0.43

U/gds, Units per gram of dry substrate. Values are mean of three replicates ± SD.

Table 3. Effect of moisture level on α-amylase production.

Moisture level (substrate: MA I) (w/v)	α- Amylase activity (U/gds)
1:1.0	24.28±0.52
1:1.5	29.35±0.89
1:2.0	36.66±0.11
1:2.5	28.99±1.66
1:3.0	25.88±0.43

U/gds, Units per gram of dry substrate. Values are mean of three replicates ± SD.

Effect of moistening agents

The nature of the moistening agent (MA) is a main factor in SSF which often determine the success of a process. MA I resulted in maximum (Table 2) α-amylase production. Similar observations were made by Babu and Satyanarayana (1995) who have reported mineral salt solution as the best moistening agent for *B. coagulans* in SSF. In contrast, Sodhi et al. (2005) have reported that amylase production by *Bacillus* sp. PS - 7 was maximum when tap water was used as moistening agent. MA I was used to moisten the substrate wheat bran, in the subsequent optimization experiments.

Effect of moisture level

The importance of moisture level in SSF and its influence on the biosynthesis of enzymes has been attributed to the interference of moisture in the physical properties of solid particles. The enzyme production attained a peak, when moistened with MA I in the ratio of 1:2 (Table 3). Enzyme production was found to decrease with lower moisture content. This might be due to an insufficient water availability preventing a good diffusion of solutes and gas, thereby resulting in slow down or complete arrest of cell metabolism. Also, it leads to lower degree of substrate swelling and higher water tension and thereby reduces the solubility of nutrients (Gervais and Molin, 2003). There was a decline in α-amylase production when the moisture level was increased above 1:2. It was suggested that higher moisture levels decreased porosity, promoted development of stickiness, reduced gas volume and exchange, changed substrate particle structure, resulted in static hindrance of the growth of the organisms through reduction in inter particle spaces, decreased diffusion and impaired oxygen transfer (Perez-Guarre et al., 2003). It also resulted in poor adsorption of enzyme to the substrate particle (Swain and Ray, 2007). The observed results are in agreement with the results of Babu and Satyanarayana (1995) and Anto et al. (2006) who have reported an optimum ratio of 1:2 for *B. coagulans* and *B. cereus* MTCC 1305, respectively in SSF; whereas, Sodhi et al. (2005) have reported maximum α-amylase production when the ratio of wheat bran to moisture level was maintained at 1:1.5 for *Bacillus* sp. PS - 7.

The moisture content of the medium changes during fermentation which is a result of evaporation and metabolic activities and so adjusting the optimum moisture level of substrate during SSF is considered most important. Hence, the substrate: initial moisture level ratio was fixed as 1:2 for the subsequent experiments.

Effect of pH

High enzyme titre (34.93 U/gds) was attained when the initial pH of the medium was maintained at 7 (Figure 2a). pH is one of the important factors that determine the growth and enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. As the metabolic activities of the microorganisms are very sensitive to changes in pH, α-amylase production was found to be affected when the pH level is above or below the optimum pH. The obtained optimum pH value is in concurrence with earlier reports of Konsula and Kyriakides (2004) and Asgher et al. (2007) with *B. subtilis.* Thippeswamy et al. (2006) and Mishra and Behera (2008) have reported maximum amylase production near neutrality for *Bacillus* sp. However, Aiyer and Modi (2005) reported pH 9 to be optimum for the production of amylase by *Bacillus licheniformis* SPT 27.

Effect of incubation temperature

Temperature control in the substrate bed is very important for SSF since growth and production of enzymes or metabolites are usually sensitive to temperature. Optimal temperature for maximum α-amylase production was found to be at 50°C (31.77 U/gds). A reduction in enzyme activity was observed at temperatures above 50°C (Figure 2b). Temperatures above 50°C resulted in lesser growth of *B. cereus* and decrease

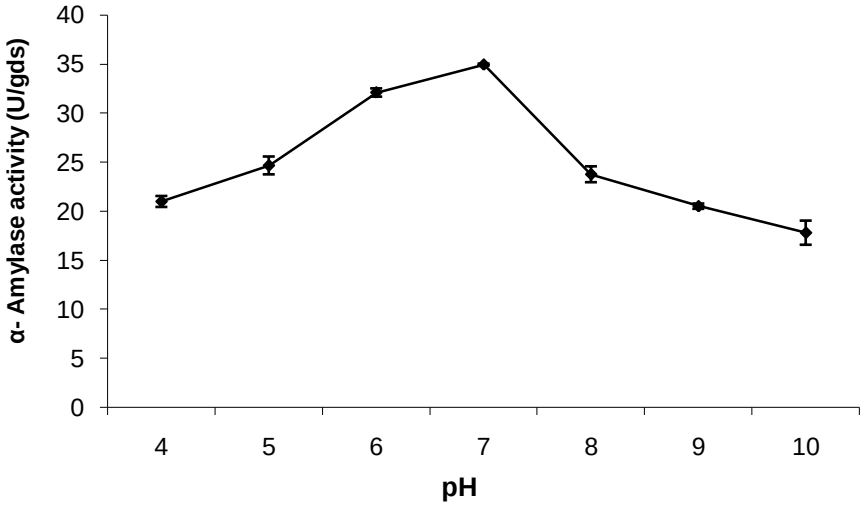

Figure 2a. Effect of initial pH on α-amylase production.

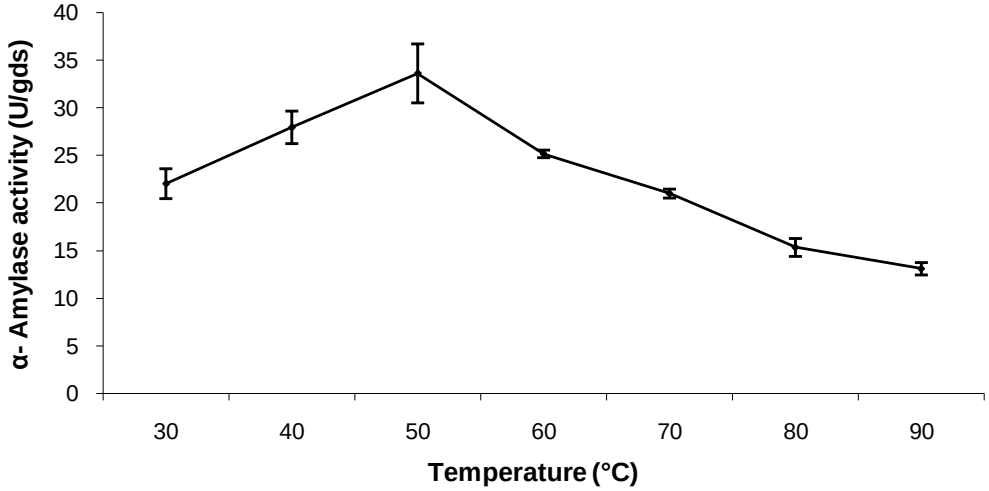

Figure 2b. Effect of temperature on α-amylase production.

in the yield of enzyme. Moisture loss in solid substrate due to increase in temperature might be the cause for lesser production of α-amylase. An identical temperature optimum for α-amylase production was reported by Asgher et al. (2007) who have reported 50°C to be the optimum temperature for amylase production by *B. subtilis* JS- 2004 strain. However, Konsula and Kyriakides (2004), Goyal et al. (2005) and Sodhi et al. (2005) have reported maximum amylase production at 40°C by *B. subtilis*, *Bacillus* sp. I-3 and *Bacillus* sp. PS - 7, respectively.

Effect of selected supplementary carbon sources

Growth and enzyme production of any organisms are greatly influenced by the nutrients available in the growth medium. α-amylase is an inducible enzyme. The carbon sources in the medium are found to exert a profound effect on the enzyme production behaviour. Some carbon sources supported good growth with low enzyme production while others supported good growth as well as enzyme secretion. Among the different carbon supplements examined, starch promoted high enzyme titre (48.53 U/gds) which was followed by maltose, lactose and sucrose (Figure 3a). The results are in agreement with the reports of Konsula and Kyriakides (2004) for *B. subtilis*, Goyal et al. (2005) for *Bacillus* sp. I-3 and Sodhi et al. (2005) for *Bacillus* sp. PS-7 who have reported maximum amylase production when starch was used as carbon supplement. Glucose and fructose supplementation resulted in the repression of enzyme

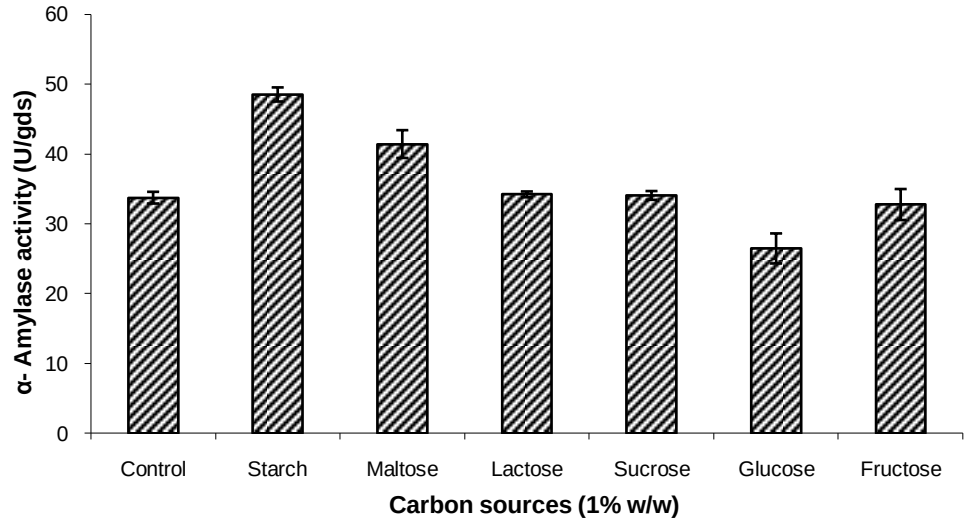

Figure 3a. α-Amylase production using selected supplementary carbon sources.

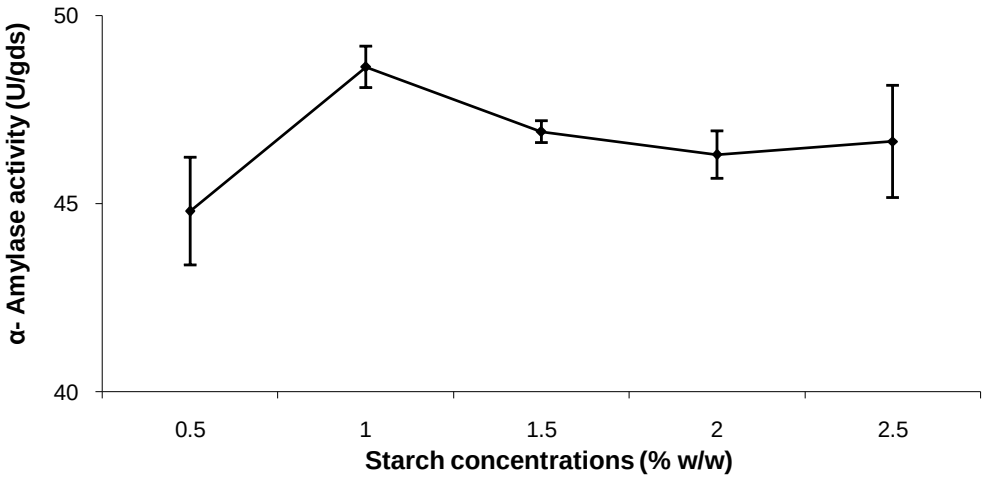

Figure 3b. α-Amylase production at different starch concentrations.

production. This might be due to the feedback inhibition caused by the presence of glucose and fructose as reported by Rama and Srivastav (1995). The repression is higher with glucose than fructose. Glucose acted as a catabolic repressor for the enzyme production.

Effect of different concentrations of starch

Among the different concentrations of starch, 1% resulted in maximum increase in α-amylase production (48.63 U/gds). The observed results (Figure 3b) are similar to the report of Gangadharan et al. (2006) for *B. amyloliquefaciens*. In contrary, Mishra and Behera (2008) observed that addition of 2% starch resulted in high enzyme yield by *Bacillus* sp.

Effect of selected supplementary nitrogen sources

Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes in SSF system. Among the various organic and inorganic nitrogen sources tested (Figure 4a), yeast extract showed maximum α-amylase yield (55.06 U/gds) followed by peptone, beef extract, tryptone, casein and urea. The influence of yeast extract on α-amylase production might be due to the presence of vitamin B group (promoting group), free amino acids and carbohydrate. It is observed that organic nitrogen compounds increased α-amylase production than when compared to inorganic compounds. Same observation was made by Qader et al. (2006) for *Bacillus* sp. AS-1. The obtained result is in concurrence with the work reported earlier by Thippeswamy et al.

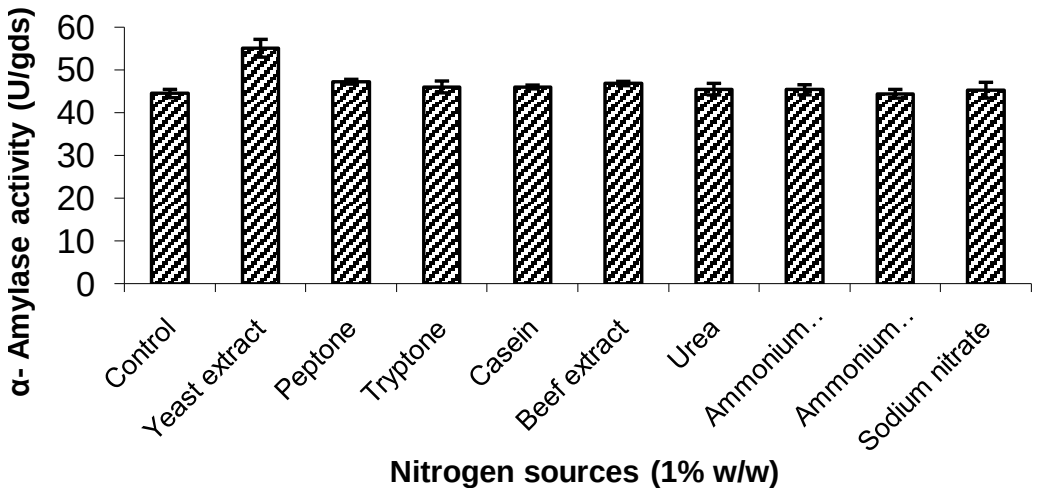

Figure 4a. α-Amylase production by selected supplementary nitrogen sources.

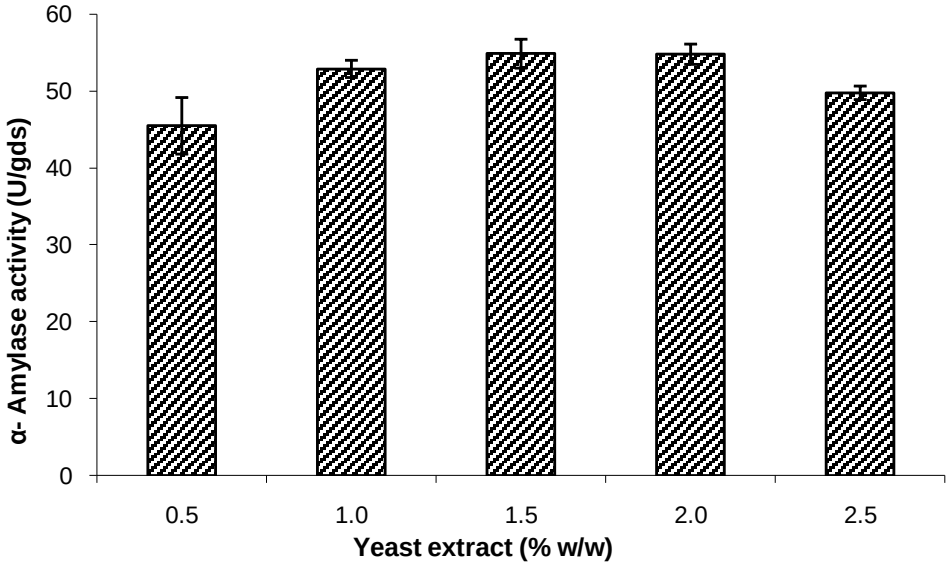

Figure 4b. α-Amylase production at different yeast extract concentrations.

(2006) for *Bacillus* sp. and Asgher et al. (2007) for *B. subtilis* JS 2004 who have reported yeast extract as the best nitrogen supplement for amylase production.

Effect of different concentrations of yeast extract

The α-amylase yield reached the peak at 2.0% concentration of yeast extract. Above 2.0%, there was a decrease in α-amylase yield (Figure 4b), which might be due to changes in C/N ratio. The nitrogen is metabolized to produce primarily amino acids, nucleic acids, protein and cellular components. Low levels of nitrogen are inadequate for enzyme production; whereas, excess nitrogen can equally be detrimental causing in some cases a complete inhibition of enzyme production (Kole et al., 1988). The observation recorded in this study is similar to the earlier report of Hamilton et al. (1999) who have recorded maximum amylase yield at 2.0% yeast extract concentration for *Bacillus* sp. IMD 435.

Effect of amount of substrate to flask volume

α-Amylase production was found to be maximum when the ratio of substrate weight -to- flask volume was 1:50 (Figure 5a). This finding is in agreement with the reports of Satyanarayana (1994) for amylase production by *B.*

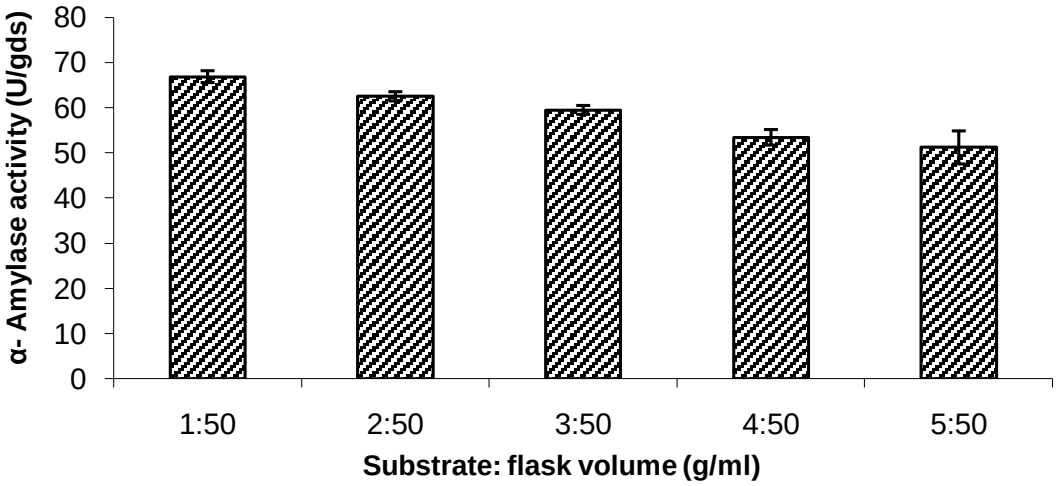

Figure 5a. Effect of amount of substrate to flask volume.

Table 4. Effect of metal salts on α-amylase production.

Metal salt (1 mM in moistening agent)	α- Amylase activity (U/gds)
Control	48.69±3.17
Calcium chloride	65.89±1.99
Magnesium sulphate	61.69±1.70
Ferric chloride	55.59±0.84
Manganese sulphate	56.01±0.95
Copper sulphate	44.33±4.19
Mercuric chloride	41.52±3.21
Zinc sulphate	48.46±2.12
Silver chloride	53.34±2.07
Sodium chloride	56.71±1.81
Potassium chloride	54.44±1.64
Lead nitrate	44.92±5.29
Lithium sulphate	61.28±2.21

Control, without metal salts. U/gds, units per gram of dry substrate. Values are mean of three replicates ± SD.

coagulans.

Effect of metal salts on α-amylase production

Supplementation of salts of certain metal ions is found to influence the growth of microorganisms and thereby stimulate or inhibit enzyme production. Among the metal ions supplemented (Table 4), $CaCl_2$ increased α-amylase production. $MgSO_4$, $LiSO_4$, NaCl, $MnSO_4$, $FeCl_2$, $AgCl_2$ and KCl stimulated α-amylase production; whereas, $PbNO_3$, $HgCl_2$, $CuSO_4$ and $ZnSO_4$ had negative effect on amylase production. α-Amylases are known to be metalloenzymes, 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. This might be due to the increased availability of calcium ions (Francis et al., 2003). The results of present study are in line with Asgher et al. (2002) who have recorded higher amylase production on addition of calcium chloride in the medium for *B. subtilis.* However, Goyal et al. (2005) have reported that $LiSO_4$ and $CaCl_2$ in the medium increased amylase production in *Bacillus* sp. I-3.

Effect of different concentrations of calcium chloride

The production of α-amylase is found to be calcium dependent. The α-amylase yield increased with the addition of calcium chloride and peaked (64.97 U/gds) with 1% (Figure 5b). Asgher et al. (2002) have recorded maximum amylase yield with 0.05% calcium chloride supplemen-

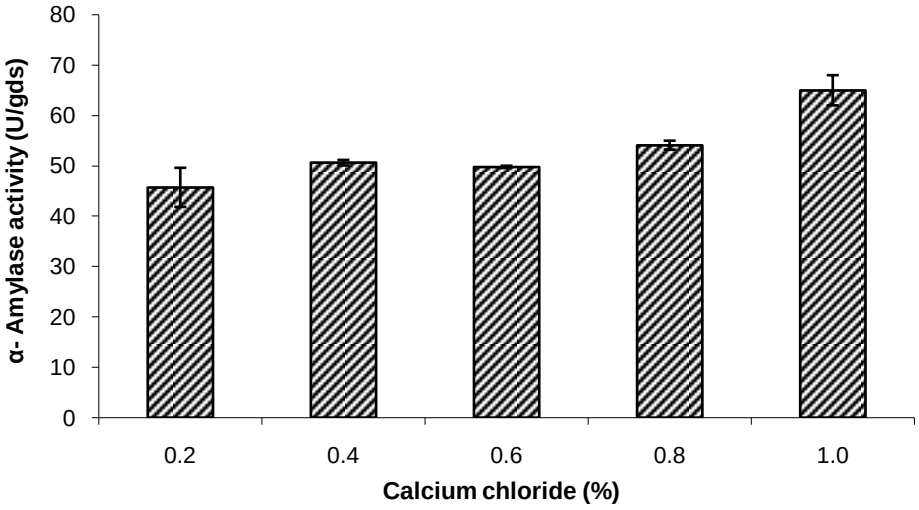

Figure 5b. Effect of different concentrations of calcium chloride.

tation by *B. subtilise*; whereas, Qader et al. (2006) have reported that a low level of calcium chloride (0.02%) was sufficient for amylase production by *Bacillus* sp. AS-1. The effect of metal ions on α-amylase production varies from microorganism to microorganism. Hence, based on the present observations, calcium chloride at a concentration of 1% was supplemented for maximum α-amylase production.

Conclusions

The present study describes the suitability of the laboratory isolate *B. cereus* for the commercial exploittation using simple, less expensive and economically feasible substrate, wheat bran with the supplementation of simple nutrients like starch and yeast extract. The maximum productivity of α-amylase was achieved by optimized process parameters such as 10% inoculum concentration, incubation time of 72 h, incubation temperature at 50°C, 1:2 (w/v) ratio of weight of wheat bran to salt solution, 1:50 ratio of substrate weight to flask volume and pH 7. The scope of the study may be explored in large scale for industrial application.

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