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Full Length Research Paper

Amylase production by moderately halophilic *Bacillus cereus* in solid state fermentation

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The production of extracellular amylase by the moderately halophilic bacterium *Bacillus cereus* was studied in solid state fermentation (SSF). Solid substrates such as rice bran, wheat bran, sugarcane bagasse, black gram husk and green gram husk were studied for enzyme production. Growth on sugarcane bagasse gave the highest amylase activity. The time course for amylase production inferred that 48 h was the optimum duration for higher amylase production. The suitable pH, temperature and inoculum level observed for higher amylase production was pH 7.0, 40.0°C and 2.0%, respectively. The amylase production of *B. cereus* was high in maltose (carbon source), yeast extract (nitrogen source), Tween 80 (surfactant) and calcium chloride (metal ion) added medium when compared to other respective sources tested. The halotolerancy of *B. cereus* for amylase production was 3% sodium chloride. The sugarcane bagasse can be utilized for the cheap production of amylase from *Bacillus cereus*. It is less expensive in comparison to synthetic media and is readily available for production purposes.

Key words: *Bacillus cereus*, amylase, solid state fermentation (SSF), sugarcane bagasse.

INTRODUCTION

Amylases are among the most important enzymes in present day biotechnology. The amylase family of enzymes is of great significance due to its wide area of potential application (Pandey et al., 2000). The extensive application of amylases in the food industry, such as baking, brewing, preparation of digestive aids, production of chocolate cakes, moist cakes, fruit juices, starch syrups etc., has paved a way for their large scale commercial production (Ramachandran et al., 2004a). Two major classes of amylase have been identified in microorganism, namely α-amylase and gluco-amylase. In addition β–amylase, which is generally of plant origin, has also been reported from a few microbial sources (Pandey et al., 2000).

α–Amylases are extra cellular endo enzymes that randomly cleave the 1, 4 –α–D-glucosidic linkages between the adjacent glucose units in a linear amylose chain. α–Amylases are enzymes with broad spectrum of applications in many sectors such as medical, pharmaceutical and analytical chemistry (Ramachandran et al., 2004b). α–amylases have been produced throughout the world by submerged fermentation; however, this process is cost intensive due to low concentrations of products and the consequent handling and disposal of a large volume of water during down-stream processing (Goes and Sheppard, 1999). The contents of synthetic media are very expensive (Haq et al., 2003). One alternative low cost production method is the use of solid state fermentation (SSF). SSF have been re-assessed as useful system for the production of protein biomass, enzymes and other valuable metabolites, and have found many important applications in the field of biotechnology (Nigam and Singh, 1994). This approach has recently been used also for the production of α–amylase (Murado et al., 1997;

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Mandhankumar et al., 1999; Mulimani and Ramalingam, 2000; Rahardjo et al., 2005).

SSF holds tremendous potential for the production of enzymes and it can be of special interest in those processes where the crude fermented product may be used directly as enzyme source. SSF has potential advantages over the submerged fermentation with respect to simplicity in operation, high productivity fermentation, less favorable for growth of contaminants and concentrated product formation (Ashokkumar et al., 2001). SSF is a process whereby an insoluble substrate is fermented with sufficient moisture but without free water. Free water does not appear to be the natural milieu for the majority of microorganisms. In this process, the solid substrate not only supplies the nutrients to the culture but also serves as an anchorage for the microbial cells (Selvakumar et al., 1998; Holler et al., 2004). Agro industrial residues are considered to be the best substrates for SSF processes including enzyme production (Ellaiah et al., 2002). Substrates traditionally used in SSF include rice bran, wheat bran, millet bran, barely bran, corn and soy bean (Krishna and Chandrasekaran, 1996). Cost and availability are important considerations and therefore the selection of an appropriate solid substrate plays an important role in the development of efficient SSF process (Selvakumar and Pandey, 1999).

The aim of the present study was to evaluate the feasibility of using easily available substrates like rice bran, wheat bran, sugarcane bagasse, black gram husk and green gram husk in SSF for the production of α–amylase with a salt pan isolate of *Bacillus cereus.* These agricultural residues are generally used as animal feed with no other useful application. In this present study, we report a number of factors that influence amylase production by *B. cereus* through SSF.

MATERIALS AND METHODS

Bacterium and enzyme activity

The bacterium used in this study was isolated from salt pan soil collected from Tuticorin Coast, Tamil Nadu, India. The salt pan soil was screened for amylase production on starch agar plates. Amylolytic isolates were selected by flooding the agar plates with iodine solution. It was identified as *B. cereus,* based on the standard key of Bergey's manual of determinative bacteriology (Shankar et al., 2011).

Amylase production on solid state fermentation (SSF)

The experiment was conducted in 250 ml Erlenmeyer flask which contain 5 g of substrate and 1 g of salt. The composition of the basal medium (g/l distilled water) for amylase production was peptone - 0.5 g, starch – 1 g, yeast extract - 0.3 g, $MgSO_4$ – 0.02 g, K_2HPO_4 – 0.01 g, at pH 7 for 48 h. Then 1 ml of enriched seed culture was inoculated into 250 ml flask containing 20 ml basal medium along with 5 g substrates. The culture was then incubated at 37°C. After incubation, 50 ml of distilled water was added and the flask was placed in a shaker at 150 rpm for 1 h. It was then filtered through a muslin cloth and cells were harvested by centrifugation at 10,000 rpm for 15 min. The supernatant was used for further assay.

Substrates

Commercial quality wheat bran, rice bran, bagasse, green gram husk and black gram husk were procured from the local market and used as solid substrates and their effect on the production of amylase was determined. The best solid substrate was selected and used in subsequent experiments (Shankar and Isaiarasu, 2011).

Effect of incubation time on amylase production

To find out the optimum time for amylase production, the amylase activity was determined for every 24 h of fermentation up to 72 h.

Effect of physical parameters on SSF

The physical parameters included for improving amylase production were temperature, pH and inoculum level. The effect of temperature on amylase production was studied by incubating the substrate inoculated with bacterial culture at various temperatures such as 10, 20, 30, 40, 50, 60, 70, and 80°C. Optimum pH for solid state amylase production was determined by using different pH in the production medium, for which the medium was individually prepared (before autoclaving) with pH 3, 4, 5, 6, 7, 8, 9 and 10 and inoculated with experimental bacterium. To find out the effect of inoculum level on amylase production by solid state fermentation, the initial inoculum level (1, 2, 3, 4, 5, 6 and 7%) were used.

Effect of supplementary nitrogen and carbon sources on amylase production

For the selection of suitable nitrogen source for amylase production by the identified bacterium, seven different nitrogen sources such as: casein, gelatin, urea, yeast extract, peptone, glycine and malt extract, were screened. They were tested individually at the concentration of 0.5% in the mineral medium (Kanmani et al., 2011a, b).

The selection of suitable carbon source for amylase production by this bacterium was performed by using seven different carbon sources like; glucose, galactose, maltose, sucrose, lactose, fructose and dextrose. They were tested individually at the concentration of 1% in the mineral medium.

Effect of surfactants on amylase production

The effect of surfactants on amylase production in solid state fermentation was performed by using four different surfactants such as: Tween 20, Tween 80, Sodium dodecyl sulphate (SDS) and Polyethylene glycol. The selected surfactants were incorporated individually into the medium at the concentration of 0.02%.

Effect of metal ions on solid state production of amylase

To study the efficiency of trace elements on solid state fermentation of amylase seven different metal ions were screened. They were: ammonium molybdate, calcium chloride, zinc sulphate, nickel sulphate, ferric chloride, potassium chromate and copper sulphate. These trace elements were incorporated into the medium individually at the concentration of 0.05% dry substrate.

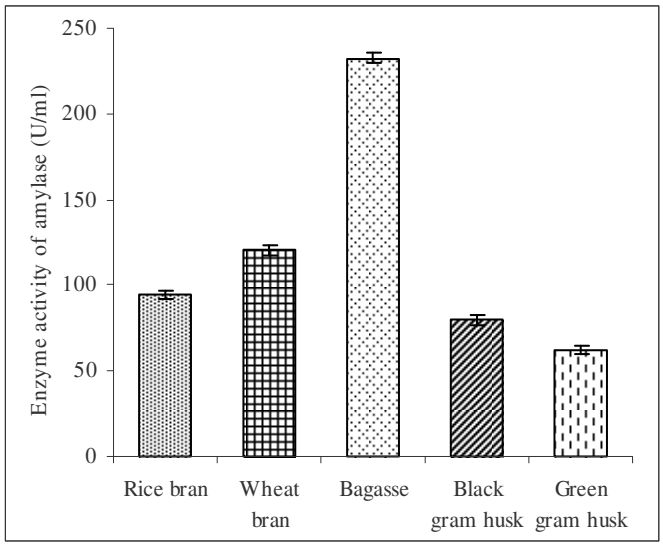

Figure 1. Effect of various substrates on *Bacillus cereus* amylase production by solid-state fermentation.

Effect of various concentration of sodium chloride on amylase production

As the bacterium is a salt pan isolate, the effect of various concentrations of sodium chloride was tested for its efficiency to produce amylase. For this the mineral medium was supplied individually with different concentration of sodium chloride and the concentrations tested were; 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0% (Mahendran et al., 2010; Sathees et al., 2011).

Effect of different starch concentration on amylase production

Different concentration of starch such as: 0.5, 1.0, 1.5, 2.0 and 2.5% were added to the basal medium with dry substrate. The efficiency was determined after 48 h of incubation.

Enzyme assay

The enzyme activity was assayed following the method of Berfeld, (1955) using 3.5-dinitrosalicylic acid. The amylase activity was measured by estimating the maltose produced during starch hydrolysis using modified dinitrosalicylic acid as a coupling reagent. The reaction mixture containing 1.0 ml of 1% soluble starch in 0.01 mol acetate buffer (pH 4.8) and 1.0 ml of enzyme solution was incubated at 40°C for 5 min. The reaction was stopped by adding 2.0 ml of 3.5-dinitrosalicylic acid solution followed by heating in a boiling water bath for 5 min. The contents were then cooled to room temperature and the volume was made up to 10 ml with distilled water. The absorbency of the reaction mixture was determined at 540 nm in a spectrophotometer. One unit of the enzyme was defined as the amount of enzyme capable of producing (1mol) of reducing sugar (as maltose) from 1% soluble starch as substrate in 1min at 40°C and pH 4.8.

Protein estimation

Total protein content was estimated by Lowry et al., (1951) method.

$$\text{Specific activity} = \frac{\text{Amylase activity}}{\text{Protein content}}$$

All the experiments were conducted in triplicate and mean of the three with standard deviation (SD) was represented as the number of Units of enzyme produced per ml of substrates.

RESULTS AND DISCUSSION

In SSF, the selection of a suitable substrate for fermentation process is critical and involves the screening of many agro industrial materials for microbial growth and product formation (Kunamneni et al., 2005). Many reports on SSF have been published in recent years supporting the application of SSF in upgrading agricultural by products and in the production of fine chemicals and enzymes (Smith et al., 1996; Gambert et al., 1999). Industrially important enzymes including; amylases have traditionally been obtained from submerged cultures because of ease of handling and greater control of environmental factors, such as temperature and pH. SSF constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly (Soni et al., 2003). There are several factors, which affect the SSF process. Among these, selection of a suitable strain, substrate and selection of process parameter are crucial (Pandey et al., 2000; Sodhi et al., 2005).

Effect of low cost substrates on amylase production

Here all the substrates support the growth and enzyme formation by the culture. Among them, sugar cane bagasse was found to be the best substrate for the production of amylase (232.65 ± 2.74 $U.ml^{-1}$).

The suitability of substrates in descending order were; sugar cane bagasse followed by wheat bran, rice bran, black gram husk and green gram husk respectively (Figure 1). It was previously reported that sugar cane bagasse was found to be the best substrate for amylase production by *Aspergillus niger* (Renato and Perez-Guerra, 2009). Kunamneni et al. (2005) reported that wheat bran was found to be the best substrate for amylase production by *Thermomyces lannuginosus.* In subsequent experiments therefore, sugarcane bagasse was used as the substrate for the production of amylase.

Effect of incubation period on amylase production

The effect of incubation time on amylase production showed that 48 h was the optimum duration for maximum amylase enzyme activity (233.23 ± 4.89 $U.ml^{-1}$). Amylase production by *B. cereus* was detected from 4 to 72 h and maximum activity reached at 48 h (85 $\mu g.ml^{-1}$) (Figure 2). Beyond this period the amylase enzyme

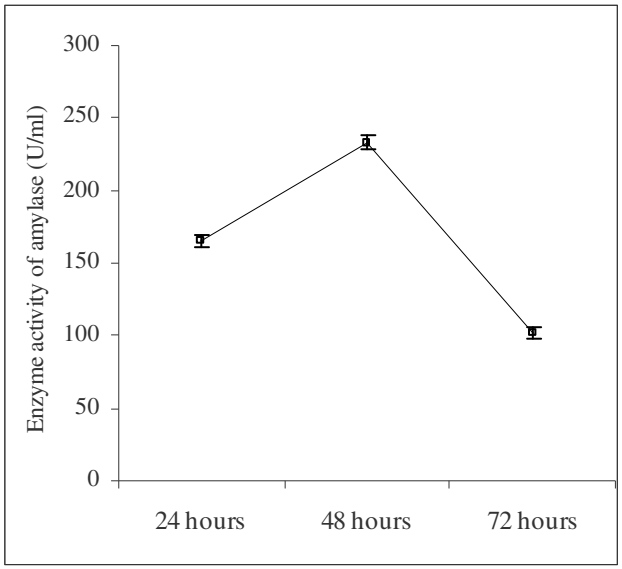

Figure 2. Effect of incubation period on *Bacillus cereus* amylase production by solid-state fermentation.

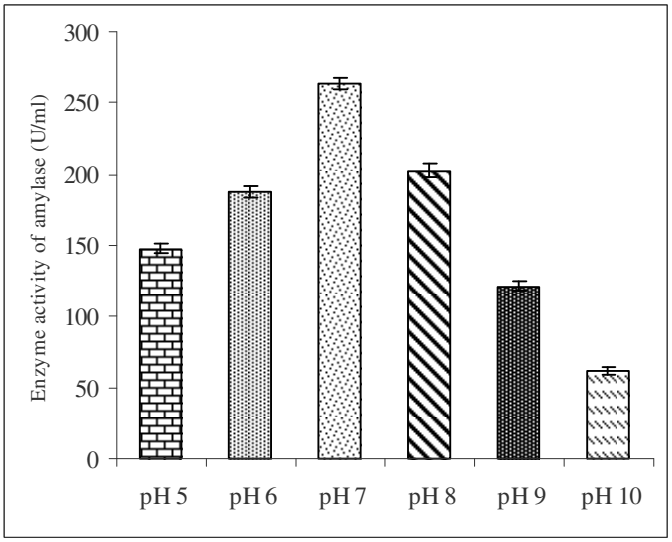

Figure 3. Effect of pH on *Bacillus cereus* amylase production by solid-state fermentation.

activity started to decrease. This is because the cells would have reached decline phase with lowered enzyme synthesis (Prabakaran and Hewitt, 2009).

Effect of pH on amylase production

The effect of initial pH on SSF of amylase showed that the pH range of 5 to 7 produced more amount of amylase and it was relatively high in pH 7.0 (264.42 ± 4.10 U.ml^{-1}) (Figure 3). Above this level, the amylase production

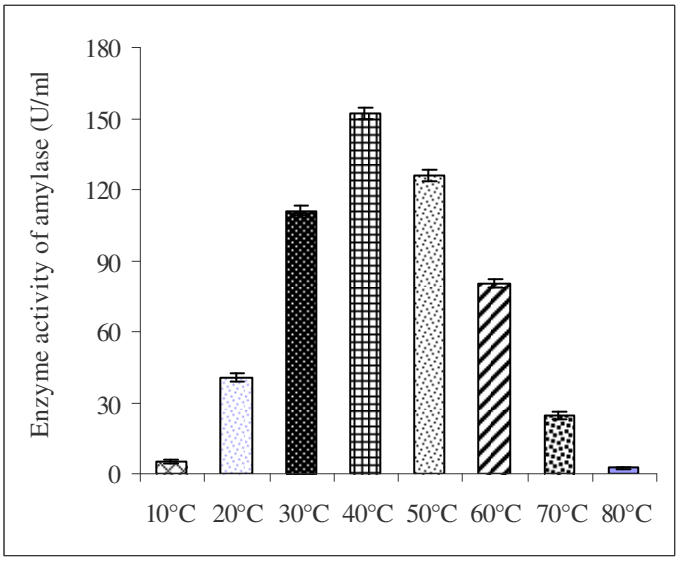

Figure 4. Effect of various temperatures on *Bacillus cereus* amylase production by solid-state fermentation.

decreased because the metabolic activities of microbes are very sensitive to rising pH. Ellaiah et al. (2002) showed that at higher pH, the metabolic activity of the bacterium would be suppressed thus inhibiting enzyme production. Renato et al. (2009) reported that amylase production by *A. niger* increased with a rise in pH to 6.0. Similarly an optimum pH of 7 for amylase production was observed with *B. cereus*.

Effect of temperature on amylase production

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the bioprocess are pH, temperature, aeration and agitation. In the present study, the effect of temperature on amylase activity by SSF revealed that 40˚C was optimum (152.13 ± 2.62 U.ml^{-1}) (Figure 4) among the tested temperatures. The enzyme production decreased beyond the temperature may be due to growth reduction and enzyme inactivation or suppression of cell viability. A similar result was reported by Francis et al. (2003). In contrast, low temperature values may reduce the metabolism of the microorganism (Mazutti et al., 2007) and consequently, the enzyme synthesis. Renato et al. (2009) reported that amylase production by *A. niger* under SSF with sugar cane bagasse is optimum at 30˚C. Previously 45˚C was reported as optimum temperature for amylase production by *Myceliophora thermophila* (Sadhukhan et al., 1990).

Effect of nitrogen sources on amylase production

Nitrogen compounds are secondary energy sources for

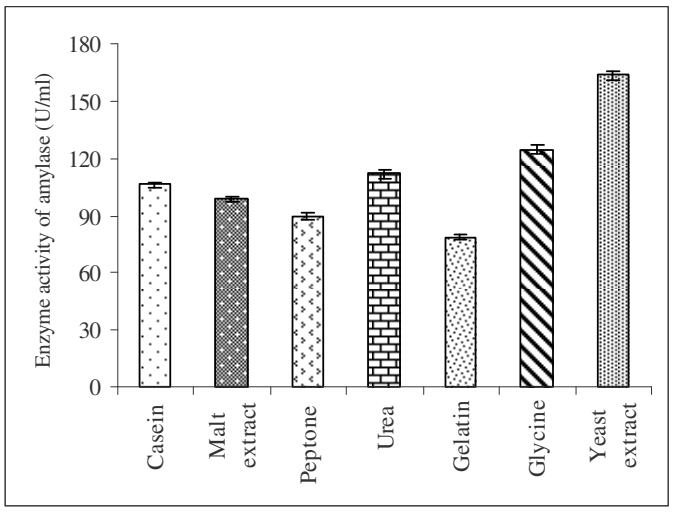

Figure 5. Evaluation of different nitrogen sources on *B. cereus* amylase production by solid-state fermentation.

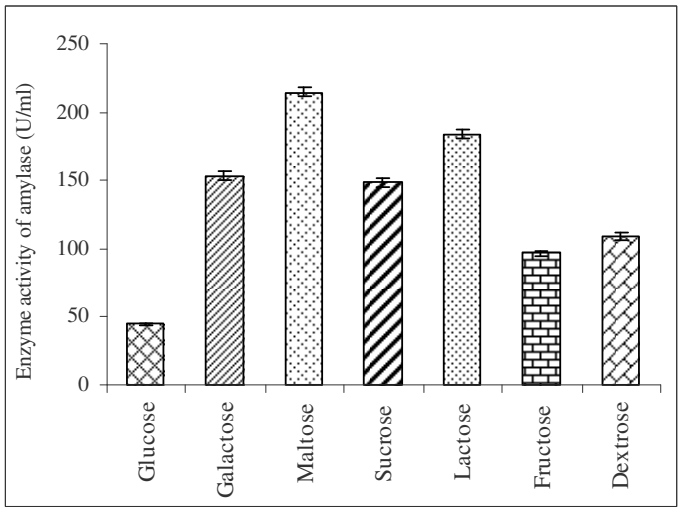

Figure. 6. Evaluation of different carbon sources on *B. cereus* amylase production by solid-state fermentation.

organisms and play an important role in growth and production of secondary metabolites. The nature of the compound and the concentration used may stimulate or down regulate the production of enzymes. In the present study experiment on the effect of supplementary of nitrogen sources on amylase production under SSF, showed that yeast extract was found to be a better nitrogen source for this isolate (163.64 ± 2.57 $U.ml^{-1}$) (Figure 5). Yeast extract was the best nitrogen source for amylase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components (Guerra and Pastrana, 2002). Renato and Perez-Guerra (2009) reported that, amylase production by Aspergillus oryzae under SSF of sugar cane bagasse was greatly influenced by organic nitrogen sources especially yeast extract. The amylase production by A. oryzae was also reported as high in yeast extract and casein (Pederson and Neilson, 2000). Ramachandran et al. (2004a) reported that peptone gave an increase in enzyme yield in SSF using coconut oil cake as substrate. Yeast extract and peptone is favored for growth and synthesis of amylase by Bacillus cereus (Teodoro and Martins, 2000).

Effect of carbon sources on amylase production

The addition of carbon source in the form of either monosaccharide or polysaccharides may influence the production of amylase enzyme. In our present study, the influence of maltose was more (214.36 ± 3.30 $U.ml^{-1}$) than the other carbon sources tested. Lactose was the second best supplementary carbon source (183.56 ± 3.37 $U.ml^{-1}$) (Figure 6). Glucose gave the lowest amylase enzyme activity (44.71 ± 1.11 $U.ml^{-1}$). Many researchers have shown that different carbon sources have varied influence on the production of extracellular enzymes especially among amylase producing strains. Ertan et al. (2006) reported high amylase production by *Penicillium chrysogenum* under solid state fermentation in wheat bran supplied with galactose. These results are similar to the findings of Heseltine et al. (1996) who observed that glucose represses the production of amylase in the hyperthermophilic archaeon *Sulfolobus solfataricus*. According to them glucose prevented amylase gene expression and not merely secretion of preformed enzyme.

Effect of surfactants on amylase production

Surfactants in the fermentation medium are known to increase secretion of proteins by increasing the cell membrane permeability. In the present study, the addition of Tween–80 increases the amylase production for *B. cereus* (Figure 7). A similar result was reported by Arnesen et al. (1998) the addition of Tween-80 to the fermentated medium increased α–amylase production by 2 fold in *T. lanuginosus* (Rao and Sathyanarayana, 2003). Surfactants such as SDS, cholic acid, Tween, etc, were reported to increase cell permeability, thereby enhancing enzyme yield.

Effect of metal ions on amylase production

Metal ions and trace elements are often required in the fermentation media for the optimum amylase production which depends on the source of enzyme. The present results showed that calcium chloride increases the amylase enzyme activity (184.11 ± 3.47 $U.ml^{-1}$) for

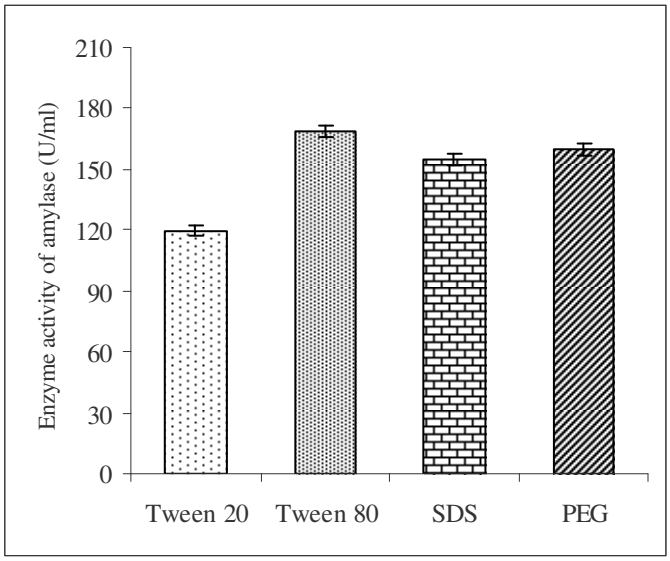

Figure 7. Evaluation of different surfactants on *B. cereus* amylase production by solid-state fermentation.

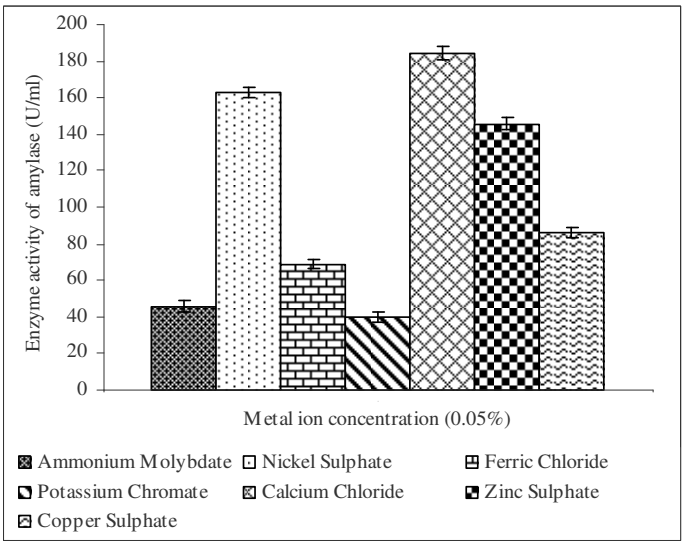

Figure 8. Evaluation of different metal ions on *B. cereus* amylase production by solid-state fermentation.

B. cereus (Figure 8). Francis et al. (2003) reported that calcium chloride stimulated enzyme activity when compared to other metal ions. Sodhi et al. (2005) reported higher amylolytic activity by *Bacillus* sp in medium supplemented with $LiSO_4$ and $MgSO_4$. Vishwanathan and Surlikar (2001) reported the positive influence of $CaCl_2$ and NaCl on α–amylase production in SSF using amaranthus grains as substrate, along with the negative influence of $FeCl_3$ and $MgSO_4$. Day et al. (2003) reported that $HgCl_2$, $CuSO_4$ and $FeCl_3$ caused total inhibition of enzyme activity by *Bacillus circulans.*

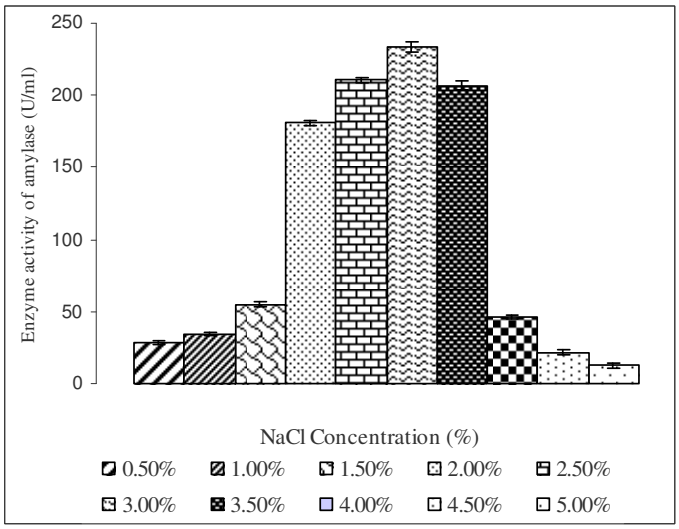

Figure 9. Evaluation of various NaCl concentrations on *B. cereus* amylase production by solid-state fermentation.

Effect of various concentration of sodium chloride on amylase production

Sodium chloride is an important nutrient factor for *Bacillus cereus* isolates from salt pan habitats with an absolute requirement of Na+ for growth and physiological activities. In our study 3% of NaCl was suitable concentration for the solid state fermentation of amylase (223.85 ± 3.40 $U.ml^{-1}$) (Figure 9). Above this concentration, the enzyme activity was decreased gradually. This study inferred that this bacterium is halophilic because it is a salt pan isolate, where the salinity influence was more due to its frequent fluctuation. The salinity induced amylase production of this study is a result of requirement of salt to maintain the osmotic balance by this bacterium. α-amylase from *Halomonas meridiana* was active in 5% NaCl, it was inactivated at temperatures above 37˚C (Coronado et al., 2000). Another amylase from *Micrococcus halobius* tolerated temperatures up to 55˚C but was inhibited by high salt concentrations, particularly in Tris-HCl buffers (Onishi and Sonoda, 1979). Khire and Pant (1992) described a thermostable salt-tolerant amylase from an unidentified species of *Bacillus.* α-amylase from the salt-tolerant archaeon *Halobacterium halobium* was progressively inactivated by increasing concentrations of NaCl (Good and Hartman, 1970).

Different concentration of starch on amylase production

Since carbon compounds provide an energy source for the growth of microorganisms and associated enzyme production it is likely that the presence of starch in the

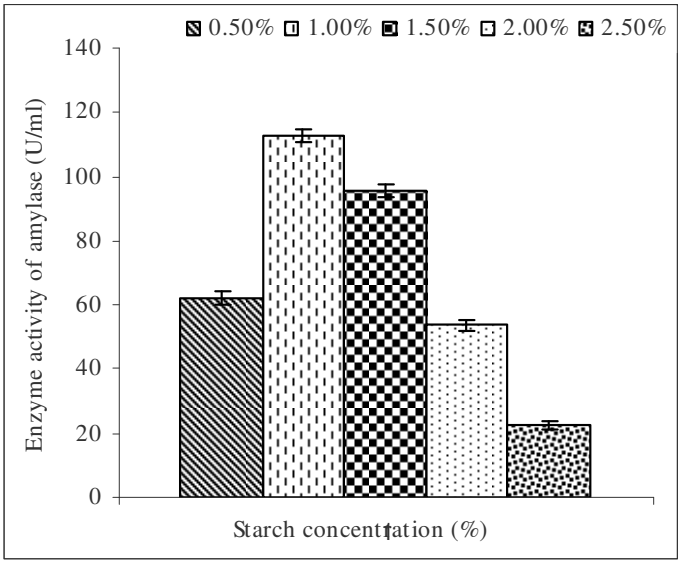

Figure 10. Evaluation of different starch concentration on *B. cereus* amylase production by solid-state fermentation.

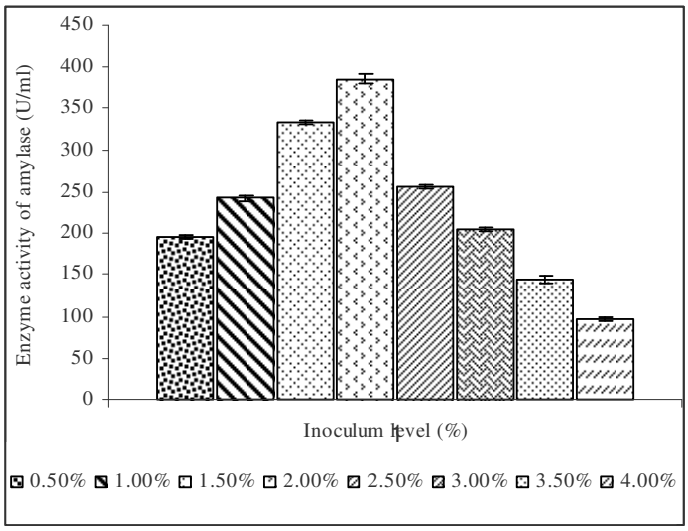

Figure 11. Effect of different concentration of inoculums on *Bacillus cereus* amylase production by solid-state fermentation.

medium stimulated increased production of amylase. In our study, 1% of starch was most suitable for enzyme production by *B. cereus* under SSF (Figure 10). Similar result was reported by Qader et al. (2006).

Santos and Martins (2003) reported that increasing starch concentration in the medium beyond 1% did not increase enzyme activity. At higher concentration, enzyme production was comparatively lower and the time required to reach the maximum enzyme level was longer. Agger et al. (2002) have reported that starch was the best inducer for α–amylase production in TAI strain of *Aspergillus nidulans* under SSF conditions. Srivastava and Baruah (1986) reported that soluble starch has been found as the best substrate for the production of α–amylase by *Bacillus stearothermophilus.*

Different concentration of inoculums on amylase production

Inoculum concentration is other important factor that influences the production of metabolites under SSF (Balkan and Ertan, 2007; Mazutti et al., 2007; Pandey et al., 2000). An inoculum concentration higher than the optimum value may produce a high amount of biomass which rapidly depletes the nutrients necessary for growth and product synthesis (Selvakumar and Pandey, 1999). On the other hand, lower inoculum level may give insufficient biomass and allow the growth of undesirable organisms in the production medium. This increases the necessary time to grow to an optimum number to consume the substrate and synthesize the desired product (Balkan and Ertan, 2007; Kashyap et al., 2002). In the present study, the highest enzyme activity (385.75 ± 4.70 $U.ml^{-1}$) was obtained at an inoculum level of 2% by *Bacillus cereus* under SSF (Figure 11). Kunamneni et al. (2005) reported on solid state fermentation of wheat bran by *A niger*, where in maximum amylase was reported at 2% inoculum level. Ramachandran et al. (2004a) reported that *Aspergillus oryzae* enzyme production was directly proportionate to the inoculum size with minimal activity at 0.5 ml and maximum activity at 2%.

Conclusion

It was concluded that from economic point of view *B. cereus* was optimized in various production parameters like pH, temperature, carbon sources, nitrogen sources, NaCl concentration, surfactant concentration, starch concentration, metal ions and low cast agricultural waste. So this *Bacillus cereus* can be used for amylase production in solid state fermentation using sugarcane bagasse.

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