International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.9, pp 111-117, 2015

Production of alpha-amylase by solid state fermentation using *Bacillus cereus* MTCC 7524 and *Bacillus licheniformis* MTCC 7445 *from* dairy sludge – A comparative study

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Abstract: Production of alpha- amylase under solid-state fermentation by *Bacillus cereus* MTCC 7524 and *Bacillus licheniformis* MTCC 7445 and the mixture of these two have been investigated using dairy sludge as substrate. in the presence of *Bacillus cereus*, highest enzyme production of 0.53±0.01 IU was observed. Production parameters were optimized as inoculum size 10%. Among different carbon sources, supplemented sucrose enhanced enzyme production of 3.94±0.03 IU. Among different nitrogen sources, supplemented ammonium chloride showed enhanced enzyme production of 0.65±0.1 IU. Optimum alpha-amylase enzyme activity was observed at 45ºC at pH 6.5.
Key words: Alpha-amylase, Bacillus cereus, Bacillus licheniformis, solid-state fermentation, dairy sludge, Optimization.

1. Introduction:

Enzyme production is one of the emerging fields in biotechnology. At industrial scale, most of the enzymes are manufactured by submerged fermentation (SmF). However, in the last decades, there has been an increasing trend towards the utilization of the solid state fermentation (SSF) technique to produce several enzymes from thermophilic microorganisms. Few important advantages of solid state fermentation over traditionally employed submerged fermentation are higher yields in a shorter time period because of better oxygen circulation, resemblance to natural habitats for filamentous fungi, less effect in downstream processing, and low energy consumption[1].

Enzymes are high molecular weight proteins and produced by microorganism, plants and animals. In this scenario eco-chemistry application of various enzymes such as amylases, glucoamylases, xylanase, pectinase, cellulases, etc. have been realized[2]. The enzyme based processes are ecofriendly as they can be carried out at moderate reaction conditions with greater specificity in contrast to chemical processes. In enzymatic reactions, the molecules at the beginning of the process, called substrates, are converted into different molecules, called products. Enzyme activity can be affected by other molecules such as inhibitors, activators etc. Inhibitors are molecules that decrease enzyme activity, activators are molecules that increase activity. Many drugs and poisons are enzyme inhibitors. Activity is also affected by temperature, chemical environment (e.g., pH) and the concentration of substrate. Enzymes are known to catalyze about 4,000 biochemical reactions[3].

Production of the α-amylases has been investigated through submerged fermentation (SmF) and solid state fermentation. The major factors that affect microbial production of α-amylase in a solid state fermentation system include selection of a suitable substrate and microorganism, particle size of the substrate, inoculum

concentration and moisture level of the substrate. Out of these, the selection of suitable solid substrate is a critical factor. The production of α-amylase by submerged fermentation using synthetic media has been reported by many workers. The contents of synthetic media are very expensive and uneconomical. Therefore, it needs to be replaced by the more economically available substrates to reduce the cost. In this regard, agro-industrial residues are generally considered as the best substrate for the production of amylases. Thus the use of solid state fermentation for α-amylase production has many advantages over submerged fermentation due to its simple techniques, low capital investment, lower levels of catabolite repression and better product recovery. The thermal stability is an important feature of most of the enzymes sold in bulk for industrial application[4]. So the selection of thermophilic microorganisms is of particular interest for the production of thermophilic α-amylases.

The purpose of the present study is to investigate the production of α-amylase under solid state fermentation process conditions. In this paper the influence of pH, temperature, initial moisture content, inoculation size and incubation time on α-amylase production by *Bacillus cereus, Bacillus licheniformis* and the mixed culture through SSF using dairy sludge has been investigated.

2. Materials and methods:

2.1. Microorganisms

Bacillus cereus MTCC ***7524*** and ***Bacillus licheniformis*** MTCC ***7445***, used in the present study was obtained from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on Nutrient Agar Medium at 20°C.

2.2. Substrate – Dairy Waste

Substrate used in the present study was obtained from Aavin Dairy Farm, Coimbatore, Tamilnadu, India. Solid organic waste in dairy processing facilities mainly originates from production processes and includes non- conforming products and product losses (e.g. milk spillages liquid whey and buttermilk), grid and filter residues, sludge from centrifugal separators and wastewater treatment, and packaging waste (e.g. discarded cuts, spent ripening bags, wax residues from cheese production) arising from incoming raw materials and production line damage[5].

2.3. Development of the inoculum

For the development of inoculum, culture was transferred from stock to 100 ml nutrient broth.100ml of nutrient broth is autoclaved at 121°C for 15min and cooled to room temperature before transferring the culture. Inoculated in 3 different conical flasks with the microorganisms *Bacillus cereus, Bacillus licheniformis,* and the mixture of *Bacillus cereus* and *Bacillus licheniformis* respectively. Then the inoculums was Incubated overnight at 35°C and at 150 rpm. it is stored for further use.

2.4. Confirmation of amylase production

α-Amylase production can be confirmed on starch agar plates Amylase production was detected after flooding the plates with iodine solution[6] Divide into 3 parts by drawing a line across the bottom of the tray / petriplate. Soak the cotton swab in respective samples (Bacillus cereus, Bacillus licheniformis and the mixture). Swab it over the respective portion of plate. Incubate for 30 mins at room temperature. Gently flood the plate with 2ml iodine solution and wait for 2 mins. Iodine reacts with starch medium and change the color of the medium. Discard the solution after 2mins and observe the zone formation.

2.5. Determination of dry weight of substrate

The substrate is not available in completely dried form. They generally contain moisture. Prior to utilize them in bioprocess, it is necessary to dry these solid substrates. Therefore, in the present study the amount of wet solid substrate was kept in the oven at 70°C for 24 h to remove the moisture from the solid substrate[7]. After drying, the mass of solid substrate was measured.

2.6. Solid state fermentation

5gm of substrate was taken into 250 ml conical flasks. To adjust percentage moisture levels (w/v), 10ml of Bushnell Haas mineral salt medium, pH 7 was added. The content of the flasks were mixed thoroughly and sterilized in the autoclave at 121°C temperature and 15psi for 15 min and then cooled at room temperature.

Each flask was incubated with one ml of inoculum and subsequently rotated in a rotary incubator shaker at (35±2) °C. Enzyme production was checked after every 24 hrs for 5 days[8].

2.7. Optimization of process parameters

Various process parameters affecting α-amylase production in solid state fermentation are optimized. The strategy was to optimize each parameter independently and subsequently optimum conditions were employed in each experimental run. The tested process parameters in this study were inoculum size (1, 1.5, 2, 2.5 and 3 ml), substrate concentration (5, 10, 15, 20 and 25 gms) incubation time (24, 48, 72, 96 and 120 hrs), temperature (4, 20, 30, 37, 45, 60 and 80°C), pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0), and supplementary carbon sources (soluble starch, sucrose, maltose and glucose and nitrogen sources (casein, ammonium chloride, yeast extract and urea)[9].

2.8. Enzyme extraction

At the end of solid state fermentation, the solid substrates were mixed thoroughly with 50 ml of 0.1M phosphate buffer (pH 7.0)[10]. The contents were mixed by shaking for 30 mins at 30°C on a rotary shaker at 250 rpm. The slurry was squeezed by muslin cloths. The extract was centrifuged at 8300 rpm and 4°C for 10 mins. The supernatant was used as a crude enzyme, i.e. α-amylase[11].

2.9. Enzyme activity

One unit of α-amylase activity is defined as the amount of enzyme that releases 1µmol of reducing sugar as glucose or maltose per minute, under assay conditions and expressed as U/g of dry substrate[12].

3. Results and discussion:

3.1. Effect of Incubation time

Table. 1. Effect of Incubation time on α-amylase production

Incubation time(hours)	*Bacillus cereus* (IU)	*Bacillus licheniformis*(IU)	Mixture (IU)
24	0.24±0.001	0.43±0.01	0.32±0.02
48	0.53±0.01	0.45±0.05	0.63±0.03
72	0.45±0.03	0.47±0.01	0.50±0.05
96	0.34±0.03	0.29±0.01	0.26±0.02
120	0.11±0.1	0.10±0.005	0.09±0.01

Table 1 shows the variation in amylase activity with incubation time at temperature 48°C in case of *Bacillus cereus* and 72 hrs in case of *Bacillus licheniformis*. The trend (Fig.1), indicates that the amylase production increases with the increase in incubation time and after 72 hrs the enzyme production decreases due to substrate inhibition. Thus, the maximum enzyme has been produced at 48 hrs of incubation time in *B.cereus* & mixed culture.

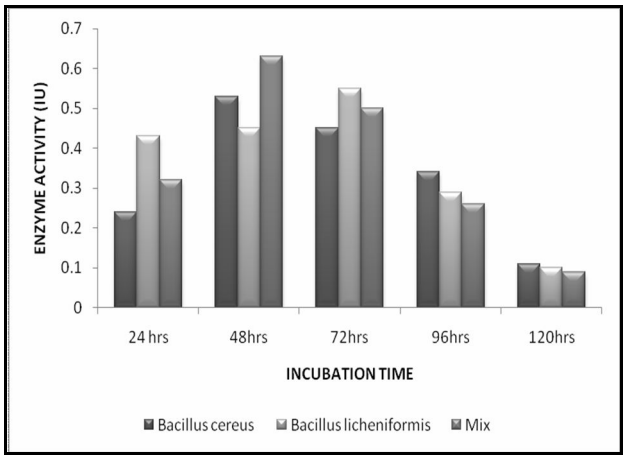

Fig. 1. Effect of Incubation time on α-amylase production

3.2. Effect of substrate concentration

The amount of substrate is an important factor for the production of α- amylase. The free excess liquid present in an unabsorbed form, therefore, gives rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate and leads to a decrease in growth and enzyme production. In the present study, Table 2, shows that the maximum amylase activity was found at 10g of substrate in case of *Bacillus cereus, Bacillus licheniformis* and mixture. After this no significant increase in enzyme activity has been found (Fig.2). This may be due to availability of limited inoculum for biosynthesis[13].

Table. 2. Effect of substrate concentration on α-amylase production

Substrate Concentration (g)	*Bacillus cereus*(IU)	*Bacillus licheniformis*(IU)	Mixture(IU)
5	0.24±0.01	0.25±0.01	0.32±0.02
10	0.79±0.01	0.43±0.01	0.76±0.01
15	0.36±0.02	0.24±0.01	0.31±0.02
20	0.17±0.01	0.18±0.01	0.15±0.02
25	0.08±0.01	0.12±0.01	0.04±0.02

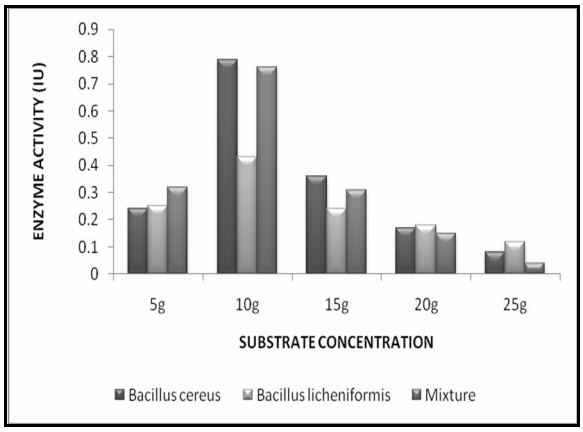

Fig. 2. Effect of substrate concentration on α-amylase production

3.3. Effect of Inoculum size:

The inoculum level is also an important factor for the production of α- amylase. Higher inoculum concentration increases the moisture content to a significant extent. Lower inoculum level results in a lower number of cells in the production medium. This requires a longer time to grow to an optimum number to utilize the substrate and to form the desired product. In the present study, Table 3 shows that the maximum amylase activity was found at 2ml in case of *Bacillus cereus* and mixture; 2.5 ml in case of *Bacillus licheniformis* and the results are tabulated in Table 3. After this inoculum concentration no significant increase in enzyme activity has been found (Fig.3). This may be due to the limiting nutrients at higher inoculum size.

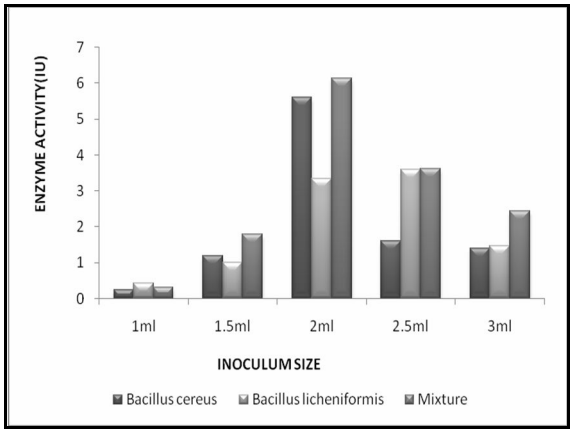

Fig. 3. Effect of inoculum size on α-amylase production

Table. 3. Effect of inoculum size on α-amylase production

Inoculum size(ml)	*Bacillus cereus*(IU)	*Bacillus licheniformis*(IU)	Mixture (IU)
1.0	0.24±0.001	0.43±0.01	0.32±0.02
1.5	1.2±0.02	1.0±0.01	1.8±0.01
2.0	5.6±0.1	3.34±0.06	6.13±0.05
2.5	1.6±0.13	3.6±0.05	3.62±0.02
3.0	1.4±0.03	1.47±0.02	2.45±0.20

3.4. Effect of additional nutrients:

The influence of four supplementary carbon sources has been studied. These carbon sources are soluble starch, sucrose, maltose and glucose. Among all supplementary carbon sources, sucrose has been found to be the best source for maximum amylase production and the results are tabulated in Table 4. The data corresponding to control in (Fig.4) indicates the production without additional carbon source[14].

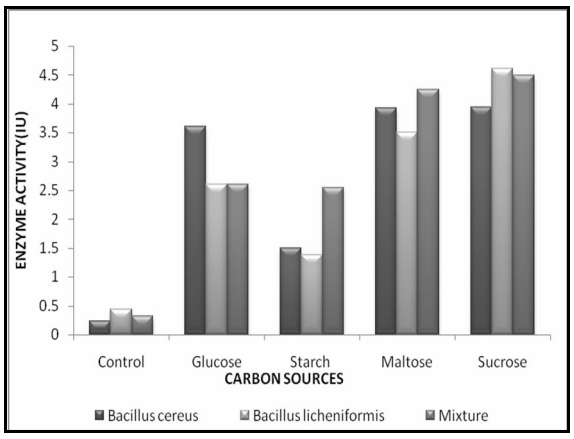

Fig. 4. Effect of supplementation of carbon sources on α-amylase production

Table. 4. Effect of supplementation of Carbon sources on α-amylase production

Carbon sources (g)	*Bacillus cereus*(IU)	*Bacillus licheniformis*(IU)	Mixture(IU)
Control	0.24±0.001	0.43±0.01	0.32±0.02
Glucose	3.6±0.02	2.6±0.01	2.6±0.01
Starch	1.5±0.1	1.38±0.06	2.54±0.05
Maltose	3.93±0.13	3.5±0.05	4.24±0.02
Sucrose	3.94±0.03	4.61±0.02	4.5±0.20

3.5. Effect of supplementation of Nitrogen sources on α-amylase activity

The influence of four supplementary nitrogen sources has been studied. These carbon sources are casein, ammonium chloride, yeast extract and urea. Among all supplementary nitrogen sources, ammonium chloride has been found to be the best source for maximum α-amylase production Table 5. The data corresponding to control in (Fig.5) indicates the production without additional nitrogen source.

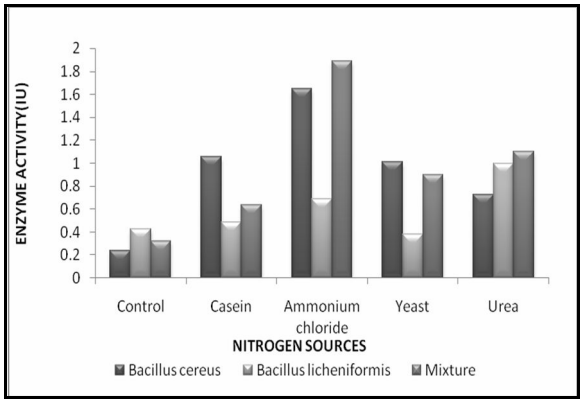

Fig. 5. Effect of supplementation of Nitrogen sources on α-amylase production

Table. 5. Effect of supplementation of Nitrogen sources on α-amylase production

Nitrogen sources(g)	*Bacillus cereus*(IU)	*Bacillus licheniformis*(IU)	Mixture(IU)
Control	0.24±0.001	0.43±0.01	0.32±0.02
Casein	1.06±0.02	0.49±0.01	0.635±0.01
Ammonium chloride	1.65±0.1	0.69±0.06	1.89±0.05
Yeast	1.01±0.13	0.38±0.05	0.90±0.02
Urea	0.73±0.03	1.0±0.02	1.1±0.20

3.6. Influence of incubation temperature on α-amylase activity

The growth of the microorganism is related to temperature which in turn influences the amylase production. (Fig.6) shows the variation in amylase activity at different temperatures varying from 4°C to 80°C. The maximum amylase production has been observed at 45°C. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial activity and thus the initial set temperature is vital[15].

3.7. Influence of pH of the medium on α-amylase activity

The pH of the growth medium is one of the physio-chemical parameters responsible for morphological changes in the organism and in enzyme secretion. In the present study the influence of pH on amylase activity has been studied by varying pH from 3.5 to 8.0. The variation in pH is carried out by adding acid or base buffer as per requirement. The trend in Fig.7 indicates that amylase activity first increases on increasing pH of medium, reaches maximum at pH of 6.5 and then decreases. The variation in amylase activity from 4 to 7 is small, indicating excellent buffering properties of dairy sludge used in SSF.

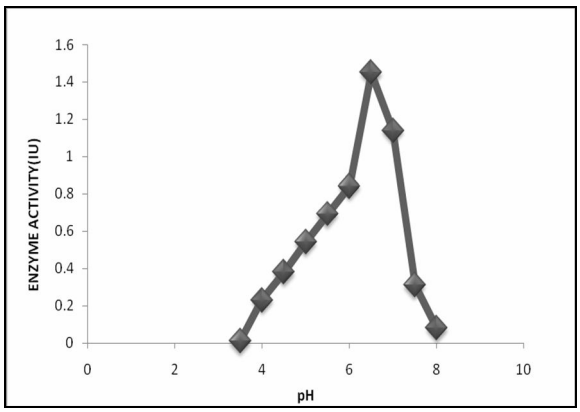

Fig. 7. Influence of pH of the medium on α-amylase production

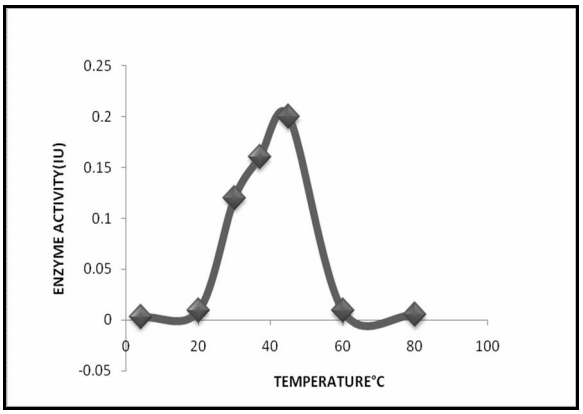

Fig. 6. Influence of temperature of the medium on α-amylase production

4. Conclusion:

It is concluded that the use of solid state fermentation for production of α-amylase using *Bacillus cereus* is an economical process and is very simple to apply. The solid substrate – dairy sludge can be used for supported biosynthesis of α-amylase using *B.cereus* under SSF. However, the other microorganism *Bacillus licheniformis* which is used in this present study did not cause enzyme productions as high as *B.cereus*. Therefore, Bacillus cereus is superior to other microbes for the synthesis of α-amylase using dairy sludge by solid state fermentation. The maximum productivity of α-amylase was achieved by utilizing dairy sludge as substrate as the solid substrate with sucrose as an additional carbon source and ammonium chloride as an additional nitrogen source at 48 hrs at 45°C and pH of 6.5, and inoculum level of 20 % (w/v). Although the results of these investigations are based on experiments conducted in flasks, they provide valuable information for the production of α-amylase by solid state fermentation process at a larger scale.

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