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Production and Optimization of Amylase from *Bacillus cereus* Using Submerged Fermentation

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A B S T R A C T

Amylase producing bacterial strain, *Bacillus cereus* MTCC 10205 isolated from soil sample was used for carrying out the present study on optimization of amylase activity. Cultural conditions and nutrients for the maximum production of amylase were optimized by altering one and keeping all other variables as constant. Out of the five media used, *Bacillus cereus* MTCC 10205 was found to produce maximum amylase (136.70 U) in starch agar medium. The medium contained starch at a concentration of 2% as a sole carbon source and the bacteria was grown for 48 h at 30ºC under submerged fermentation conditions. Optimization of cultural conditions indicated that amylase activity got increased to 294.63 (2.15 fold) when the bacterial strain was cultured at 35ºC for 36 h under static condition with inoculum size 3 per cent (7.5×108 CFU/ml) and using the corn starch as C-source in place of starch and casein hydrolysate as N-source in BOD incubator.

Introduction

Amylase is one of the most important enzymes in various industries, that catalyses the breakdown of starch into sugar. It can be derived from several sources such as plants, animals and microbes (Rao *et al.,* 2006). Among microbial, plant and animal enzymes, microbial amylases have immense applications in various fields in world market because of their wide application in starch based industries especially food, paper, textile, baking, detergent, pharmaceutical industries etc. (Anupama and Jayaraman, 2011). The major advantage of using microorganisms for the enzyme production is that the process is economically viable and microbes are easy to manipulate to obtain enzymes of desired characteristics (Aiyer, 2005). However, microbial sources are the most preferred one for large scale production meeting industrial demands. Amylases are of ubiquitous occurrence and holding maximum market share of enzyme sales

(Sivaramakrishanan *et al.,* 2006). Amylases were produced by number of microorganisms such as *Bacillus subtilis* (El-Banna *et al.,* 2007), *Bacillus* sp. VS04 (Vishnu *et al.,* 2014) and *Bacillus* sp. (Parmar and Pandya, 2012).

These microbial amylases are now available commercially and they have almost completely replaced acid hydrolysis of starch in starch processing industry (Gupta *et al.,* 2003) because of number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Satyanarayana *et al.,* 2005).

Amylases are useful in a broad range of industrial applications ranging from baking, brewing, fermentation, textile, paper and detergents industries (Singh *et al.,* 2009). Due to the industrial importance of amylases, there is an ongoing interest in the isolation of new bacterial strains producing enzymes suitable for industrial applications such as alkaline amylases for the decrement industry and starch saccharifications (Ben-Ali *et al.,* 1999).

The production of amylases has been found to be influenced by growth conditions and nutrients (Gupta *et al.,* 2003). The present study was mainly focused on the production of amylase from microbial source isolated from soil and optimizing various parameters to enhance the amylase production.

Materials and Methods

Samples collection

For isolation of microorganisms producing amylase, soil samples were collected from different places like the vegetable and grain market of Hisar and Rohtak, Haryana. The bacteria were isolated by serial dilution and streak plate methods. The isolates were maintained on starch agar medium.

Screening of isolates

The bacterial colonies were grown on starch agar medium (SAM) plates. After appearance of bacterial colonies, plates were washed with sterilized distilled water and 10 ml of lugols iodine (prepared by dissolving 1 g iodine and 2 g potassium iodide in 300 ml distilled water) was poured in each plate. After 15 min, the Petri plates were washed with sterilized water to wash off the excess of dye. Formation of the clear zones of hydrolysis around the colonies showed the production of amylolytic enzymes. The isolates showing clear zones of hydrolysis on starch agar medium were further purified by streak plate method and tested for their capability to produce amylase.

Identification of isolate

The selected isolate was grown at 30ºC for 2 days on starch agar media (SAM) slants. The slants were submitted to Institute of Microbial Technology (IMTECH, CSIR), Chandigarh for identification. It was identified as *Bacillus cereus* and was added to their collection centre MTCC with accession number MTCC 10205.

Optimization of fermentation conditions for amylase production

The fermentation conditions for amylase production were studied for different parameters. The experiments were carried out systematically in such a way that the parameters optimized in one experiments was maintained at its optimum level in the subsequent experiments. The different parameters that enhance the production of amylase were studied by taking one factor at a time. The parameters such as different media (Starch agar, Nutrient broth, Luria bertani and M9 minimal media); incubation period (6-48 h); incubation temperature (25-45ºC); inoculum size (1-5%); pH (5-7.5); carbon

sources-2% (fructose, amylose, amylopectin, sucrose, xylose, D-glucose, sorbital and corn starch) and nitrogen sources-1% (tryptone, casein hydrolysate, beef extract, ammonium chloride, ammonium sulfate, ammonium nitrate and ammonium persulfate) of the medium.

Extraction of enzyme

After 36 h of growth at 35ºC, the modified starch agar medium broth was filtered through 4 layers of muslin cloth and centrifuged at 10,000 rpm for 15 min at 4ºC in refrigerated centrifuged. The supernatant was used to assay the enzyme activity.

Enzyme assay

The most common way to follow the amylase activity is to determine the reducing sugars. Amylase in the sample hydrolyzes the substrate and the amount of released reducing sugar is determined spectrophotometrically using dinitrosalicylic (DNS) acid (Miller, 1959). A standard curve of maltose ranging from 0 to 1000 µg/ml was constructed and then determined the released maltose in the samples from standard curve. One unit of amylase activity is defined as the amount of enzyme that liberates 1nmole of maltose equivalent under the experimental conditions in 1 min.

Results and Discussion

In order to achieve maximum amylase production by *Bacillus cereus* MTCC 10205, a proper combination of various cultural conditions and nutrients was established. One single independent culture variable was altered while others were maintained at a constant level and level of extracellular amylase production was monitored. The results of present study are presented and discussed under different headings as under:

Effect of media on amylase production

The media optimization is an important aspect to be considered in the development of fermentation technology. The isolate *Bacillus cereus* MTCC 10205 was grown on different media (Fig.1) and it was found that enzyme activity was maximum (136.70 U) when grown in starch agar medium. Among other media, nutrient broth (NB) medium showed enzyme production (105.31 U), next to SAM while LB medium exhibited the minimum enzyme production (56.05 U).

Effect of incubation period

The effect of incubation period on amylase production was studied by growing the isolate in SAM over a period of 48h and at a temperature 30ºC in BOD incubator. The amylase activity was detected only after 6h of incubation.

The results presented in figure 2 clearly demonstrate that maximum amylase production occurs after 36h of incubation with the yield of 146.52 U. After 36h, decline in the activity was observed with a value of 141.43 U after 42h and 137.01 U after 48h of incubation.

Effect of incubation temperature

Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this is usually varied from one organism to another. In order to find out suitable and optimum temperature of incubation for the isolate MTCC 10205, the culture was grown at different temperature ranging from 25 to 45ºC under submerged conditions in SAM.

The results presented in figure 3 revealed that this isolate yielded maximum amylase at 35ºC (157.96 U).

Fig.1 Production of amylase by *Bacillus cereus* MTCC 10205 on different media

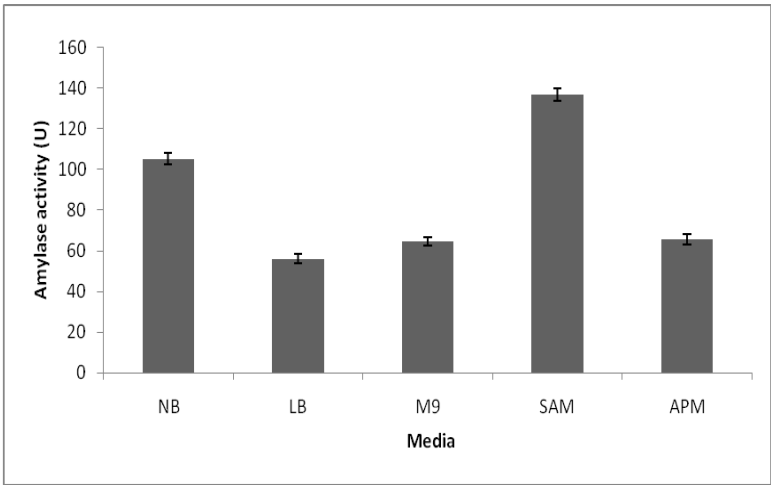


Fig.2 Effect of incubation time on amylase production by the isolate MTCC 10205

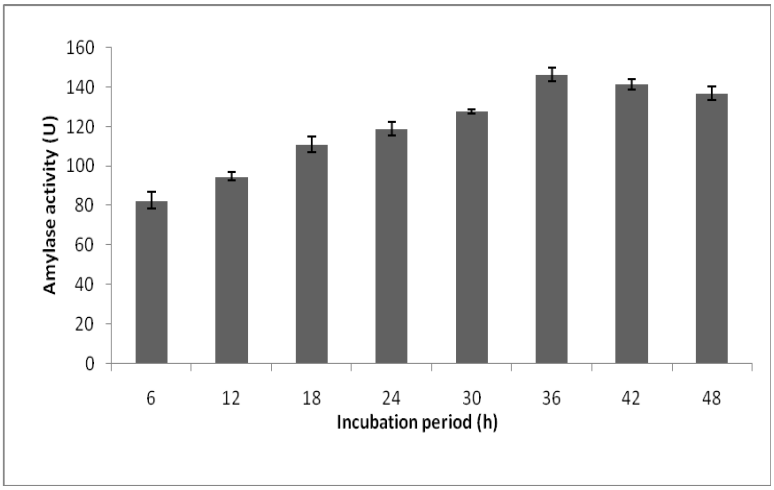


Fig.3 Effect of incubation temperature on amylase production by the isolate MTCC 10205

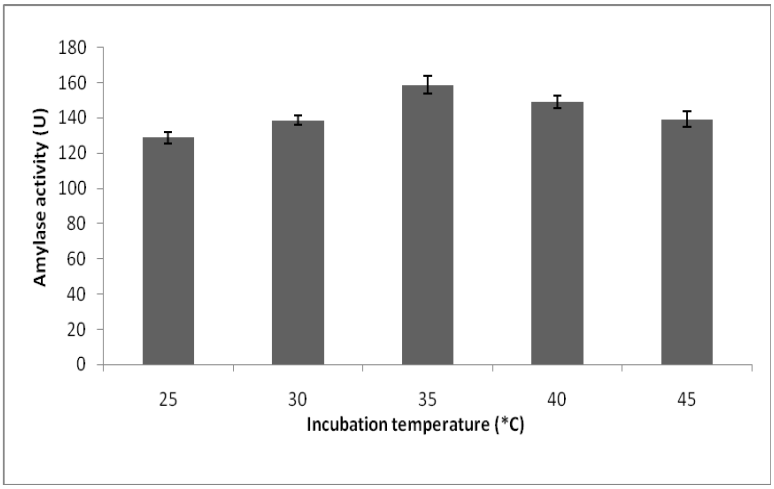


Fig.4 Effect of inoclum size on amylase production by the isolate MTCC 10205

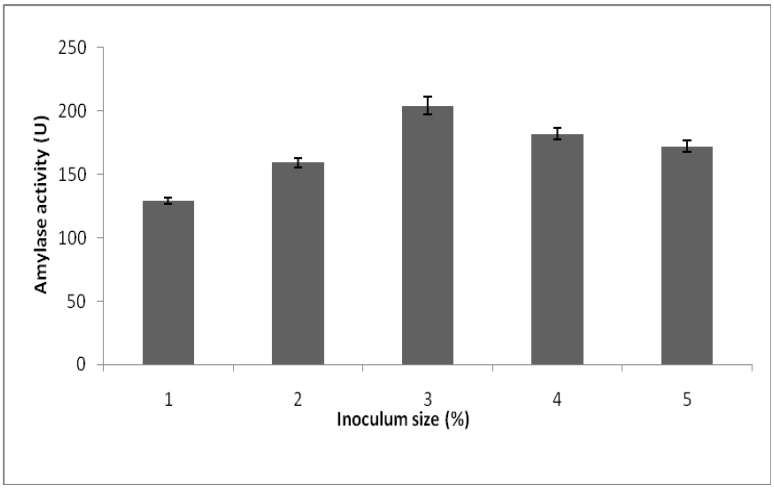


Fig.5 Effect of pH on amylase production by the isolate MTCC 10205

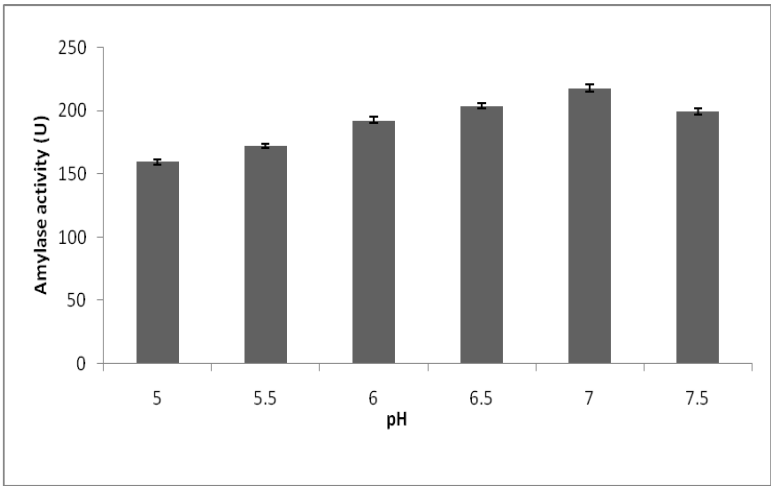


Fig.6 Effect of different carbon source on amylase production by the isolate MTCC 10205

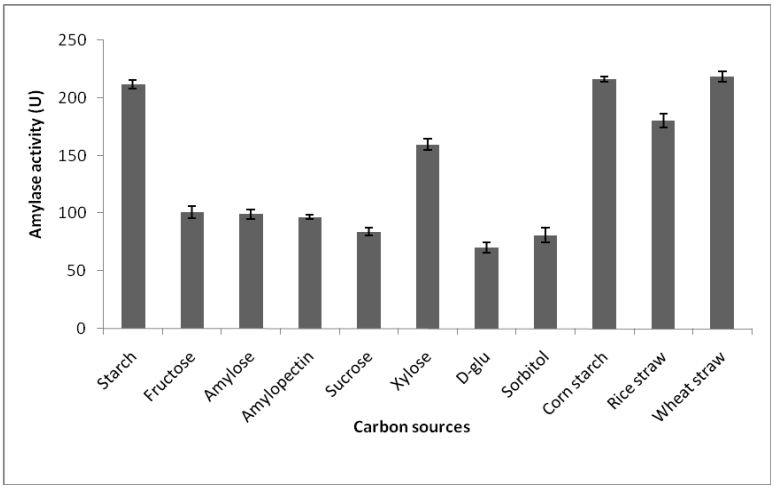
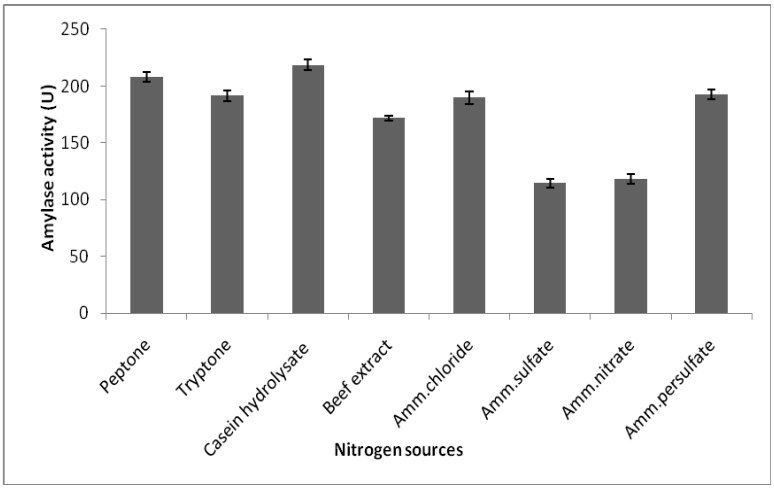


Fig.7 Effect of different nitrogen source on amylase production by the isolate MTCC 10205



Effect of inoculum size

The amylase production from the isolate under study was studied at different inoculum size from 1 % to 5%. As shown in figure 4. Maximum amylase production was recorded with 3 per cent inoculum (204.16 U). An inoculum concentration lower or higher than 3 per cent produced less amylase.

Effect of Ph

The influence of broth pH on amylase production by isolated bacterial strain was studied by growing the *Bacillus cereus* in SAM with pH varying from 5.0 to 7.5 in SmF. As it is clear from results presented in figure 5, the maximum amylase production was observed at pH 7.0 (203.71 U). On either side of the optimum pH (7.0) of the medium, the enzyme production decreased.

Effect of carbon source

To investigate the effect of various carbon sources on amylase production the present isolate was grown in different media containing starch, fructose, amylose, amylopectin, sucrose, xylose, D-glucose, sorbitol and corn starch as carbon source. Medium containing starch as carbon source was taken as a reference having 211.63 U of amylase activity.

Figure 6 shows that highest amylase production (216.28 U) was obtained in medium containing corn starch. It was also observed that xylose also favoured amylase production with yield of 159.66 U, whereas D-glucose inhibits the amylase production with yield of 70.43 U.

Effect of nitrogen source

The effect of various nitrogen sources on amylase production by the isolate, MTCC 10205 was studied by replacing peptone with alternative nitrogen sources. Among the different nitrogen sources, casein hydrolysate was found to be the best because it supported the maximum enzyme production (218.54 U) (Fig. 7). Beef extract, ammonium chloride, ammonium per sulfate and tryptone also supported the enzyme production with yield of 171.77 U, 189.67 U, 192.50 U and 191.36 U, respectively, but not as efficient as peptone (207.78 U). Ammonium sulphate and ammonium nitrate proved to be the poorest.

The isolate *Bacillus cereus* MTCC 10205 was

grown on different media and maximum enzyme activity was found to be 136.70 U when grown in starch agar medium as compare to nutrient broth and LB medium. Similarly, Anto *et al.,* (2006) reported the production of amylase from *Bacillus cereus* NY-14 and *Bacillus cereus* MTCC 1305 respectively. The effect of incubation period on amylase production was studied by growing the isolate in SAM medium. The maximum amylase production (146.52 U) was observed after 36h of incubation period. These results are in accordance with Panda *et al.,* (2008) who observed highest amylase production after 36h of incubation of *Streptomyces erumpens*. Similarly, Bole *et al.,* (2013) and Vishnu *et al.,* (2014) reported the highest amylase production after 48h and 72h of incubation of *Bacillus* sp. and *Bacillus* sp. VS04, respectively. Likewise, the maximum amylase production was achieved at 35ºC (157.96 U) temperature by isolate MTCC 10205. Similar observations were also recorded in *Bacillus* sp. AS-1 (Qader *et al.,* 2006) and *Bacillus* sp. (Bole *et al.,* 2013).

The higher inoculum concentration increases the moisture content to a significant extent. This leads to a decrease in growth and enzyme production Baysal *et al.,* (2003). Lower inoculum size 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. Maximum amylase production was recorded with 3 per cent inoculum. Similarly, Malhotra *et al.,* (2000) and Vishnu *et al.,* (2014) reported 2% (v/v) inoculum size to be optimum for amylase production by *B. thermooleovorans* NP5 and *Bacillus* sp. VS04, respectively.

The pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium (Sivaramakrishanan *et al.,* 2006). The maximum amylase production was observed at pH 7.0. Similar observations were recorded in *Bacillus* sp. at pH 7.0 (Parmar and Pandya 2012, Bole *et al.,* 2013) and *B. subtilis* KIBGE-HAR (Riaz *et al.,* 2009). The effect of various carbon and nitrogen sources on amylase production the present isolate was grown in different media. Medium containing starch as carbon source was taken as a reference having 211.63 U of amylase activity. Starch is known to induce amylase production in different bacterial strains (El-Banna *et al.,* 2007). Agricultural wastes are used for both liquid and solid fermentation to reduce cost of fermentation media. These wastes consist of carbon sources necessary for the growth and metabolisms of organisms. These sources include orange waste, pearl millet, potato, corn, tapioa, wheat and rice as flours Haq *et al.,* 2005). Among the different nitrogen sources, casein hydrolysate was found to be the best because it supported the maximum enzyme production. Similar observations were recorded by Anto *et al.,* (2006) in *B. cereus* MTCC 1305. In contrast, Qader *et al.,* (2006) observed that the amylase production was maximum when yeast extract was used as a nitrogen source in *Bacillus* sp. AS-1

Therefore, the use of submerged fermentation for production of amylase using *Bacillus cereus* MTCC 10205 is an economical process and is very simple to apply. The maximum amylase production (216.28 U) was obtained in starch agar medium containing corn starch as carbon source and casein hydrolysate was found to be the best nitrogen source with maximum enzyme production (218.54 U) in 36 h at temperature 35 0C, pH of 7.0, and inoculum level of 3 %.

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