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Production of a thermostable extracellular amylase from thermophilic *Bacillus* species

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Abstract:
Isolation and identification of thermophilic *Bacillus* sp was carried out from a soil sample. The cells were cultivated in a medium containing soluble starch as sole carbon source. The addition of calcium (10 mM) or peptone (1%) and yeast extract (0.5%) to the medium shortened the lag period and improved the growth and amylase synthesis. The optimum temperature for amylase production was detected as 35°C. Amylase production occurred at pH 5.0-9.0 with a maximum at pH 7.0. The optimal pH and temperature values for extracellular activity were 7.5 and 50°C respectively. Effects of different salts were noted and it was found that $CaCl_2$ with concentration of 0.2g/l played an important role for optimum production and stability of alpha amylase in the fermentation medium. Starch with a concentration of 20 g/l was a good source for the enzyme synthesis. The levels of the amylase production detected in culture supernatants varied greatly with the type of carbon source used. Lactose, soluble starch and glucose stimulated amylase production. Effect of different nitrogen sources revealed that peptone increase the enzyme yield. The concentration of yeast extract was an important factor for the synthesis of amylase by the isolate. The activity of the enzyme increased between 2 and 4 g/l yeast extract concentrations with an optimum of 4 g/l. The optimal concentration of peptone for the production of amylase was detected as 10g/l.
Key words: Amylase, *Bacillus* sp., thermostable enzyme.

Introduction:

Amylases [α-amylase, β-amylase and glucoamylase (GA)] are among the most important enzymes in present-day biotechnology. The enzymes of amylase family have great significance due to its wide area of potential application. The spectrum of amylase application has widened in many other fields, such as clinical, medical and analytical chemistry. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (1, 2). Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market (3, 4). Amylases have potential application in a number of industrial processes such as in the food, textiles, paper industries (5), bread making (6), glucose and fructose syrups, detergents, fuel ethanol from starches (7), fruit juices (8), alcoholic beverages (9), sweeteners (10), digestive aid and spot remover in dry cleaning (11). Thermostability is a feature of most of the enzymes sold for bulk industrial usage and thermophilic organisms are therefore of special interest as a source of novel thermostable enzymes. Recent research with thermostable a-amylases has concentrated on the enzymes of thermophiles and extreme thermophiles (12-18) and little is known about the properties of the enzymes produced by these organisms. The present study deals with the isolation and identification of a bacterium and describes the effects of culture conditions on the activity of amylase.

Materials and Methods:

Isolation & screening of the organism

The *Bacillus* sp. used in this study was isolated from soil. The primary screening of the strain KCPSS-12ss was done by starch agar plate method (19). Selection of thermophilic bacteria was done by growing them on a medium containing 2% Bacto-tryptone, 1% Bacto-yeast extract, 1% NaCl and 2% agar at pH 7.0. The screening of bacteria capable of producing starch digesting enzymes was done by growing them on a medium containing 1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar at pH 7.0. The plates were stained with Gram's iodine solution (2%I2

and 0.2%potassium iodide), and largest halo-forming zone was considered as the most promising strain and was chosen for further investigation.

Media composition

The growth medium used for amylase production was composed of (g/l): 20.0 Soluble Starch, 4.0 Yeast Extract, 10.0 Bacto Peptone, and 0.5 MgS4.7H O, 0.5 NaCl, and 0.2 CaCl2. The pH of the medium was adjusted to pH 7.0 with 1N NaOH and was autoclaved at 121°C for 15 minutes.

Production of Amylase

Five ml starch broth was inoculated with a loop- full of growing culture of Bacillus strain and was incubated at 35°C for 24 hrs. This 5 ml of 24 hrs old culture was then transferred into 45 ml of sterile starch broth medium and was incubated for 35°C for 24 hrs. After incubation the crude enzyme was obtained by centrifugation of the culture broth at 10,000 rpm for 10 min at 0°C and this Cell Free Filtrate (CFF) was stored at -20°C.

Enzyme assay

The amylase assay was based on the reduction in blue colour intensity resulting from enzyme hydrolysis of starch (20). The reaction contained 1 ml enzyme (cell free supernatant) and 10 ml of 1% starch solution incubated at 50°C for 10 min. The reaction was stopped by adding 10 ml of 0.1N HCl. One millilitre of this acidified solution was added to 10 ml of 0.1N HCl. From this, 1 ml was added to 10 ml iodine solution (0.05% iodine in 0.5% KI). The optical density of the blue-coloured solution was determined at 660 nm. The same procedure was repeated using 1 ml-distilled water instead of the enzyme sample in order to measure the optical density without the enzyme. One unit of amylase activity is defined as the quantity of enzyme that causes 1% reduction of blue colour intensity of starch-iodine solution a 50°C in 1 min.

Protein determination

The protein concentration of the CFF was determined by the Lowry method (21), with bovine serum albumin (BSA) as standard.

Results and Discussion:

Time for growth and production of amylase

At different time courses the production of amylase and cell mass are shown in Fig.1a & 1b. Maximum amylase production was obtained at 24 hrs of incubation. After 24 hrs cell mass increased but enzyme production declined and after 72 hrs there was no activity.

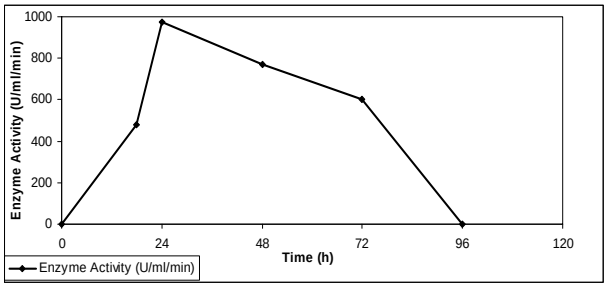

Figure 1a: Effect of incubation time on enzyme production

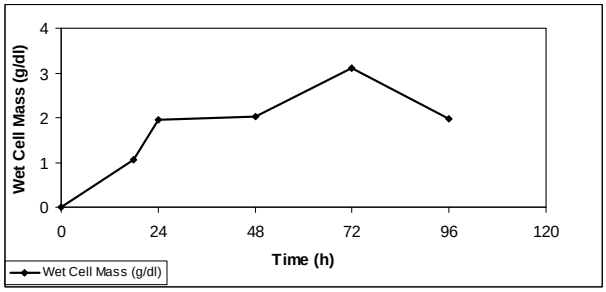

Figure 1b: Effect of incubation time on growth.

Effect of substrate concentration on amylase production

Carbon sources greatly influence amylase production and the most commonly used substrate is starch (20). In this research, the effect of different concentrations of soluble starch on amylase production was studied (Fig.2a & 2b).

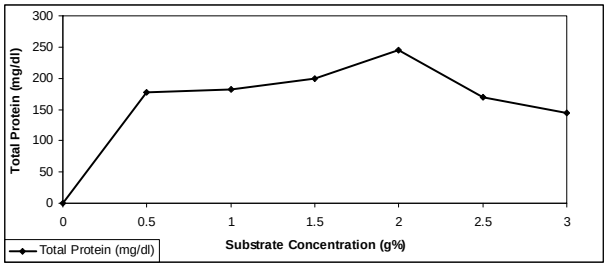

Figure 2a: Effect of substrate concentration on enzyme production

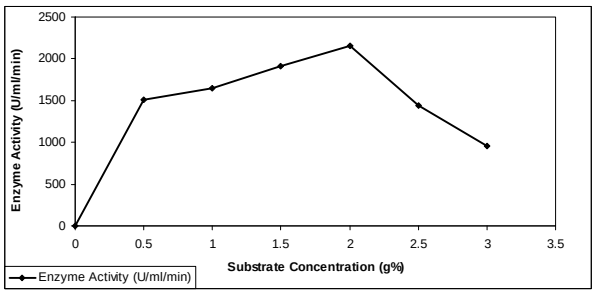

Figure 2b: Effect of substrate concentration on enzyme activity.

It was reported earlier that starch concentration beyond 1%in fermentation medium did not increase the enzyme production (22) but the strain used in this research showed that 2% starch in a fermentation medium can also increases enzyme production while 3% starch decreased the same.

Effect of temperature on amylase production

The effect of temperature on bacterial growth and amylase production from *Bacillus* strain KCPSS-12ss was studied. The production of enzyme and bacterial growth was studied at different temperatures ranging from 25°C to 45°C and optimum enzyme production was observed at 35°C (Fig. 3). After 35°C both growth and amylase production decreased, which indicated that the optimum temperature for maximum bacterial growth and amylase production were the same.

Effect of pH on amylase production

Enzyme synthesis and bacterial growth of *Bacillus* sp. KCPSS-12ss was observed between pH 4.0 to 11.0 (Fig.4). The results suggest that there is a spur in enzyme synthesis at pH 7.0 and the higher enzyme production at this pH was considered, probably, as a result of increased cell growth. The organism did not grow at pH 4.0, 10.0 and 11.0. In acidic medium results are insignificant. This may be due to the fact that bacteria required slightly alkaline pH for the production of amylase. Increasing the initial pH of the medium up to pH 9.0 resulted in a reduction in amylase production. The effect of pH on extracellular amylase activity was determined by using 50 mM phosphate buffer in a pH range of 6.0 to 8.0. The optimum pH was found to be 7.5 (Fig.5).

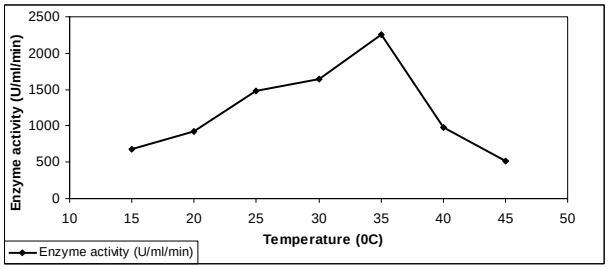

Figure 3: Effect of temperature on enzyme activity.

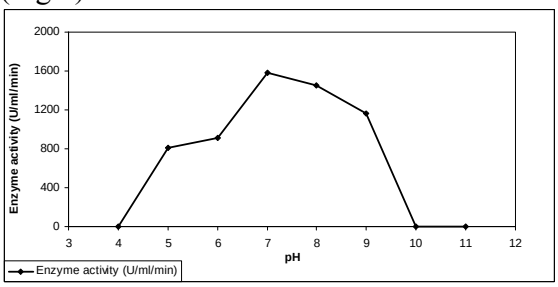

Figure 4: Effect of pH on amylase production.

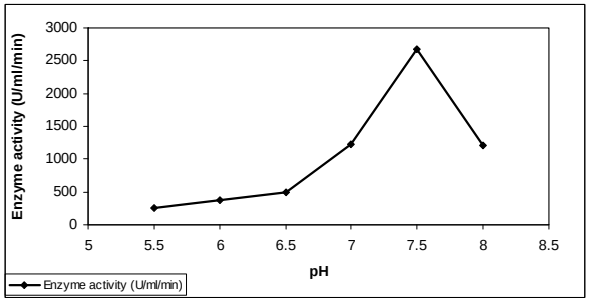

Figure 5: Effect of pH on enzyme activity.

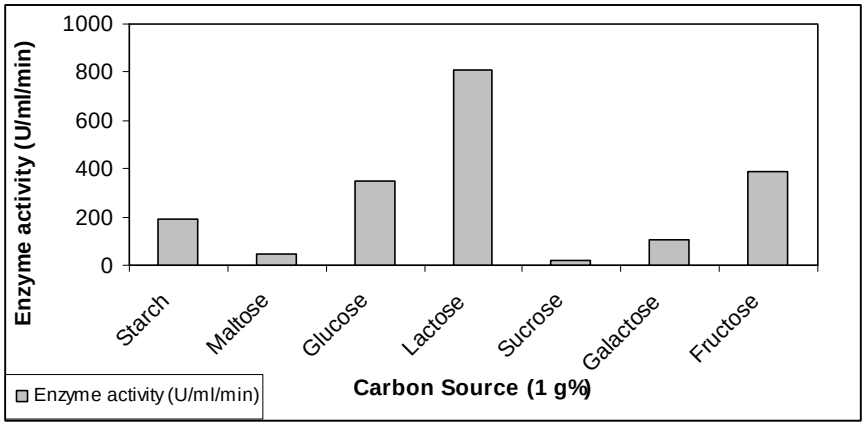

Figure 6. Effect of supplemented carbon source on enzyme production.

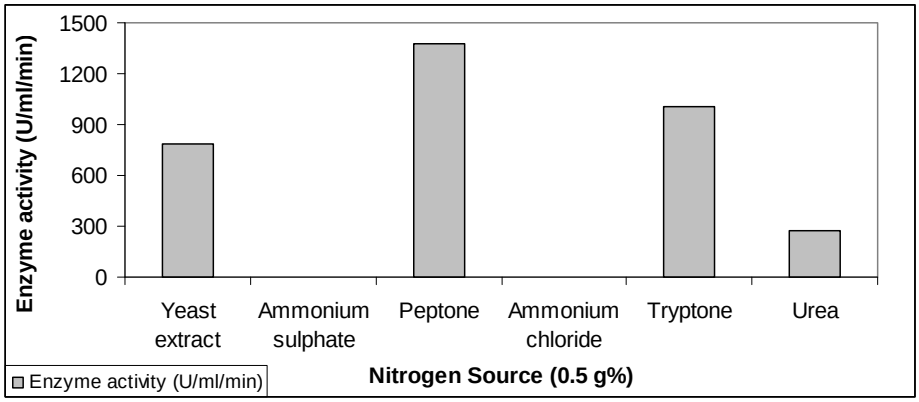

Figure 7. Effect of supplemented nitrogen source on enzyme production.

Effect of carbon source on amylase production

To investigate the effects of various carbon sources on amylase production, *Bacillus* sp. KCPSS-12ss strain was grown in different media containing starch, galactose, lactose, dextran, fructose, sucrose, glucose and maltose as carbon sources. Starch is a generally accepted nutritional component for induction of amylolytic enzymes. This material was considered as a reference. Fig.6 shows that highest amylase production was obtained in medium containing lactose. It was also observed that starch, fructose and glucose favored amylase production, whereas sucrose inhibited it. In case of *B. flavothermus* the highest amylase activity with maximum biomass was obtained when lactose was used as a carbon source; but presence of sucrose, fructose and glucose in the media gave rise only to good bacterial growth with little or no amylase production (23). It has been reported that the synthesis of carbohydrate degrading enzymes in most species of the genus Bacillus is subjected to catabolic repression by readily metabolisable substrates such as glucose and fructose (18).

Effect of nitrogen source on amylase production

The influence of organic and inorganic nitrogen sources on amylase production was determined (Fig.7). It has been reported that more amylase was produced when organic nitrogen compounds were used. Maximum enzyme production was found with peptone as the nitrogen source (18, 20). It has also been reported that the optimum production of amylase for *Bacillus* sp. was found when yeast extract was used (22). The results of

this present study suggested that optimum peptone concentration for amylase production was 1.0% (Fig.8). Yeast extract also seems to be suitable as well. Inorganic sources inhibit amylase synthesis.

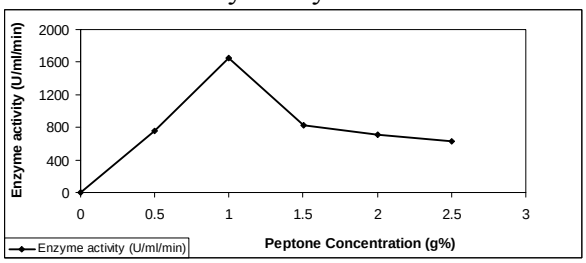

Figure 8: Effect of peptone concentration on enzyme production.

Effect of yeast concentration on amylase production

The concentration of yeast extract was found to be important factor in the amylase synthesis by several organisms (24) and thus the influence of this compound on amylase synthesis by *Bacillus* sp. was investigated and 4 g/l was found to be the optimum concentration (Fig.9). It has been reported that increasing the concentration of yeast extract to a level of 5.0 g/l lowered the pH significantly and this resulted in the complete repression of the enzyme (24). In this study it was observed that the pH of the broth increased from 6.0 to 6.8 at the end of the fermentation.

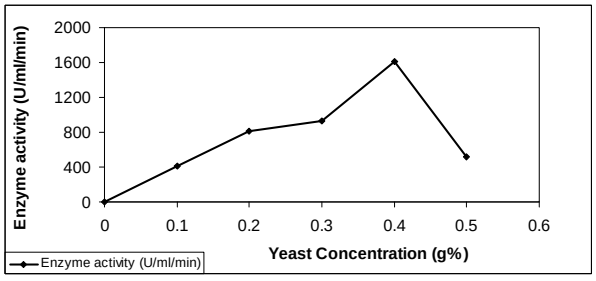

Figure 9: Effect of yeast concentration on enzyme production.

Effect of Ca^{2+} ions on amylase production

The production of amylase is Ca^{+2} dependent. In the case of *Bacillus Licheniformis* induction of calcium salt in the medium increased the amylase production (25). The stability of amylase is calcium dependent (26). In the present study different concentration of $CaCl_2$ were evaluated. Fig.10 shows that 0.02%was found to be optimum for the production of amylase. With the increase in calcium ions there was a slight reduction in enzyme production. When calcium ions were not added in the medium, the results were insignificant. This may be due to the fact that calcium ion was the best binder, stabilizer and activator of amylase. Therefore, efficiency of the enzyme was enhanced when calcium ion was present in the medium.

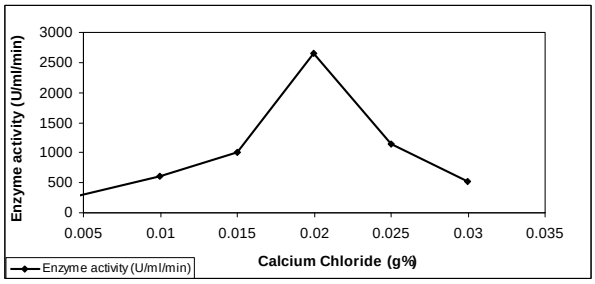

Figure 10: Effect of calcium chloride on enzyme production.

Conclusion:

The results obtained in this study show that there is appreciable high production, activity and stability of the enzyme at high temperatures. This suggests that KCPSS-12ss can be a potential producer of extracellular thermostable amylase which could find applications in industry and biotechnology. The enzyme thus produced is presently under characterization.

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