Influence of growth conditions on enhanced production of alpha amylase from Penicillium species in solid state fermentation

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The present study deals with the production of alpha amylase by Penicillium sp. employing solid state fermentation. Seven different media were evaluated. Out of all media tested wheat bran with distilled water gave maximum production of alpha amylase. Different parameters such as substrate concentration, moisture content, temperature, incubation time, size of inoculum and pH were optimized for the enzyme production. The maximum enzyme production was obtained by using 7.5 g of wheat bran, 100% of moisture content, incubation period 72 h, temperature of 30°C, inoculum size of 13.3% and pH 6. Addition of 1.5% starch as carbon source and 0.2% NH4NO3 and 2% yeast extract as inorganic and organic nitrogen sources respectively gave maximum production of alpha amylase. In addition to this effect of metal ions was also investigated CaCl2 act as stimulator for enzyme production by Penicillium sp.

Keywords: Alpha amylase, Penicillum, growth conditions, solid state fermentation

Introduction

Alpha amylase (E.C. 3.2.1.1) is an extracellular starch hydrolyzing enzyme which hydrolyzes starch into a number of different products such as glucose, maltose and malto-oligosaccharide. Alpha amylase has many applications in numerous industries such as food processing plants, paper manufacturing units, bread making and fruits pulp factories, detergents production units, textile sectors, etc.1-2. By the expansion of more and more dimensions in the sphere of biotechnology the applications of alpha amylase has been expanded in many other fields such as analytical chemistry, medical and clinical chemistry3. Alpha amylase can be produced from plants, animals, fungi and bacteria. However enzyme from fungal, and bacterial origin has prevailing applications in industry4. Filamentous fungi have been famous for the starch hydrolyzing enzymes that are secreted naturally by them. Because filamentous fungi sufficiently secrete proteins, this ability has made them important to the industrial production of enzymes. Fungi that were commonly used for the production of alpha amylase included Penicillium sp, Aspergillus niger, Themomyces lanuginosus, Trichoderma sp5-6. Alpha amylase can be produced by using different techniques such as submerged and solid state fermentations. Generally synthetic medium is used in submerged fermentation to produce alpha amylase. The synthetic media are too much expensive, so they are replaced with more inexpensive agricultural by-products to minimize the expenses. The solid state fermentation has many advantages for enzyme production over submerged fermentation because of it has simple procedure and technique, low capital investment and improved product recovery7. Another significant benefit of solid state fermentation is that filamentous fungi are highly well adapted for solid state fermentation. The hyphal mode of fungal growth and their excellent acceptance to low water activity and high osmotic pressure conditions make fungi capable and feasible in natural micro flora for utilizing the solid substrates8. Due to the constantly rising demand of this enzyme, researchers are trying to boost the productivity of amylases by a range of methods like efficient downstream processing, optimization of enzyme, use of low cost substrates, etc9.

Materials and Methods

Fungal Organism, Spore Inoculum Preperation

Penicillium sp. was obtained from the culture collection of Department of Biotechnology, Lahore College, for Women University, Lahore, Pakistan. The Penicillium spores were used for the preparation of inoculum. Ten ml of sterilized water was added in the 3-4 days old slant having enormous growth of
**Penicillium** sp. The sterilized inoculating needle was used for mixing the spores for the preparation of homogenous suspension.

**Solid State Fermentation**

Ten gram of solid substrate like wheat bran was moistened with 10 ml Mineral Salt Medium (MSM) in 250 ml Erlenmeyer flask and sterilized. After sterilization 1 ml of the spore suspension was added into each flask. The flasks were incubated at 30°C for 72 h. All the experiments were conducted in triplicates. After pre-determined period of incubation, the 100 ml of distilled water was added to each flask having fermented bran. The flasks were placed in an incubator shaker at 160 rpm for one hour. After one hour contents of flasks were filtered and filtrate was used for the estimation of enzyme.

**Fermentation Media**

Different fermentation media were screened for the production of alpha amylase by *Penicillium* sp. These included:

- **M1**: Wheat bran, 5 g, 8 ml mineral salt medium (MSM) containing mg/l: Zn SO₄·7H₂O, 6.2 mg; FeSO₄·7H₂O, 6.8 mg; CuSO₄·7H₂O, 0.8 mg; distilled water, 1000 ml.
- **M2**: Banana peel 20 g, 50 ml MSM containing g/l: (0.8 g NaCl, 0.8 g KCl, 0.1 g CaCl₂, 2.0 g Na₂HPO₄, 0.2 g MgSO₄, 0.1 g FeSO₄, 8.0 g glucose, 0.2 g NH₄Cl.
- **M3**: Rice bran 5 g, 10 ml MSM containing g/l: KH₂PO₄, 28; NH₄NO₃, 58; NaCl, 1.08; MgSO₄·7H₂O, 1.0g, distilled water, 1000 ml.
- **M4**: Rice husk 20 g, 50 ml MSM containing g/l: (0.8 g NaCl, 0.8 g KCl, 0.1 g CaCl₂, 2.0 g Na₂HPO₄, 0.2 g MgSO₄, 0.1 g FeSO₄, 8.0 g glucose, 0.2 g NH₄Cl.
- **M5**: Wheat bran 5 g, 5 ml distilled water.
- **M6**: Soyabean meal 10 g, 10 ml distilled water.
- **M7**: Mixed vegetable waste 25 g, distilled water 25 ml.

**Enzyme Assay**

Estimation of alpha amylase was carried out according to Haq *et al.*. One ml of enzyme was incubated with 1 ml of 1% Litner soluble starch solution for 30 min at 40°C. After incubation, 1.0 ml of dinitrosalicyclic acid (DNSA) reagent was added in each test tube. A blank containing 1.0 ml of distilled water in place of enzyme was also run parallel. The test tubes were placed in boiling water bath for 5 min. The test tubes were then cooled at room temperature and their volume was raised up to 10 ml by adding distilled water in each of them. The absorbance was taken at 546 nm by using a spectrophotometer, against maltose as standard. Reducing sugar released was measured by DNSA method of Miller. Activity of one unit of enzyme is defined as the quantity of enzyme which releases 1mg of reducing sugar from 1% Litner soluble starch corresponding to 1 mg maltose hydrate in 30 minutes.

**Protein Estimation**

The total protein content was estimated according to method of Bradford.

**Statistical Analysis**

Data was tabulated and statistically analyzed. Standard deviation and Duncan multiple comparison test was calculated using SPSS version 16.

**Results and Discussion**

**Screening of Fermentation Media**

Figure 1 depicted the screening of seven different fermentation media for the biosynthesis of alpha amylase by *Penicillium* sp. Out of all these media M3 consist of wheat bran along with distilled water gave the highest production of alpha amylase. The cause might be that the competency of wheat bran for the optimal production of enzyme depends upon its chemical characteristics because it constitutes a suitable fraction of carbohydrates, fiber, fats and proteins which are needed for the growth of microbes and alpha amylase biosynthesis. The fall in production of the enzyme in other media compared to wheat bran was either due to the deficiency of some substances in the media that were indispensable for growth of fungi and increased in enzyme production or may be because repressor compounds which were present in the component of media.

**Impact of Different Wheat Bran Concentration**

Figure 2 revealed the effect of different wheat bran concentration ranging from 2.5-12.5 g on production of alpha amylase by *Penicillium* sp. The wheat bran at the level of 7.5 g produced maximum enzyme (8 ± 0.1 U/ml/min). The production of alpha amylase was reduced.
decreased gradually with any change from optimal level. The reason might be that by increasing the concentration of substrate up to optimal level increased the activity of enzyme and afterward reached to a saturation point. At high level of substrate concentration, inadequate complexes were produced in between substrate and enzyme. In addition to this molecules of substrate were so closely associated around the molecules of enzyme, they might be attached to the regions on the enzyme, which might not be the active site or blocked the active site, hence, enzyme production become cease\textsuperscript{16}.

**Impact of Incubation Time on Alpha Amylase Production**

Different times of incubation (0–96 h) were screened for the biosynthesis of alpha amylase (Fig. 3). The optimum time of incubation was observed at 72 h. The basic cause might be that the growing fungus managed to reach at the ending point of the log phase and came into the stationary growth stage. This was witnessed by Prescott and Dunn\textsuperscript{17} who found that the yield of alpha amylase by fungal strain attained the optimum level in the stationary growth phase. Incubation period above 72 h consequently decreased the yield of alpha amylase. The reduction in alpha amylase production might be due to decrease in sugar contents and other nutrients. Furthermore, fungal generated inhibitors could also be another possible reason\textsuperscript{18}. Our findings are more encouraging in this regard the optimum incubation time was 72 h which is less than reported time period (120 h) of many works\textsuperscript{19,20}. So our findings are made the process more economical by reducing the energy requirements.

**Influence of Moisture Content**

Figure 4 indicates influence of varying moisture contents (50-120%) on the alpha amylase production by Penicillium sp. optimal enzyme production (8.5 ± 0.1 U/ml/min) was obtained at 100% moisture contents. Any increase or decrease in moisture contents from optimal level resulted reduction in alpha amylase production. The reason might be that the greater moisture content caused reduction in the production of enzyme because of difficulty of the growth of the producer strain. It caused particles to stick together thus interfering critically the oxygen diffusion in the substrate\textsuperscript{21}. Lesser moisture content caused a reduction in nutrients solubility of the substrate, low level of swelling and high water tension\textsuperscript{22}.

**Effect of Incubation Temperature**

Incubation temperature is also very critical for biosynthesis of enzyme. In current research influence of varying incubation temperature (25°C-50°C) on biosynthesis of alpha amylase was studied (Fig. 5).
The maximum production of enzyme (8.76 ± 0.02 U/ml/min) was achieved at 30°C. Reduction in enzyme production was found above or below this optimal state. Alpha amylase production was decreased with the increase in temperature the reason might be that it caused vaporization which consequently impact on cell growth. Reduction in moisture content, in turn lowered the rate of growth of the fungi leading to decreased enzyme production. Our findings were in accordance to Varalakshmi et al. who reported optimal temperature for alpha amylase biosynthesis was 30°C.

**Influence of Inoculum Size**

Size of inoculum is one of vital parameter among physical factors. In current study effect of various inoculums size (6.6-40%) was studied (Fig. 6). The 13.3% of inoculum size (1 ml = 2.6 × 10^6 CFU) gave optimal production of alpha amylase (9±0.1 U/ml/min) by *Penicillium* sp. The protein contents at 13.3% inoculum were found to be 0.31 ± 0.01 mg/ml. Increase in inoculum size results reduction in alpha amylase production. The reason might be that larger size of inoculum caused high levels of water content which eventually dropped the growth of fungi and also production of alpha amylase. Below 13.3% a decrease in enzyme production was also noted. Probably it was due to the reason that low level of inoculum caused in a lesser amount of spores in production medium because it took a longer period to grow in an optimum quantity to use substrate and made the desirable product.

**Influence of pH**

Figure 7 depicted the impact of different initial pH (4.0-7.0) on the production of alpha amylase by *Penicillium* sp. The maximum production of enzyme (12 ± 0.1 U/ml/min) was found at pH 6.0. The reason might be acidic pH support the fungal growth and in turn secretion of enzyme. Below or above the optimal level fall in the production of enzyme was recorded. This is due to the reason that enzymes act more efficiently over a narrow range of pH and is extremely sensitive to minute changes in pH.

**Evaluation of Additional Carbon Sources**

The effect of different carbon sources such as glucose, sucrose, xylose, lactose, fructose and starch on the production of alpha amylase by *Penicillium* sp. was evaluated. These additional carbon sources were added at 1% level and screened. Among all above mentioned carbon sources starch gave maximum alpha amylase production ((17 ± 0.1U/ml/min) along with 0.9 ± 0.05 mg/ml total protein (Fig 8). It might be ascribable to the reason that starch was gradually metabolized in the media with enhanced production of enzyme. Different concentrations of starch (0.5-3%) were also screened of which 1.5% found to be optimum for enzyme production (Fig. 9). Further increase and decrease in starch concentration resulted reduction in enzyme production. Probably it was due to the reason that surplus amount of carbon was evenly unfavorable and caused catabolic repression resulted reduction in enzyme production. Contrary to this at lower concentrations of starch decrease in

![Fig. 6 — Influence of inoculums size α–amylase production by Penicillium sp.](image)

![Fig. 7 — Influence of pH on α–amylase production by Penicillium sp.](image)

![Fig. 8 — Evaluation of additional carbon sources for α–amylase production by Penicillium sp.](image)
alpha amylase production was due to the lesser amount of carbon, which was insufficient for the growth and also for production of enzyme.\textsuperscript{30-28}

**Evaluation of Inorganic Nitrogen Sources**

Nitrogen sources play a key role in enzyme production as well as growth of fungi. Different inorganic nitrogen sources such as NH\(_4\)Cl, NH\(_4\)NO\(_3\), and (NH\(_4\))\(_2\)SO\(_4\) were screened for production of alpha amylase (Fig. 10). Ammonium nitrate (NH\(_4\)NO\(_3\)) at the concentration of 0.2\% (Fig. 11) showed highest enzyme production (24 ± 0.1/ml/min). Our findings were in accordance to Irfan \textit{et al.}\textsuperscript{31} who found that ammonium nitrate showed maximum enzyme production.

**Evaluation of Organic Nitrogen Sources**

The effect of different organic nitrogen sources such as peptone, tryptone, urea and yeast extract on the production of alpha amylase by 	extit{Penicillium} sp. was evaluated (Fig. 12). These additional organic nitrogen sources were added at 1\% and screened. Among all above organic nitrogen sources yeast extract gave maximum alpha amylase production (28±0.1 U/ml/min). Different concentrations of ammonium nitrate (0.5-3\%) were screened for further optimization (Fig. 13). Optimal level of alpha amylase production (33±0.1 U/ml/min) by 	extit{Penicillium} sp. was achieved at 2\% of yeast extract. Our finding coincides with the finding of Irfan \textit{et al.}\textsuperscript{31} who found yeast extract was good nitrogen source for optimal enzyme production.

**Evaluation of Different Metal Ions**

The effect of different metal ions such as MgSO\(_4\), CaCl\(_2\), FeSO\(_4\), ZnCl\(_2\) and CuSO\(_4\) on the production of

![Fig. 9 — Effect of different starch concentration on α–amylase production by 	extit{Penicillium} sp.](image)

![Fig. 10 — Evaluation of inorganic nitrogen source on α – amylase production by 	extit{Penicillium} sp.](image)

![Fig. 11 — Effect of different concentration of Ammonium nitrate on α–amylase production by 	extit{Penicillium} sp.](image)

![Fig. 12 — Effect of additional organic nitrogen source on α–amylase production by 	extit{Penicillium} sp.](image)

![Fig. 13 — Effect of different concentration of yeast extract on α–amylase production by 	extit{Penicillium} sp.](image)
alpha amylase by *Penicillium* sp. was evaluated (Fig. 14). These metal ions were added at the level of 0.1% and screened. Among all above metal ions CaCl₂ gave maximum alpha amylase production (36 ± 0.1 U/ml/min). The reason behind this might be that alpha amylase was metallic enzyme which in any case had one activating calcium ion. The affinity of calcium ion to alpha amylase was much powerful than that of other ions tested. Moreover, the ions other than calcium ions act as inhibitor and prevent the enzyme production. Our finding is also more successful because maximum production of alpha amylase (7.68 U/ml min) by *Penicillium* sp. is according to Ahmed et al.

References


Fig. 14 — Impact of different metal ions on α–amylase production by *Penicillium* sp.


