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Production and Optimization of α -Amylase from *Aspergillus flavus* under Solid State Fermentation

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ABSTRACT

*An important and well-known industrial enzyme that breaks down starch or glycogen is alpha amylase. It is cost-effective to use microorganisms to produce amylase because they may be easily engineered to produce enzymes with specific properties. The filamentous structure of fungi makes them an ideal alternative to bacteria in enzyme synthesis. The synthesis of enzymes might benefit greatly from agricultural waste byproducts. The amylase production by Aspergillus flavus was carried out by solid state fermentation using two naturally occurring carbon sources, namely rice bran and wheat bran. The enzyme activity that was obtained was lowest from rice bran ($428 \pm 24 \mu\text{mol}/\text{min}/\text{mL}$) and greatest from wheat bran ($670 \pm 36 \mu\text{mol}/\text{min}/\text{mL}$). At a temperature of 30°C , with an optimal inoculum size of 10% ($660 \pm 36 \mu\text{mol}/\text{min}/\text{mL}$), an incubation duration of 120 hours ($670 \pm 28 \mu\text{mol}/\text{min}/\text{mL}$), and a pH of 6 ($680 \pm 42 \mu\text{mol}/\text{min}/\text{mL}$), the maximum yield of enzyme synthesis was achieved. By adding 0.2 percent MgSO_4 and 0.022 percent CaCl_2 , the amylase production yield was maximized. Based on the findings of this investigation, *Aspergillus flavus* might be a good candidate for using in industrial α -amylase production.*

Parameter optimization, solid state fermentation, α -amylase, Aspergillus flavus, and keywords.

INTRODUCTION

Amylases have potential applications in the food, fermentation, textile, paper and pharmaceutical industries. Amylase can be derived from several sources, such as plants, animals and microorganisms. Amylases are the hydrolytic enzymes, widely spread in nature having varied application in different industrial processes and constitute a class of industrial enzymes and representing approximately 25-33% of the world market. α -amylases (endo-1,4 α -D-glucan glucohydrolase, E.C3.2.1.1) are extracellular enzymes that randomly cleave the 1, 4 α -D flucosidic linkages between adjacent glucose units inside the linear amylose chain. [1- 3] The enzymes from microbial sources generally meet industrial demands and made significant contribution to the production of foods and beverages. The microbial amylase has almost completely replaced chemical hydrolysis of starch in starch processing industry. [4] Selection of the right organism plays a key role in high yield of desirable hydrolytic enzymes especially amylases. Amylases from fungal sources

have gained much attention because of the cost effectiveness, consistency, less time, space, high productivity, amenable to genetic manipulation, ease of process modification, optimization and also suitable for industrial application. [5] Amylase of fungal origin was found to be more stable than the bacterial enzymes on a commercial level. Several attempts have been made to optimize culture conditions and suitable strains of fungi. [6-7] Fungal α -amylases are produced by different fermentation techniques. Production of α - amylases has been investigated through submerged (SmF) and Solid-state fermentation (SSF). [8] The use of SSF for enzyme production has numerous advantages over SmF, including simple technique, low capital investment, inexpensive substrate high volumetric productivity, relatively higher concentration of the products, less effluent generation, lower energy requirements and use of simpler downstream processing. [9-10] Recently, it has gained importance in the



production of microbial enzymes owing to several economic advantages over conventional submerged fermentation. The content of synthetic media is very expensive and uneconomical, so they need to be replaced with more economically available agricultural by-products to reduce the costs. Most agricultural wastes contain three major components; cellulose (30-50%), hemicellulose (20-35%) and 4-35% lignin. Starch based agro industrial residues are generally considered the best substrates for the SSF of α -amylases. [11-12]

The selection of suitable substrates for SSF has mainly been centered on agro industrial residues due to their potential advantages for filamentous fungi. Which are capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium. Optimization of various parameters and manipulation of media are one of the most important techniques used for the over production of enzymes in large quantities to meet industrial demands. [13] Production of α -amylase in fungi is known to depend on both morphological and metabolic state of the culture. Growth of mycelium is crucial for extracellular enzymes like α -amylase. [14] Various physical and chemical factors have been known to affect the production of α -amylase such as temperature, pH, period of incubation, carbon sources acting as inducers, surfactants, nitrogen sources, phosphate, different metal ions, moisture and agitation. Interactions of these parameters are reported to have a significant influence on the production of the enzyme. The present study is undertaken to enhance the production of α -amylase capacity by endophytic fungus *Aspergillus flavus* under SSF fermentation using agro industrial waste and optimization of the parameters, favoring the maximal production of α -amylase.

MATERIALS AND METHODS

Microorganism and culture maintenance

Fungal strain of *Aspergillus flavus* was obtained from Industrial Biotechnology Research Laboratory (IBRL) culture collection, USM Penang. The culture was maintained on potato-dextrose-Agar (PDA) slants. The slants were grown at 27°C for 5 days and stored at 4°C. Starch hydrolyzing activities were detected as clear zones after exposure to Iodine. [15]

Inoculum preparation

A volume of 10 mL of sterilized distilled water was added to a sporulated (5-day-old) PDA slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and then it was shaken thoroughly to prepare homogenized spore suspension. A volume of 1 mL suspension contained about 10⁶ spores.

Substrate

Wheat bran and Rice bran were procured from local market of Thanjavur City. These substrates were dried and ground into coarse powder with an electronic blender, and was used as a substrate for amylase production in solid state fermentation.

Solid state fermentation

Agricultural wastes such as wheat bran, and rice bran were used as substrate for α -amylase production. The substrates were ground into coarse powder with a blender. SSF was carried out using wheat and rice bran states individually. Starch content of individual substrates was checked by Anthrone method. [16] 5 g of substrates amended with 10mL of mineral salt solution containing (composed of (g/L); (NH₄)₂SO₄ 4MgSO₄ .7H₂O, FeSO₄ .7H₂O 0.02, K₂HPO₄ 1.4 and KH₂PO₄ 0.6, at pH 5.5), were taken into 250 mL Erlenmeyer flasks, mixed homogeneously and sterilized at 121°C for 15 min in an autoclave. Thereafter, the flask material was cooled a room temperature and inoculated with 1 mL of inoculum. The flasks were then incubated at 27°C for 48 hour.

Optimization of cultural and nutritional parameters

Various process parameters affecting enzyme production during SSF were optimized. The strategy was to optimize each parameter independently of the others and subsequently optimal conditions were employed in all experiments. Optimization experiment was carried out at different incubation period (2, 4, 6, 8 and 10 days), inoculum level (1, 3, 5, 10 and 15%) incubation temperature (30, 40, 50, 60, 70°C), pH (2, 4, 6, 8 and 10; adjusted with 0.1 N HCl and 0.1N NaOH) and moisture content (10, 20, 40, 60, 80, and 100%). Different metal salts MgSO₄ (0.2, 0.4, 0.6, 0.8 and 1%) and CaCl₂ (0.02, 0.06, 0.06, 0.08 and 0.1%) concentration were supplemented with the production media to determine their effect on amylase production under the optimized fermentation conditions.



Protein assay

Protein content was determined by the method of Lowry *et al.* (1951) [17] with crystalline Bovine Serum (BSA) as the standard.

Determination of dry mass of substrates

Dry mass of substrates was determined by drying them in an oven at 80°C for 24 hour.

Enzyme extraction

After the incubation period from submerged fermentation the fermented broth was centrifuged at 8 000 rpm for 15 min. Supernatant was filtered through Whatman No. 1 filter paper and filter was used the enzyme source. From the solid state fermentation, 50 mL of distilled water was added in each flask containing fermented mash and placed on a rotary shaker at 200 rpm for 60 min. The mixture was filtered through two fold cheese cloth and then filtering through Whatman No.1 filter paper. The filtrate was used as source of amylase. [18]

Enzyme assay

The amylase activity was assayed by measuring the reducing sugar liberated in the reaction mixture. The reaction mixture (3 mL) consisted of 0.5 mL of 1% (w/v) soluble starch and 0.5 mL appropriately diluted enzyme source in 2 mL of 0.1 M phosphate buffer (pH 6.5). After incubation at room temperature for 20 min the reaction was stopped by addition of dinitrosalicylic method (DNS). Then boiling tubes in a water bath for 15 min and thus the reducing sugars released by enzymatic hydrolysis of starch were determined. [19] One unit of amylase activity was defined as the amount of enzyme that releases one micromole of reducing sugar as glucose per minute under assay conditions and enzyme activity is expressed as the specific activity, which is represented as U/mL of protein. The experiments were carried out in triplicates.

Statistical analysis

The results were expressed as mean \pm standard deviation.

RESULTS AND DISCUSSION

Two indigenous carbon sources namely wheat bran and rice bran were used as substrate for α -amylase synthesis by *A. flavus* through SSF. α -amylase production by using different conditions, such as inoculum level, incubation period, pH, temperature,

different carbon source, nitrogen sources, moisture content, MgSO₄, and CaCl₂ concentration were analyzed by SSF method.

Effect of substrate

Different substrates i.e. wheat bran and rice bran were used in the fermentation for the production of α -amylase at 30°C using *A. flavus* for 5 days. Wheat bran gave the highest (670 \pm 36 μ mol./min/mL) and Rice bran (428 \pm 24 μ mol./min/mL) gave the lowest enzyme activity as shown in Fig. 1. Highest activity in Wheat bran may be due to its high carbohydrate contents and suitable texture.

Effect of inoculum level

Inoculum level is an important factor for the production of enzymes. Effect of inoculum size was checked by varying the concentration of *A. flavus* in SSF of wheat bran. Various inoculum levels (1, 3, 5, 10 and 15% (v/w)) were tried to study their effect on amylase production. Optimum inoculum size of 10% (660 \pm 36 μ mol./min/mL) of *A. flavus* gave highest yield of enzyme production as shown in (Fig. 2). Increasing the size of inoculum resulted in decreased enzyme production. Increased inoculum size resulted in increases moisture level, which ultimately decreased the fungal growth and enzyme production. [20] The free excess liquid present in an unabsorbed form creates an additional diffusion barrier together with that imposed by the solid nature of the substrate and lead to a decrease in growth and enzyme production. On the other hand, low inoculum level introduces 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 form the desired product. Highest yield of amylase production from *A. flavus* using inoculum size of 0.0637 \times 10⁶/mL-1. [21] Zambare (2010) obtained highest enzyme production (1672-1693 U/gdfs) at inoculum level of 5-8% v/w) in SSF using *A. oryzae*. [22] The reason behind such a result may be that a small quantity of inoculum might have postponed mycelia growth and reduced enzyme productivity while any higher inoculum volume resulted in inadequate heat transfer, although water-holding capacity of media might have been better.

Effect of incubation time

The incubation period varies with enzyme production. Short incubation period offers potential for inexpensive production of enzymes. [23] In the present study, amylase production was 190 \pm

28 $\mu\text{mol./min/mL}$ in 24 hr and gradually increased up to $670 \pm 22\mu\text{mol./min/mL}$ in 120 hour of incubation. After 120 hr there was gradual decrease in enzyme production (Fig. 3). Kathiresan *et al.* (2006), reported, that maximum activity was detected in 96 hour by *Penicillium fellutanum* under sub merged fermentation and as against a short duration of 24 h in the case of bacteria. [24]

Effect of pH

The enzyme an activity of the wheat bran containing media crude enzyme extract was assayed at various pH was studied. Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion (Fig. 4). The pH change observed during the growth of microbes also affects product stability in the medium. Most of the earlier studies revealed the optimum pH range between 6 and 7 for the growth of bacterial strains and enzyme production. [25-27] The maximum enzyme activity was obtained at pH 6 ($680 \pm 42\mu\text{mol./min/mL}$) and the minimum was obtained at pH 4 ($380\pm 24 \mu\text{mol./min/mL}$).

Effect of temperature

SSF is usually carried out in the temperature range of 25 to 40°C. Amylase production was optimum at 30°C ($660 \pm 46\mu\text{mol./min/mL}$) (Fig. 6). Temperature optimum for amylase was found to be in a range between 25 and 37°C for the mesophilic fungi. [27] Increases in temperature will lead to an increase in activity reaction kinetics, but also accelerate the denaturation induced by higher physiological temperatures (Fig. 5).

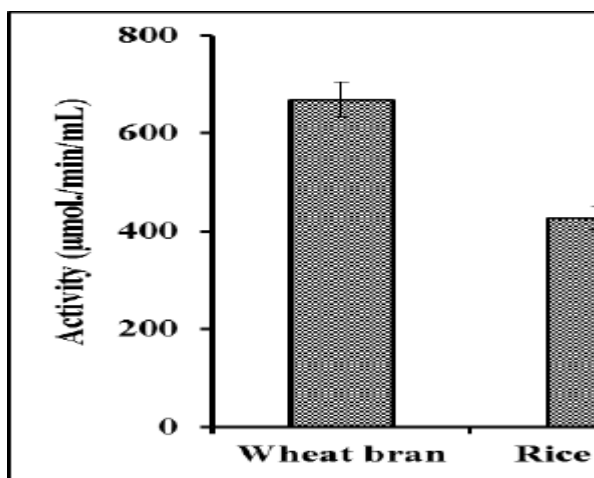


Fig. 1: Effect of substrate on α -amylase production

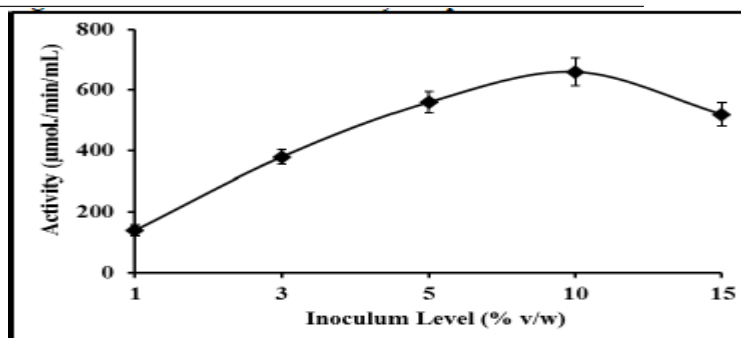


Fig. 2: Effect of different inoculum levels on α -amylase production

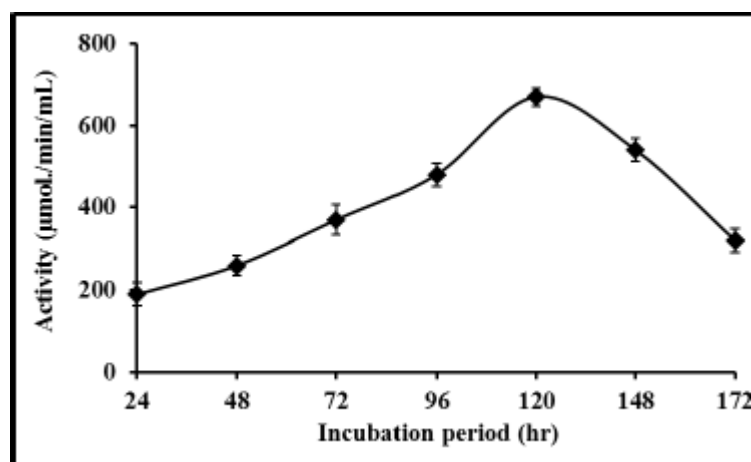


Fig. 3: Effect of different incubation period on α -amylase production

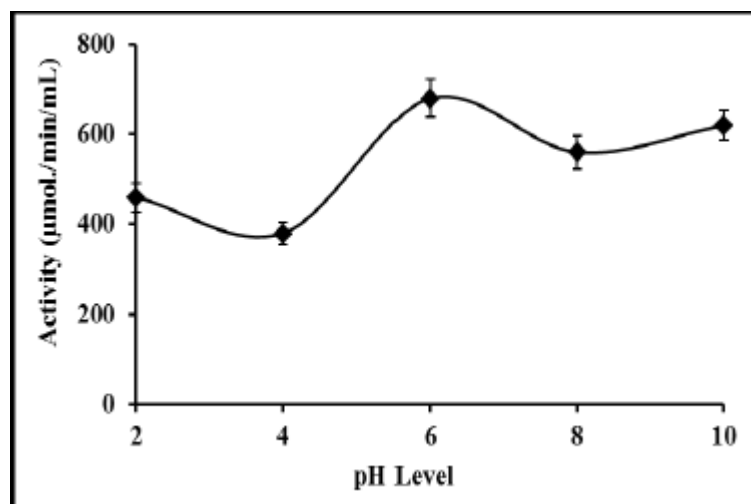


Fig. 4: Effect of different pH level on α -amylase production

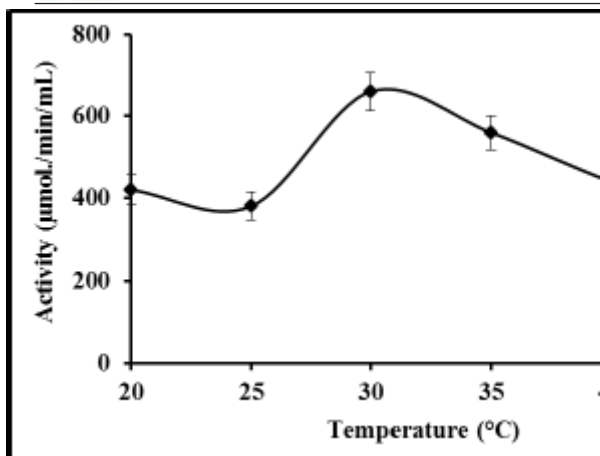


Fig. 5: Amylase activity for enzyme extract at various temperatures

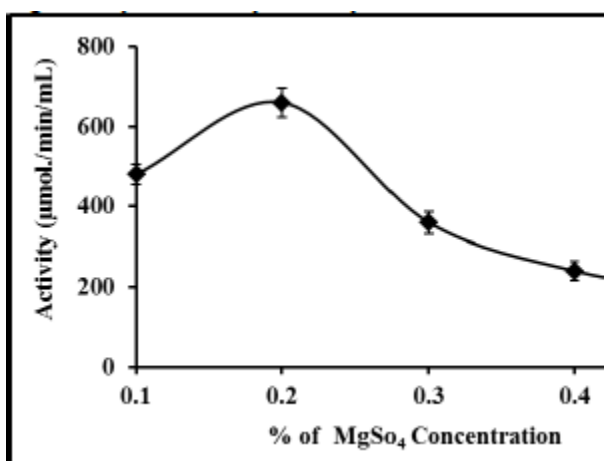


Fig. 6: Effect of different percentage of MgSO₄ on α-amylase production

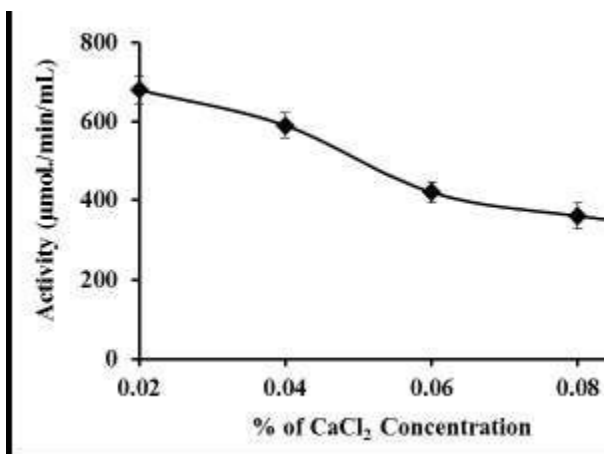


Fig. 7: Effect of different percentage of CaCl₂ on α-amylase production

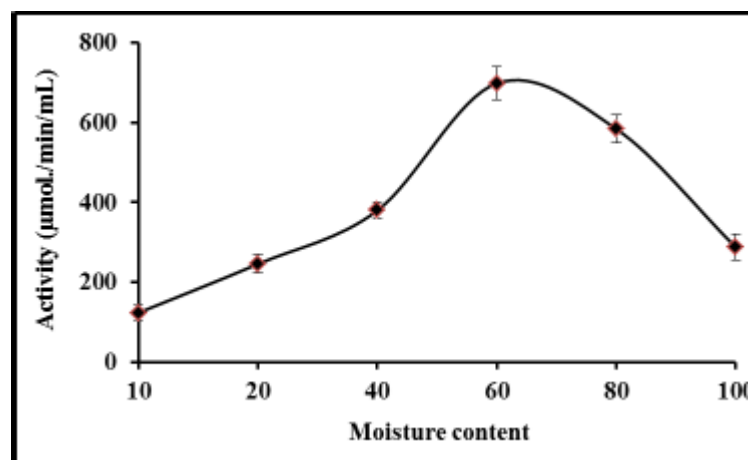


Fig. 8: Effect of different moisture content on α-amylase production

This must be fully reflected in industrial applications, in which the enzyme is expected to be useful for long operating duration. It is widely known that at high temperatures enzymatic activity can be destroyed because enzymes are proteinaceous molecules.

Effect of metal ions

These ions play an important role for the production of α-amylase. The underlying reason for this is that most α-amylases are metalloenzymes. Ca²⁺ and CaCl₂ ions are significantly important for the production of these enzymes. [28-29] In the present study effect of different concentrations of CaCl₂ on α-amylase production was evaluated. 0.02% was found to be optimum (680 ± 36µmol./min/mL) for the production of α-amylase. With the increase in calcium ions there was a slight reduction in enzyme production. The addition of MgSO₄.7H₂O into the wheat bran medium enhanced amylase production by *A. flavus* and 0.2% MgSO₄ resulted in maximum α-amylase activity (660 ± 36µmol./min/mL) (Fig. 6 & 7).

Effect of moisture content

The results presented in Fig. 8 indicate that moisture content of 60% was optimum for maximum enzyme yield. Optimum yield was observed as (698 ± 42µmol./min/mL) at 62% moisture content. Highest content moisture in solid state systems could increase the processes of diffusion. [30] Increase in moisture content resulted



in clumping of the solid particles and consequent reduction in enzyme yield and microbiological activity on a substrate will progressively decrease at lower water contents. [31] Fungal enzyme α -amylase was produced using a specific culture *A.flavus*. Various parameters viz. temperature, pH, incubation time and inoculum level, various mineral salts were studied to optimize the conditions to carryout SSF of wheat bran by *A. flavus*. Hence from the present study we conclude that SSF of the wheat bran for the optimal production of enzyme is technically feasible. Further utilization of wheat bran for the production of enzyme in SSF would reduce the cost considerably. The results will be useful for industrial production of this important enzyme with low input cost.

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