**Production of amylase by *Aspergillus fumigatus* under State Fermentation conditions**

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**Abstract**

The study aimed at isolation and screening of fungal amylase producer, optimization of solid state fermentation conditions for maximum amylase production by the best amylase producer, and characterization of the crude amylases, so produced. *Aspergillus fumigatus* showed the highest amylase activity in secondary screening under SSF conditions and was selected for further studies. The test strain showed maximum amylase production and supernatant protein concentration for incubation period (6 days), temperature (35°C), initial pH (6.0), and beef extract as nitrogen source. Wheat bran was chosen for further studies. The amylase produced to be *α*-type and 60 kDa was the molecular weight of the partially purified amylase. The enzyme showed maximum enzyme activity at pH 6.0, temperature of 55°C, and incubation time of 60 minutes.. The study indicates that *Aspergillus fumigatus* can be an important source of amylase and the crude enzyme, hence obtained, can be cost effectively applied in multiple sections.

 **INTRODUCTION**

Enzyme technology is an off-shoot of fundamental science related to cellular metabolism. With the development of science of microbiology, biochemistry, a better understanding of the wide range of enzymes present in living cells and their mode of action was achieved . Although enzymes are formed only in living cells, many can be isolated without loss of catalytic function in vitro . This unique ability of enzymes to perform their specific chemical transformations in isolation has led to an ever-increasing use of enzymes in industrial processes, collectively termed as “enzyme technology”.

Microbial enzymes are widely used in several industries, notably in detergent, food processing, brewing and pharmaceuticals. In fact, their use has been recorded since ancient times without known the functional utility in oriental countries .They are also used for diagnostic, scientific and analytical purposes . Amylases are starch degrading enzymes that catalyze the hydrolysis of internal alpha 1-4 glycosidic bonds in polysaccharides. They are ubiquitous enzymes produced by plants, animals and microbes where they play a dominant role in carbohydrate metabolism . Amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Amylases from plant and microbial sources are employed for centuries as food additives . Barley amylases are used in Brewing industry. Fungal amylases are widely used in preparation of oriental foods. Fungal amylases are mainly used for industrial applications due to their cost effectiveness, consistency, less time and space requirement for production and ease of process optimization and modification. Filamentous fungi have been widely used for the production of different enzymes including amylases. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of amylase. Production of enzymes by solid state fermentation using these moulds turned a cost effective production technique. In the present study, we report the isolation of amylase producing fungus from soil samples of agricultural field. Fermentation conditions were optimized to achieve high enzyme production using cheap vegetable waste as substrate.

Kirchhoff discovered the first starch degrading enzyme in 1811, which was followed by studies of digestive amylases and malt amylases. In 1930, Ohlsson. proposed the groups of starch digestive enzymes in malt as alpha and beta amylases based on the anomeric form of sugars generated by the enzyme reaction.

Amylase plays a pivotal role in industries. It is naturally produced by plants , animals and microorganisms however microbial amylase production is considered to be very effective. Broad spectrums of industries ranging from fermentation, food, brewing, pharmaceuticals and textiles to paper all are focusing to improve the microbial production of amylase using different biotechnological aspects (Saxena R, *et al*., 2011). Microbial produced amylase inoculated into the media is considered more reliable and effective for industrial processes and applications (Gopinath SCB, *et al*., 2017). To meet the requirements of the developing industrial sector low cost and effective production of amylase is necessary.

**Solid State Fermentation (SSF)** holds good potential for the production of enzymes and special in the process where the crude fermented product may be directly used as the source. In this fermentation process, natural raw material/ agricultural waste can be used as the source. SSF can also constitute a solid matrix that requires supplementing nutrient solution containing carbon source and other nutritional components. However, solid state (matrix) must contain enough moisture. Depending upon the nature of substrate, the amount of water absorbed could be one or several times more than its dry weight, which leads to relatively high-water activity on solid/gas interface in order to allow high rate of biochemical process. Maintenance of adequate moisture level in the solid matrix along with suitable water activity is essential for SSF. Solid substrate have generally large surface area per unit volume. Small substrate particles provide enough space for microbial attack but pose difficulty in aeration due to limitation in inter-particle space availability. Large particles provide better aeration opportunities but less surface area. In bioprocess optimization, sometimes it may be necessary to use a compromised size of particles (generally mixed range of particles) for cost effectiveness. For example, Wheat bran (constituting of coarse and fine particles) can be used as substrate for solid state fermentation. In SSF process, mixture of two forms of particles (coarse and fine particles) is used at different ratios for optimal production.

Solid substrate generally provide good environment to the microbial colonies (comprising bacteria and fungi), for their growth. Among these, filamentous fungi are considered best for SSF due to hyphal growth, which have the ability to penetrate through the substrate particles. Several agro-crops such as barley, fruit pulps, various oil cakes (e.g. ground nut oil cake, soya beancake, coconut oil cake, etc.), coffee husk, corn cobs, tea waste, brewing grains, etc. are the most commonly used substrate hydrolytic exo-enzymes are synthesized and excreted outside the cells that create and help in obtaining simple products (nutrients and carbon source) by the cells. This in turn promotes biosynthesis and microbial activities. Apart from these, several other factors that affects the SSF process includes physio-chemical and biological factors such as pH of the medium, temperature, incubation period, nature of substrate, etc. With the increasing harmful effect of traditional chemical pesticides now researchers are working on the production of biological control agents through SSF by using agro-industrial waste as substrates. Production of entomopathogens of fungal origin is being produced by SSF for adopting sustainable production method and for effective biological control method to reduce the pest eradication in the agricultural practices. Now novel approaches are developing for agricultural waste treatment and further being used for up scaling of SSF processes using bacterial culture for the production of poly γ glutamic acid in continuously stirred solid state bioreactors (Fang *et al*., 2020).

 **Objectives:**

The project work has been performed considering the following main objectives:

1. Isolation of amylase producing *Aspergillus fumigatus* from the soil.

2. Characterization of Amylase.

3. Optimization of production parameter in most promising isolates.

  **Methodology**

 **Collection of soil sample**

The soil sample was collected under sterile conditions to avoid contamination from paddy fields in a village nearby Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh, India.

 **Isolation of fungal strain:**

Isolation of potential fungal strain is important before proceeding for the production of enzyme of interest. For this project *Aspergillus fumigatus* was used for the production of amylase enzyme under Solid State Fermentation condition. The fungus *Aspergillus fumigatus* was isolated from the soil sample using serial dilution method. The soil sample was diluted 103,104,….…times. 0.5 ml of each diluted suspension was then transferred to petri dishes containing Potato Dextrose Agar (PDA) medium (Chloramphenicol that is a broad spectrum antibiotic, can also use in PDA to inhibit bacterial growth) through dilution plating methods. The petri plates were kept on incubation for 2-3 days at 28°C.

 **Screening of isolation fungal amylase activity:**

For amylase production, the fungal isolates were tested using starch hydrolysis. The modified starch agar media (Soluble starch-2g, Peptone-2g, Yeast extract-1g, Agar-2g, Distilled water 100ml at pH6) were inoculated with the isolates and incubated for 48 hrs, at 28°C. Once the incubated period was completed the petri plates were flooded with iodine solution. The zone of clearance formed around the microbial growth indicated the production of amylase. The young colonies of fungal culture were aseptically picked and transferred to Potato Dextrose Agar (PDA) slant with 1% starch. The slant was then incubated at 30°C for 4 days. After sufficient growth, they were stored at 14°C in refrigerator.

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**IMAGE:1 Aspergillus Fumigatus colonial growth on Potato Dextrose Agar(PDA) media.**

 **Inoculum Preparation:**

After screening the amylase activity, the fungal isolate was further used by harvesting the spores. After incubating the culture for 10-14 days the spores were harvested. The culture was fully sporulated at this stage. The petri plate was flooded with a mixture of distilled water (0.5ml), and 0.05% Tween-80 (5.0ml), then the culture was rubbed gently using a sterile bent glass rod. The spores were released. Spore suspension was filtered using sterilized glass wool placed in the funnel into Erlenmeyer flask (50ml). Then the spore suspension was transferred into screw cap tubes and centrifuged again. After repeating the process three times, the spores were finally resuspended in 5.0 ml of distilled water containing 0.1% Tween-80 and shake it for 30 sec before storage. The spore suspension was finally stored in darkness at 4°C.

**Substrate:**

For the production of microbial amylase, the agro-industrial waste is used as substrate. In this project we have used wheat bran as substrate, collected from the local market and grinded in small particles with the help of grinder. Solid State Fermentation was performed and the enzyme production was checked.

 **Fermentation Condition:**

There are two types of fermentation processes that are used for microbial production of amylase. One is Solid State Fermentation (SSF) and another is Submerged Fermentation (SmF). Traditionally, submerged fermentation process is used because in the technique, it is easy to control the environmental factors such as temp. and pH and also ease of sterilization.Therefore, the process is used in SmF is always in liquid state containing nutrients required for the growth. In Solid State Fermentation (SSF) process microorganism grow on solidified medium. SSF is preferred over SmF for the production of amylase because of the economic advantage.

 **Solid State Fermentation Process:**

Five grams of substrate was taken in 250 ml Erlenmeyer flask and a pre-determined quantity of water was added to the flask to maintain the moisture content. The isolate was subjected to fermentation medium containing (KH2PO4-0.14g, NH4NO3-1g, KCL-0.5g, MgSO4.7H20- 0.01g, FeSO4.7H20-0.001g, soluble starch-2g, distilled water-100ml at pH6.5). The content was thoroughly mixed and kept for autoclaving at 121°C for 15 min. After cooling the flask to room temperature, it was inoculated with fungal spore suspension and then was incubated at 28 °C for 48 hrs. at 200 rpm in BOD incubated under static condition. To investigate the optimum inoculum level, the concentration of inoculum, was increased accordingly. Initial enzyme concentration was checked with the same substrate for SSF. Experimental work was carried out in such a way that the parameters optimised in one experiment were maintained its optimum level in further experiments.

 **Enzyme Extraction:**

For extraction the amylase enzyme from SSF medium, 100ml of sodium phosphate buffer pH6.9 was added to each flask after a specified incubation time. The flasks were shaken for half an hour and the suspension was filtered using what man filter paper No.1. The fermented broth was then centrifuged for 30 min at 7000 rpm. The cell free supernatant was carefully collected and used as crude enzyme extract.

 **Screening of Microbial Amylase Production/ Enzyme Assay:**

There are different methods to screen out the production of microbial amylase including solution-based or solid-based techniques. The solution-based method is carried out using Di nitro Salicyclic Acid (DNS) and Nelson-Somogi (NS) technique. In solid based method, pinpoint inoculation of the strain (fungal strain) is done at the centre of the petri plate on starch containing media (PDA-Potato Dextrose Agar). After incubation the plates for an appropriate period, iodine solution is poured into the plates. Dark bluish colour is developed on the substrate region and around the inoculum clear region (due to hydrolysis) is observed which indicates the utilization of starch by microbial amylase.

In solution-based method, a mixture containing 20mM Phosphate Buffer (pH-7), 1% soluble starch and fermentation extract is prepared and incubated at 37°C for about 20 min followed by addition of Di-nitro Salicyclic Acid. Using UV-visible spectrophotometer, colour development is observed at 540nm and the amount of reducing sugar was estimated.

Spectrophotometric method was used to determine the amylase activity Bernfield (1955). In assay mixture containing enzyme extract, DNSA (coupling agent) and starch (substrate); one unit of amylase activity is defined as the amount of enzyme that release one micromole (µm) of reducing sugar as glucose per minute under the assay conditions.

**3.3..Evaluation of factor affecting enzyme activity**

**3.3.1 Effect of Incubation period:**

To investigate the effect of incubation period on enzyme production, the fermentation medium was incubated at regular intervals of 18, 36, 54, 72 and 90 hours at pH 5.6 and temperature 35°C.

**3.3.2.Effect of temperature:**

The fungal strain was grown on production media with different initial temperature or incubating the fermentation medium at temperatures ranging from 25°C-45°C for 90 hours at pH 5.6.

**3.3.3. Effect of Carbon Source:**

Potato Dextrose/ starch were used in the basal medium at 1% level as carbon source to support the microbial growth. Flask was incubated at 30°C for 5 days. The content was filtered and enzyme activity was assayed.

**3.3.4. Effect of Nitrogen Source:**

ln enzyme production, the effect of different nitrogen source was observed by adding ammonium chloride, ammonium nitrate, ammonium sulphate and sodium nitrate separately to the fermentation medium and incubation them for 90 hours at room temperature.

**3.3.5.Effect of Phosphate concentration:**

By adding 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1.4% of phosphate in the medium at pH 5.6 for 90 hours of incubation, phosphate concentration in enzyme production can be investigated.

 **Results and Discussion**

Amylases are the enzymes that are involved in starch hydrolysis. There can be derived from plants, animals, and microbes as well. But the main advantage of using micro organisms for amylase production is in that the enzyme will be more heat resistant and pH optima. Another reason is in economical bulk production capacity. Microbes are also easy to manipulate to obtain enzymes of desired characteristics (Aiyer, *et al*.2005, Despoulain *et al* 1990). Amylase produced from microbes meet industrial demands and therefore they almost replaced chemical hydrolysis of starch in starch processing industry (Bernfield, 1955)

For microbial production of amylase, Solid State Fermentation (SSF) was preferred. SSF process employs natural raw material as carbon or energy source. An inert material was used as solid matrix that requires nutrient solution containing necessary nutrients and carbon source as well. Depending upon the nature of substrate, the amount of water absorbed could be one or several times more than its dry weight that leads to relatively high water activity on solid/gas interface in order to allow higher rate of biochemical process. Low diffusion of nutrients and metabolites take place in lower place water activity conditions whereas compaction of substrate occurs at higher water activity. Hence, maintenance of adequate moisture level in the solid matrix along with suitable water is essential for SSF process. Solid state should have generally large surface area per unit volume. Smaller substrate particles provide large surface area for microbial attack but pose difficulty in aeration/respiration opportunity but provide lesser surface area. In bioprocess optimization, sometimes it may be necessary to use a comprised size of particles (usually a mixed range) for the reason of coast effectiveness. For instance, here wheat bran has been used as substrate SSF, in two forms, fine and coarse particles. Former contains particles of smaller size and the latter larger than these. Most of SSF processes use a mixture of these two forms at different ratios for optima production.

Solid substrates generally provide a good environment to microorganisms for their growth. Filamentous fungi are considered best for SSF due to their hyphae growth, which have the capability to penetrate through them. During the microbial growth on substrate hydrolytic exo-enzymes are synthesized by microbes and excreted outside the cells that create and help in accessing simple products (nutrients and carbon source) by the cells. This in turn promotes biosynthesis and microbial activities. Apart from these there are several other factors that must be considered. These include physio-chemical and biological factors such as pH of the medium, temperature and period of incubation, type of inoculum, nature of substrate, microorganisms used, etc.

Assay of enzyme production was studied for incubation period from 5 to 13 days and observed that on 8 day maximum (34.24 1U/ml) production of amylase enzyme. All reading was noticed on 8 days of different parameters.

Assay of enzyme production was carried out at temperature ranges 30°C to 50°C for 3 days. It was found that *Aspergillus fumigatus* showed considerable amount for growth at 30°C. However, the optimum temperature for amylase production 45°C was noticed that was maximum 21.88 1U/ml on 8 day and it was low on 12 day due to decrease of culture growth.

The initial pH of medium was adjusted to variable pH range by adding the 0.IN HCI. Crude enzyme was tested in the pH range of (pH 4.0-80). The enzyme production however started after 24 hours of incubation and showed maximum production on 8 day of incubation period at 45°C.

Flask containing production supplemented with Carbon source (glucose, sucrose, starch, fructose, xylose, galactose and dextrin). The influence of these carbon sources was tested. Starch, sucrose, dextrin, and galactose were good carbon source of amylase production. Xylose and fructose could be considered as moderate source. It was observed that maximum production of amylase on added substrate starch (35.72+1U/ml) on 8 day

Effect of different nitrogen sources on the production of amylase was studied, it was observed that Casein and Gelatin caused poor production of enzyme whereas urea produced considered amount of amylase, while peptone give optimum production. Adding source in the substrate peptone produced the maximum amount of amylase (37.06+1U/ml).

 **Conclusion**

The fungal strain *Aspergillus fumigatus* was isolated from the soil sample and for the production of amylase under solid state fermentation conditions. Production profile of amylase was observed, and characterization experiments were performed with the crude enzyme extract. The experiment results are summarised below:

*Aspergillus fumigatus* was found to be the best amylase producer.

Maximum production of amylase (35.18 1U/ml), least production was observed on glucose.

Effects of nitrogen sources were studied, and peptone was found to give maximum production (36.06 IU/ml) whereas Gelatin gives poor production of amylase.

 **Acknowledgement**

I feel pleasure in expressing my profound sense of gratitude and indebtedness to the management, **Meerut Institute of Engineering and Technology N.H. 58, Delhi-Roorkee Highway, Baghpat Road Bypass Crossing , Meerut,** who provided me the opportunity to utilize the facilities available in labs.

I am highly obliged to **Dr. Hirdesh Kumar (Assistant Professor, Department of Biotechnology and Microbiology), Meerut Institute of Engineering and Technology, Meerut** to arrange for my project in their esteemed organization.

I express my heart-felt gratitude to my guide **Dr. Hirdesh Kumar,** asst. professor (Project Mentor) whose unique guidance , all embracing help, valuable suggestions and encouragement have enabled me to complete this task which would not have been a success without them.

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