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Production and optimization of Amylase from *A. niger* isolated from legume seeds.

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ABSTRACT

Legumes are type of plant whose fruit is enclosed in a pod. Chick peas, cow peas, peas, broad beans and kidney beans all are type of legumes. Legume seeds are important source of protein. Legume seeds are more proteinacious than non legumes. Many fungi are pathogens of legumes they infect during storage. *Penicillium, Aspergillus, Mucor* and *Rhizopus* are common genera isolates from seeds. Whereas *A.niger* is predominant and most recovered isolate of legume seeds. *A.niger* is widely used organism and now it is extensively used for the productivity of amylase enzyme. Amylase enzymes have vast applications at industrial level where it is used for the production of fructose and glucose syrup from starch degradation, biofuel formation, and production of dough, juices and cakes and in brewing industry. Amylases are also used as diagnostic tool. The ability to degrade starch is used as a standard for the determination of α-amylase production by *A.niger.* The determination of optimum time, pH, temperature, carbon and nitrogen sources are important for the better yield of the enzyme. *A.niger* produce amylase enzyme with greater extent at day 6 with pH 6 and at temperature 25 °C. It uses about all carbon and nitrogen sources but in maltose and ammonium sulphate production of amylase is higher. Submerged fermentation method is chosen technique for the production of amylase at laboratory level although it is not used widely at industrial level.

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INTRODUCTION

Legumes are type of plant whose fruit is enclosed in a pod. Chick peas, cow peas, peas, broad beans and kidney beans all are type of legumes. Legumes are rich in fiber, carbohydrates, and protein and micro nutrients such as folate, thiamin, manganese, magnesium, potassium and iron. Legumes are easy to grow, they grow rapidly and they are not only proteins abundant but also they contain minerals and vitamin B.

During storage Legume crops are introduce to infection by fungal pathogens, which often cause the accumulation of mycotoxins (1). These fungi are of saprophytic or pathogenic nature which affects seed germination (2). Fungi infect legumes easily because they are dry and fungus can grow in low moisture content. Worldwide, many studies have shown the biology of the seed borne and plant diseases which are related to these leguminous crops (3-6). Isolation of mycoflora from legumes can be done by standard blotter method and agar plate method. *Alternaria*, *Aspergillus*, *Curvularia*, *Eurotium*, *Fusarium*, *Penicillium*, *Rhizoctonia*, *Mucor*, *Cephalosporium*, *Acrothecium*, *Curvularia*, *Pythium*, *Trichoderma*, *Rhizopus*, *Sclerotium* and *Gliocladium* are the most common fungal genera which isolates from legume seeds, including broad beans (*Vicia faba*), kidney beans

(*Phaseolus vulgaris*), cowpeas (*Vigna sinensis*), chickpeas (*Cicer arietinum*) and peas (*Pisium sativum*) (7,8,9). In seeds, starch is hydrolyzed for utilization by the growing seedlings to fulfil its energy requirement.

Whereas amylase is an enzyme that catalyses the hydrolysis of starch into sugars. Amylases are the key enzymes and are of great importance, constituting a class of industrial enzymes having approximately 25% of the world enzyme market (10,11). Now a day's microorganisms are use to produce enzymes. Amylase enzyme from microbial sources has been used for centuries as food additives. Fungal amylases have been broadly used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources are used at the industrial level due to its cost effectiveness, pliability, less time and space required for production and ease of process modification and optimization (12).

α-amylase from fungal sources, especially *Aspergillus* specie has possessed more importance because of the easy availability and high productivity of the *Aspergillus*, which are also suitable for genetic manipulations. Some species are pathogenic for humans and animals, where they invade various tissues and produce *Aspergillosis* (18). Many species are reported to produce mycotoxins. Some species are used in the food industry and some species are use as biotechnologically to produce enzymes and organic (19). Different species of the genus *Aspergillus* such as *A.niger, A.oryzae, A.flavaus* and *A.fumigatus* have been frequently used for the production of α-amylase (13).

To prepare these extra cellular α-amylase enzymes on a commercial scale, many experiments have been done to specify cultural conditions and to select superior strains of the fungus (16). Methods which are used for production of α-amylase are submerged fermentation and solid State fermentation. The second one is a fairly new method while the first one is a traditional method of enzyme production which has been in use for a longer period of time from microbes (14,15).

The ability to degrade starch is used as a standard for the determination of α-amylase production by fungi. In the laboratory it is tested by performing the starch test to determine the presence of starch in the medium by using Iodine solution as an indicator. Starch and iodine complex produces a dark blue coloration of the medium and a yellow zone around the colony of fungus if α-amylase is produced.

Amylase activity is estimated by measuring the optical density of reducing sugar released during hydrolysis of starch. It is also measured by dinitrosalicylic method (DNS), Nelson – Somogyi (NS) Method, Dextrinizing Activity, Indian Pharmacopoeia Method and Reduction in Viscosity of Starch Suspension. The determination of optimum time, pH , temperature , carbon and nitrogen sources are important for the better yield of the enzyme.

The most useful and important applications for α-amylases are in the starch industry, Food industries, Biofuel industries, Detergent industries, Paper industries: Clinical and medicinal applications and Elimination of environmental pollutants:

Reporter gene assays have become essential for the study of gene regulatory elements and gene expression. In molecular biology, the presence of amylase can work as a supplementary method of selecting for successful integration of a reporter buildup in addition to antibiotic resistance. Introduction of foreign DNA into this gene result in a loose of amylolytic activity in the host cell that can be analysed using a simple and low cost iodine staining technique (17).

MATERIALS AND METHODS

Sample collection:

Legume seeds were collected for the isolation of fungus.

Isolation of Fungi:

Fungi were isolated from legume seeds, using potato dextrose agar containing streptomycin to avoid bacterial contamination. Seeds were placed on Petri dishes under hygienic conditions containing growth media. Five different types of seeds used and five samples were collected for each type of seed. A total of 25 samples were assayed for their fungal content. All dishes were placed on room temperature for 6 days.

Identification of Fungi:

At the end of incubation period fungi were identified on the basis of macro and microscopic characteristics.

Identification was confirmed by staining of recovered fungal culture.

Screening of Fungi for α-amylase Production: *Aspergillus.niger* was selected for screening of enzyme production and optimization of enzyme. *Aspergillus.niger* was sub cultured on starch yeast extract agar medium. After growth of *A.niger* starch hydrolysis test was performed for amylase production.

Cultural Optimization of α-amylase

Effect of Incubation period:

α-amylase production was estimated by the change of different incubations days. A colony of *A.niger* was inoculated in starch yeast extract liquid medium and incubated at room temperature. Cultures were filtered after the intervals of 2-days. Clear supernatants obtained after centrifugation of filtrates were assayed for α-amylase activity.

Effect of Temperature:

α-amylase production was estimated by change of different temperatures. For each temperature separate culture broth was used. A colony of *A.niger* was inoculated in starch yeast extract liquid medium and incubated at room temperature, 42 C and 37 C for 6 days. Cultures were filtered after the interval of 6-days. Clear supernatants obtained after centrifugation of filtrates were assayed for α-amylase activity.

Effect of pH:

The influence of different pH on α-amylase production was tested. pH of media was adjusted to 4, 5, 6, 7, 8 and 9. A colony of *A.niger* was inoculated in starch yeast extract liquid medium and incubated at room temperature for 6 days. Cultures were filtered after the interval of 6-days. Clear supernatants obtained after centrifugation of filtrates were assayed for α-amylase activity.

Effect of Different Carbon sources:

α-amylase production was estimated by the influence of different carbon sources. Six carbon sources (maltose, glucose, sorbitol, fructose, lactose and sucrose) were added in the liquid medium individually in a 1% ratio. A colony of *A.niger* was inoculated in the medium and incubated at room temperature for 6 days. Cultures were filtered after the interval of 6-days. Clear supernatants obtained after centrifugation of filtrates were assayed for α-amylase activity.

Effect of Different Nitrogen sources:

α-amylase production was estimated by the influence of different nitrogen sources. Six nitrogen sources (peptone, KNO3, (NH4)2SO4, NH4CL, NaNO3 and NH4NO3) were added in the liquid medium individually in a 0.3% ratio. A colony of *A.niger* was inoculated in the medium and incubated at room temperature for 6 days. Cultures were filtered after the interval of 6-days. Clear supernatants obtained after centrifugation of filtrates were assayed for α-amylase activity.

Assay of Extracellular α-amylase:

A reaction mixture containing 1ml of 0.5% soluble starch in acetate buffer and 1ml of fungal filtrate was incubated at 30 C for 30 mints. The amount of reducing sugar maltose was estimated by determining the optical density at 700 nm wave length in the spectrophotometer to obtain the assay of extracellular α-amylase.

RESULTS

Most common genra of fungi found on legume seeds were *Penicillium, Aspergillus, Mucor* and *Rhizopus*. Whereas the prevalent species were *A. niger, A. flavus, A. fumigatus, M. racemosus*, *P. chrysogenum, R. stolonifer*. Broad beans were recorded with highest fungal growth (95%) followed by chick peas (88%), kidney beans (70%), peas (67%) and then cowpeas (57%) (Table I). A.niger was best α-amylase producer. Clear zone around colonies were observed which indicates positive results. On the reaction mixture of iodine and starch the tube turned blue within 5 mints which confirmed the enzyme production (Table II). Amylase enzyme at different incubation days produced in different amount. Its production was increased at optimum day 6 further incubation decreased the production rate (Figure 1). Enzyme production rate is greatly affected by

temperature. At different temperature different concentration of amylase was produced. Enzyme production was increased at room temperature which was 28^{0}C, at 37^{0}C and 42^{0}C enzyme was produced but in low amount (Figure 2). pH plays an important role in enzyme production. Different pH affects differently on α-amylase production. Enzyme production was increased at optimum pH 6 and varies with other PH (Figure 3). It shows that enzymes production from *A.niger* is highly susceptible to Ph of media. *A.niger* utilizes all form of sugars as a carbon source. It can grow by utilizing different carbon sources but the growth rate is varying among these sugars. It utilized maltose, sorbitol, sucrose and fructose chiefly as compare to glucose and lactose (Figure 4). Nitrogen is an important element for livings. *A.niger* also uses nitrogen for the synthesis of its amino acids and other important compounds. Ammonium sulphate was the inorganic nitrogen source which was utilized at greater extent and thus enzyme production was high in the media supplemented with ammonium sulphate (Figure 5). Other nitrogen sources were also consumed with different rate.

Table I.: the percentage of Aspergillas niger in different seeds

Legume seeds	Percentage of A.niger
Broad beans.	95%
Chick peas	88%
Kidney beans	70%
Peas.	67%
Cow peas.	57%

Table II: Screening of A.niger for α-amylase production

Reaction mixture	**Iodine**	**Discoloration of blue color**	**Positive result**	

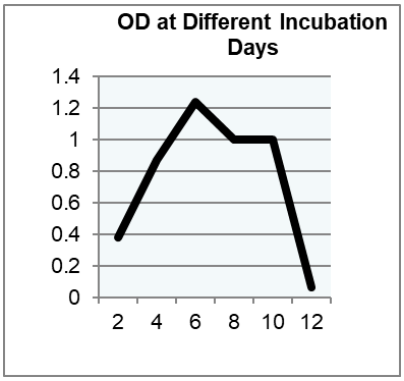

Figure 1: Graphical representation of OD of α-amylase enzyme at different incubation days. Higher production achieved on day 6.

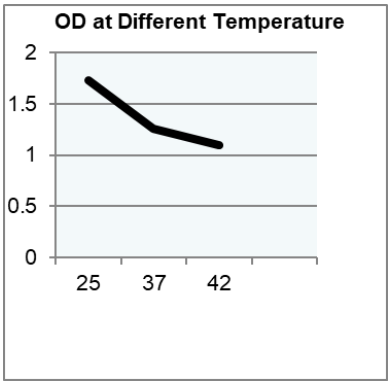

Figure 2: Graphical representation of OD of α-amylase enzyme at different temperature. Higher production achieved at 25 C.

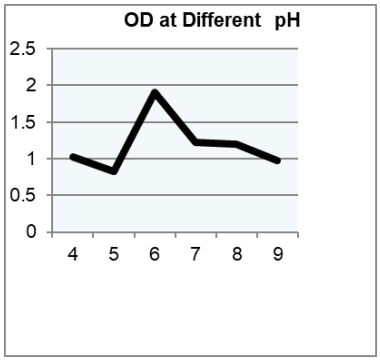

Figure 3: Graphical representation of OD of α-amylase enzyme at different pH. Higher production achieved at Ph 6.

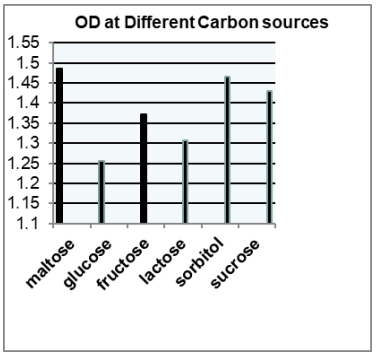

Figure 4: Graphical representation of OD of α-amylase enzyme at different carbon sources. Higher production achieved in maltose.

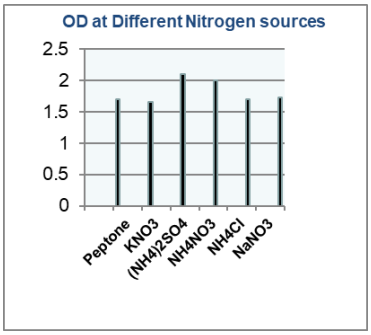

Figure 5: Graphical representation of OD of α-amylase enzyme at different nitrogen sources. Higher production achieved in ammonium sulphate.

DISCUSSION

Legume seeds are important source of protein. The legumes belong to the family Leguminosae. Fungal genera are pathogenic group of microorganisms which isolates from legumes. Fungi infect legumes easily because they are dry and fungus can grow in low moisture content. *A.niger* is most common recovered isolate of all seeds. Broad beans were recorded with highest fungal growth (95%) followed by chick peas (88%), kidney beans (70%), peas (67%) and then cowpeas (57%). α-Amylases are most common and important type of amylase enzymes produced by plants, animals and microbes. *Aspergillus niger* is a filamentous fungus growing aerobically it produces α- amylases with greater extend. The most useful and important applications for α-amylases are in the processing of starch industry, food industry, biofuel industry, detergent industry, paper industry, elimination of pollution and, clinical and medicinal.

Enzyme production from *A.niger* is estimated by starch hydrolysis test and further it was confirmed by observing the discoloration of blue color (Table II) from reaction mixture which contained complex of starch, iodine and supernatant of centrifuged in which enzyme was present. Starch and iodine complex gives blue coloration, in the presence of amylase starch is degraded in maltose that is why blue color is disappeared. α-amylase enzyme in the laboratory can be produced by submerge fermentation because of easy to control physic-chemical parameters.

α-amylase enzyme produce by *A.niger* can be optimize at different cultural and environmental conditions such as at different pH , carbon and nitrogen sources , incubation days and temperatures. At different conditions amylase production in *A.niger* is change this shows that enzymes are highly susceptible to environmental and cultural conditions. pH of the medium is very crucial for the growth of any organism and enzyme production is direct proportional to the growth of organism. At optimum pH *A.niger* grows with high degree thus production of α-amylase was increased at pH 6. At pH 7and 8 growth of *A.niger* was moderate that is why enzyme production was also moderate at pH 9, 5 and 4 enzyme production was decreased due to high acidity and alkalinity of media *A.niger* growth may be reduced. Temperature and incubation period are another physical factors which play a significant role for α-amylase enzyme production. At room temperature *A.niger* grows well within 6 days of incubation so the enzyme was also produced with greater extent at room temperature in 6 days whereas initially and after 6 days of incubation α-amylase production was decreased.

A.niger utilizes different carbon and nitrogen sources to fulfill its nutritional requirements. Maltose was commonly used carbon source for A.niger whereas other sugars were also utilized with different proportional thus at different carbon sources α-amylase production were vary.

Sorbitol is a sugar which metabolize slowly but *A.niger* possessed sorbitol dehydrogenase gene (sdhA) which is involve in sorbitol catabolism thus sorbitol can be use as sugar source for *A.niger.* Ammonium sulphate is the inorganic nitrogen source which was utilized by *A.niger* at high amount thus higher amount of amylase enzyme was produced in the medium containing ammonium sulphate. There is no specific nitrogen and carbon source for *A.niger* consumption it utilizes all forms of sugars for carbon source and nitrogen compounds for nitrogen source. As in the graph it is clearly understand that all nitrogen sources were used by *A.niger* for its growth and enzyme production.

CONCLUSION

From this current study and results of my research work I found that amylase enzyme can be easily produce in the laboratory where all the conditions for enzyme production meets. Microbes are efficient and cost effective source of amylase enzymes where as from fungal genra *A.niger* is predominant and most common fungus which produces extracellular α-amylase enzyme. Enzymes are highly susceptible to the physic-chemical parameters thus at optimum conditions production rate will be high. Now a day's α-amylase are widely used in industries due to its wide application α-amylase production is becoming prior at industrial level.

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