

Starch Hydrolyzing Enzyme Production from Fruit Peels using *Aspergillus Niger*

ABSTRACT

Amylase is one of the important industrial enzymes due to its application in detergents, food and textile industries. In the present study, the production of amylase, a starch hydrolysing enzyme, was optimized using *Aspergillus niger* under various conditions i.e., incubation time, carbon source, pH and temperature. Amylase production by *Aspergillus niger* using submerged fermentation of mixed fruit peel wastes such as sweet lime, banana, cucumber, orange, pomegranate, pineapple, and watermelon was used. The cultural and nutrient requirements of *Aspergillus niger* for the production of amylase in production media containing different pH, temperature, incubation period, metal ion concentrations, surfactants, carbon sources and nitrogen sources were quantified in the present study. The optimum temperature and incubation period for enzyme production was 37°C and 5th day, respectively. The main objectives of the present study were to use a suitable fungal strain for the production of extracellular alpha-amylase and to determine the time course for the production of alpha-amylase. Thus, this enzyme can be produced cost-effectively using low-cost fruit peel waste and could be utilized in the detergent or textile industry.

Keywords: Amylase, *Aspergillus niger*, mixed fruit peel waste, submerged fermentation, incubation

INTRODUCTION

An amylase is an enzyme that catalyses the hydrolysis of starch into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Amylase is an enzyme that occurs naturally in the saliva of some mammals and humans that aids in the process of digestion. It accelerates the breakdown or hydrolysis of starch into simple sugars. The pancreas and the salivary glands mainly synthesise amylase to hydrolyse dietary starch into disaccharides and trisaccharides that are

converted into glucose and used as energy. Amylase was one of the first enzymes to be discovered in the 1800s. It was initially named diastase but later renamed amylase in the late 20th century. The first industrially produced enzyme was an amylase from a fungal source in 1994, which was used for the treatment of digestive disorders (1).

Nowadays, *Bacillus*, *Aspergillus*, *Rhizopus* and *Rhizobium* isolates are specified and considered to be the most important sources of industrial amylases. Nevertheless, various other sources of microbial amylases have also been investigated throughout the world. Growth parameters and other nutrients that promote high yields of microbial amylases were also studied extensively. The usual carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for commercial production of amylase. These expensive products can be replaced in the medium with economically available agricultural by-products or industrial amylaceous substances as carbon substrates which are cost-effective (2–4).

Thus, the present study was designed in search of cheaper carbon sources for the production of alpha-amylase enzyme by using fungal strains. Amylase produced from the fungal cultures was found to be more stable than that produced by bacteria on a commercial scale. Many suitable strains of fungi have been optimized for their culture conditions. Moulds are capable of producing high amounts of amylase. *Aspergillus niger* is used for commercial production of amylase. Studies on fungal amylases especially in developing countries have concentrated mainly on *Aspergillus niger*, probably because of their ubiquitous nature and non-fastidious nutritional requirements (5,6).

Amylase constitutes an industrial enzyme that represents approximately 30% of worldwide production. It is also used in fields that are related to biotechnology such as: removing environmental pollutants, by using micro-organism conversion of starch into the desired substrate, infiltration of waste containing starch and production of biochemical material to help the starch substrate. The spectrum of amylase application has expanded into many other fields due to the advent of biotechnology such as medical, clinical and analytical chemistry. Generally, amylase can be derived from various sources from plants, animals and various micro-organisms. The microbial enzymes generally meet industrial demands. The alpha-amylases are metalloenzymes means for their stability, activity and structural integrity they require calcium ions (7,8).

Fermentation is the metabolic breakdown or biological conversion of complex substrates into simpler compounds by using various microorganisms such as bacteria and fungi. Fermentation has been widely used for the production of a wide variety of substances that are highly beneficial to individuals and industries. Over the years, this technique has gained immense importance due to its economic and environmental advantages (9,10).

The main types of fermentation are solid state and submerged fermentation based on the substrate used during the fermentation process. The outcome of fermentation highly varies for each substrate. Hence, it is extremely important to choose the right substrate. Fermentation techniques have to be optimized for each substrate. This is primarily due to the reason that an organism reacts differently to each substrate. The rates of utilization of various nutrients differ in each substrate, and so does productivity. Fermentation is used as a primary technique for the production of enzymes (11,12).

Several industrial important enzymes are produced from fungi belonging to the genus *Aspergillus*. *Aspergillus niger* is the single largest fungal source of enzymes. *Aspergillus* is widespread in the growing environment in soil, plants, decomposing organic matter and air. There are 200 species of *Aspergillus*. The cell wall of *Aspergillus niger* is composed of 6 sugars: glucose, galactose, mannose, arabinose, glucosamine, galactosamine. Sixteen amino acids are found in the cell wall of *Aspergillus niger* namely aspartic acid, glutamic acid, glycine, alanine, valine, tyrosine, phenylalanine, proline, cystine, arginine, lysine, histidine (13–15).

Agro-industrial wastes have been reported to be good substrates for the cost-effective production of alpha-amylases and are thus attracting researchers to use agro-industrial waste as a substrate for alpha-amylase production. Fungal species have been studied a lot for the production of alpha-amylase because of the low cost of substrates used for the production of alpha-amylases (16).

Aspergillus niger is a mould classified within the *Nigri* section of the *Aspergillus* genus. The *Aspergillus* genus consists of common moulds found throughout the environment within soil and water, on vegetation, in faecal matter, on decomposing matter, and suspended in the air. Species within this genus often grow quickly and can sporulate within a few days of germination. A combination of characteristics unique to

A. niger makes the microbe invaluable to the production of many acids, proteins and bioactive compounds. Characteristics including extensive metabolic diversity, high production yield, secretion capability, and the ability to conduct post-translational modifications are responsible for *A. niger*'s robust production of secondary metabolites. *A. niger*'s capability to withstand extremely acidic conditions makes it especially important to the industrial production of citric acid (17,18).

A. niger causes a disease known as "black mould" on certain fruits and vegetables such as grapes, apricots, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly found in indoor environments, where its black colonies can be confused with those of *Stachybotrys* (species of which have also been called "black mould") (19).

There are two ways in which *Aspergillus niger* can be grown for industrial purposes: solid-state fermentation (SSF) and submerged fermentation (SmF). SSF uses a solid substrate with nutrients and minimal moisture to grow microorganisms. Nutrients such as nitrogen and carbon come from agricultural by-products such as wheat bran, sugar pulp, rice husks, and corn flour. SSF gives a better yield of microbe products and is more cost-effective than SmF due to using agricultural by-products. SSF is predominantly used over SmF. In SmF, microbes are grown in a liquid medium inside large aseptic fermentation vessels. These vessels are expensive pieces of equipment that provide more water for growth and allow for tight control of environmental factors, such as temperature and pH, that affect microbial growth (20,21).

A. niger produces many useful enzymes for the catabolism of biopolymers to obtain nutrients from its environment. The production of specific enzymes can be increased for industrial purposes. Another use for *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for NMR analysis (22–24).

Aspergillus is a group of filamentous fungus that can be easily cultivated in laboratory conditions – meaning that they are, therefore, one of the most extensively studied groups of fungus.

The *Aspergillus* group can, once cultivated, be used to synthesize useful industrial compounds such as 'glycoside hydrolases. These enzymes are used in the process of converting biomass into biofuels – by breaking down cellulose and hemicellulose from plant cell walls, into a substance that is later converted into second-generation ethanol. This species can also be used to produce bioactive metabolites, as well as other pharmaceutical products (25,26).

Microscopic examination revealed the dark brown rough-edged conidia spores and brown conidiophores. Cultural examination using Potato dextrose Agar and Malt Yeast Agar. Genomic sequencing of the fungi can be done for identification and differentiation from other fungi. Thin-layer chromatography can be used for the identification and quantification of ochratoxin mycotoxin (27,28).

Aspergillus niger causes a black mould of onions and ornamental plants by infecting onion seedlings. It can disseminate and become systemic and manifest when conditions are favourable. *A. niger* causes a common post-harvest disease of onions, in which the black conidia can be observed between the scales of the bulb. It can also cause disease in peanuts and grapes (29).

MATERIALS AND METHODS

1. Sample Collection:

- Sample is collected from the local market of Mohaan, Kakori and Krishna Nagar, Lucknow.
- Various fruit peels were collected from Banana
Cucumber
Orange
Pomegranate
Pineapple
Sweet
Lime
Watermelon

2. Sample Preparation:

- The collected fruit peels were chopped into small pieces using scissors.
- The finely chopped pieces were washed thoroughly with water for at least 5-6 times.
- After washing they were kept for air dry for 2-3 days on blotting paper.
- The peels were then transferred to a hot air oven for tray drying at 60°C for 2-3 hours.
- After proper drying the peels were ground into fine powder.

3. Raw Material Preparation:

- Broth was made in the ratio of 1:10 by soaking 20 grams of fruit peel powder into 200 millilitres of double distilled water.
- Then it was kept overnight in a BOD incubator shaken to allow proper broth formation.
- Then the broth formed was filtered using a muslin cloth to remove any solid that may be present in the broth.
- The filtered broth was measured and autoclaved at 121°C for 15 minutes.
- The prepared broth was used further for the fermentation process.

4. Media Preparation:

Media used for fungal growth is usually Potato Dextrose Agar (PDA). It contains all the nutritional requirements relevant to the growth of fungi. This media is made from extracts of finely used potato and contains laboratory-graded dextrose and agar. It is a solid media that is utilized on petri plates.

Procedure:

- Using a weighing balance, 3.9 grams of Potato Dextrose Agar powder was measured in butter paper.
- This was mixed in a conical flask using distilled water and the volume was made up to 100 millilitres.
- This was then sealed with a cotton plug and placed on a hot plate to allow the prepared media to dissolve.
- Once the media dissolves, the media must be autoclaved at 121°C at a pressure of 15 pounds per square inch, with a holding time of 15 minutes.
- The sterilized media is then taken to a Laminar Air Flow cabinet and poured into Petri Plates.
- The media in the plates is allowed to solidify before inoculation is conducted.

5. Isolation of fungus (*Aspergillus niger*):

The fungi species of *Aspergillus niger* selected from the black gram mould disease that occurs in an onion bulb. Therefore, the spores of *Aspergillus niger* can be isolated from that. This can be done by suitable inoculating and the picking of a pure colony as mentioned in the steps given below.

Procedure:

- An onion bulb containing black gram mould disease was collected from a local vendor in Banthara, Lucknow.
- The onion bulb was taken in the sterilized environment of the Laminar Air Flow Cabinet and was peeled to expose the infected inner layers.
- 20 microlitres of autoclaved double distilled water was taken in an Eppendorf tube.
- Using a flame-sterilized inoculation loop, the black mould on the inner layers of the onion is lightly scraped.
- This was then dissolved in the water contained within the Eppendorf tube.

6. Microbial Culture:

Microbiological cultures are multiplied microbial organisms that are grown in a pre-set culture medium in a laboratory. Growing a microbial culture requires an appropriate physical environment with controlled pH, atmospheric gases, temperature, and pressure. Other nutritional requirements of the microbes include carbohydrates, proteins, salts, vitamins and growth factors. Microorganisms are cultured to determine the species of the organism, to detect its presence in a sample and for diagnostic purposes.

Procedure:

- Pour the autoclaved PDA media on sterilized petri plates and leave it to solidify for 30 minutes.
- Then using a micropipette add 2-3 drops of the water contained within the Eppendorf tube which has scraps of black mould prepared earlier.
- After that with the help of L-shaped spreader it is spread smoothly on plate containing solidified PDA media. Then the plates were sealed using parafilm tapes.
- Then it was incubated for 2-3 days at 37°C to achieve proper growth of fungus.

7. Sub-Culturing for fungal growth:

This is done in order to obtain a pure culture that will ensure that a single strain of the fungal organism has been isolated. This will help determine the exact organism used for the production of the starch hydrolysing enzyme.

Procedure:

- Prepared media is poured into petri plates as described in the procedure above. It was then allowed to solidify.
- In a Laminar Air Flow, the plates initially inoculated were opened in the vicinity of a spirit lamp.
- From here an isolated colony was picked using a flame sterilized inoculation loop.
- The streak-plate method was used to create a streak on the solidified media gently in S shape.

- The plates were then sealed using parafilm tapes and were allowed to incubate at 37°C for 48 hours.



Figure 1: Sub-Culturing of *Aspergillus niger*

- Once isolated colonies were obtained, the colonies from the plates were further sub-cultured using the streaking method with a sterilized cotton bud.
- The colonies were allowed to grow at 37°C for 48 hours and eventually pure isolated colonies of *Aspergillus niger* was isolated.

8. Detection of isolated species (*Aspergillus niger*):

Since the isolating source could have contained a large number of fungal species, it is necessary to determine which species has eventually been isolated from the source. In order to perform this, Lactophenol Blue staining is conducted. This can help to identify the morphology of the fungus. When this is studied under the microscope, it can help to identify the strain that has actually been isolated. Lactophenol Blue contains phenol, lactic acid and methyl blue. Phenol kills the live microbes that are not fungal on the slide so that they can be properly stained. Lactic acid preserves the fungal strains. Methyl Blue stains the chitin on the cell walls of the fungus and helps them be visible under the microscope.

Procedure:

- In the vicinity of a lit spirit lamp, the petri plate containing the growth culture was opened.
- Then the microscope slide and coverslip were thoroughly cleaned using 70 percent ethanol.
- A drop of lactophenol blue was placed on the centre of the slide using a micropipette.

- Then a flame sterilized inoculation loop was used to lightly pick up a fungal spore.
- The spore was placed on the drop of lactophenol blue on the slide and it was covered with a coverslip gently, avoiding air bubbles.
- This was then studied under the microscope to view morphology.

9. Suspension of the fungal Cells:

A suspension is formed using the Tween 80 solution in order to count the spores using a hemocytometer. Tween 80 is a detergent that helps detach the spores from the media on the plate. It dislodges the spores. This solution can be used for the counting of the spores which can be done using a hemocytometer.

Procedure:

- A dilution of Tween 80 was prepared in 1 percent - which was done by putting 100 microliters in 10 milliliters of distilled water.
- This solution was then poured onto a plate containing isolated colonies of *Aspergillus niger*.
- That solution was allowed to sit on top of the petri plate for about 5 minutes.
- Using a flame sterilized inoculation loop, the fungal colonies were gently scraped and a suspension of the spores was formed along with the Tween 80 solution.
- This solution was then used to count the spores or measure concentration.

10. Counting of the fungal spores:

A hemocytometer is a suitably divided chamber which can be used to count the spores that are present in the solution. The volume of the chamber is known which is 0.1 cubic millimeters. The spores are counted in boxes containing 16 squares each and an average is taken to establish whether a suitable count of spores is present proceed for fermentation or not.

Procedure:

- The hemocytometer and coverslip were wiped with 70 percent ethanol.
- Using a micropipette, the chambers were filled with the suspension of the spores.
- This was then placed under a microscope for visualization.

- Thenthe spores were counted.

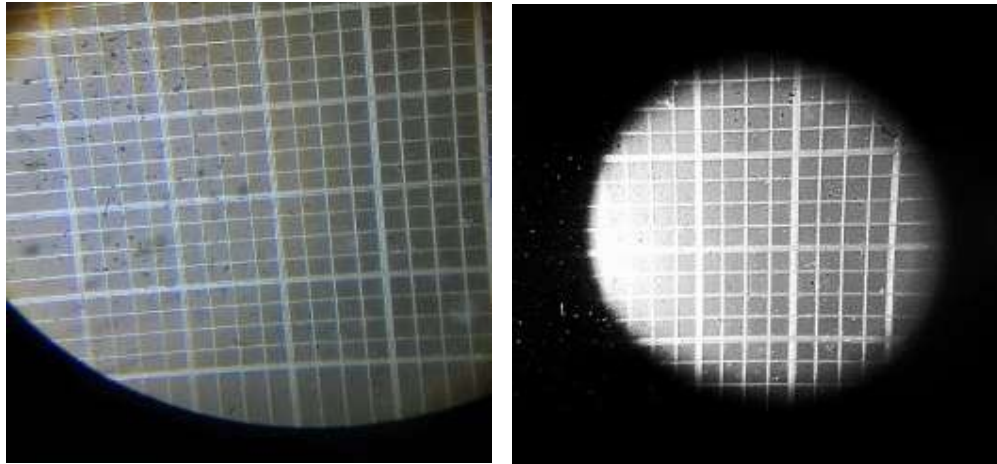


Figure 2: Spores visualized on the haemocytometer using a microscope.

11. Establishment of suitable dilution for fermentation:

In order to perform fermentation for a desired volume, a suitable volume of inoculum solution is added. In order to estimate this quantity, optical density is performed to measure the appropriate concentration of the spore solution used for growth. In order to create this suspension Tween 80 is utilized. Tween 80 dilutions are used. According to the Clinical and Laboratory Standards Institute, an absorbance of 0.08 to 0.1 at a wavelength of 530 nanometer is equivalent to 10^6 spores. This amount is said to be suitable for inoculation.

Procedure:

- The dilution prepared earlier are used in the amounts of 300 microlitres and 500 microlitres.
- Then volume is made up to 4 millilitres in each tube.
- Each of these were used to measure the optical density of the formed solution at 530 nanometers.
- Then desired concentration was chosen as the inoculum.

12. Fermentation:

This outlines the entire process utilized for the preparation of the suitable fermentation broth for the production of the starch hydrolyzing enzyme and the fermentation process.

12.1 Preparation of the fermentation broth:

This includes the collection, washing, drying, grinding and desolvation of fruit peels to form a suitable broth for the fermentation. Fruit peels were taken from different sources as that would result in a better substrate for *Aspergillus niger* to produce the desired enzymes.

Procedure:

- Fruit peels were collected from banana, cucumber, orange, pomegranate, pineapple, sweet lime, and watermelon.
- Those were then thoroughly washed with water.
- The peels were cut into small pieces and were allowed to air dry for 24 hours on a blotting paper.
- Then they were transferred to a hot air oven where they underwent further drying at 60 degrees Celsius for 48 hours.
- Once completely dried the fruit peels were grinded to form a fine powder.
- Of this 20 grams was used to make a broth by adding 200 millilitres of distilled water to it.



Figure 3: Broth kept in overnight BOD incubator shaker and filtered using muslin cloth.

- That was kept overnight in a BOD incubator shaker and allowed for the proper formation of broth.
- Then the broth formed was filtered using a muslin cloth twice to remove any solids that may be present in that broth.
- The filtered broth was measured and then autoclaved at 121 degrees Celsius for 15 minutes.
- This broth was used further for fermentation process.

12.2. Fermentation Process:

The fermentation process constitutes for 5 days at a temperature of 25 degrees Celsius at an rpm of 120. Fermentation is the chemical breakdown of a substrate by an organism which is *Aspergillus niger* in this case. The optical density is measured at regular intervals in order to check the growth of the microbes.

Procedure:

- The fermentation broth prepared using the fruit peels was divided into two sterilized conical flasks, one containing 50 millilitres and the other 150 millilitres.
- The prepared dilution of the 1 percent spore containing solution explained earlier was used for the inoculation.
- In the flask that contains 150 millilitres of fermentation broth, 500 microlitres of the prepared dilution of inoculum was added using a micropipette.
- This flask was then sealed with cotton plugs and placed in an incubator shaker at 25 degrees Celsius at a rpm of 120 for a period of 5-6 days.
- The flask containing 50 millilitres of fermentation broth was also placed in the same condition as a control.
- Every alternate day, the optical density of the media was taken at 540 nanometres using the control as a blank.

13. Microbial Count:

The microbial count is performed on fermentation broth in order to estimate the amount of *Aspergillus niger* grown. This aids in the estimation of the production of the enzyme in fermentation broth. This is conducted by estimating the wet and dry mass of the cells. It is also a step in the purification process of starch hydrolysing enzyme which is produced in the broth.

Procedure:

- While filtering the fermentation media, the fungal mass was weighed. That was the wet mass.
- The fungal mass was placed in a hot air oven at 60 degrees Celsius for 12 to 16 hours.
- That was then recovered and the dry mass was taken.

14. Crude Enzyme:

The liquid fermentation broth left behind after the filtration of microbial growth are collected and considered as Crude Enzyme. In order to purify it, the crude enzyme was centrifuged at 1000 rpm for 5 min. After centrifugation supernatant part was collected and pellet part which contain cell debris were discarded and further assays were performed on the resulting solution in order to detect the activity of the enzyme.

15. Enzyme Assay:

Enzyme assays are laboratory techniques that measure enzyme activity within a sample. They are used for a variety of purposes, which include identifying the presence of an enzyme, study of enzyme kinetics and enzyme inhibition.

15.1 Starch assay of starch hydrolysing enzyme:

This includes the use of citrate buffer and starch solution in order to modulate the activity of the produced starch hydrolysing enzyme. The amylase acts on the starch solution under the suitable conditions that are maintained by the citrate buffer. The resultant activity can be measured by taking the optical density at 540 nanometers.

Procedure:

- Starch solution was prepared by adding 0.25 grams of starch to 25 millilitres of

distilled water and was allowed to dissolve evenly by placing on a hot plate.

- Citrate buffer of normality 0.1 was prepared by adding 1.34 grams of Sodium Citrate and 1.095 grams of Citric acid in 100 millilitres distilled water. The pH must be maintained at 4.5.



Figure 4: Freshly prepared Citrate buffer and Starch solution.

- The enzyme assay was performed by adding 2 millilitres of the prepared starch solution into two cuvettes in which one acted as control or blank and the other as sample.
- Then 200 microlitres of the prepared Citrate buffer was added to each cuvette.
- In the blank, 200 microlitres of distilled water was added.
- In the other cuvette, which was for sample enzyme from fermentation media was added.
- The optical densities were measured at 540 nanometres.

15.2. Assay of Enzyme activity by using DNS method:

Enzyme activity is the process used to determine activity for enzyme by DNS method. In this method 3,5 Di-nitro salicylic acid is reduced to 3-amino 5-nitro salicylic acid and this is done in the presence of reducing sugars such as maltose. The activity of amylase on starch can be measured using this method by checking the absorbance of the solution at 540 nanometres.

Preparation of required reagents for DNS:

- Potassium sodium tartrate: Dissolve 9g of Potassium sodium tartrate in 15ml distilled water.
- NaOH Solution: Take 1.6g of NaOH pellets and properly dissolve in 200ml distilled water.
- 3,5-DNS solution: Dissolve 0.3g of DNS reagent in 6ml distilled water.

DNS Reagent:

- Prepare fresh racemic solution by mixing reagents Potassium sodium tartrate and 3,5-DNS and make up the volume up to 150ml NaOH.

Preparation of Standard Sugar solution:

- Stock standard sugar solution: 250g of Glucose in 100ml of distilled water.
- Working standard solution: Take 10ml from stock and make volume up to 100ml.



Figure 5: DNS Reagent and Working Glucose

Procedure:

Preparation of Standard Solution:

- To a test tube 500 microlitres of distilled water was added.
- Then 500 microlitres of working glucose solution was added.
- This was then incubated at 25 degrees Celsius for 3 minutes.
- DNS reagent of 1 millilitre was added to the test tube.

- This was then placed in boiling water bath for 5 minutes.
- Then 10 millilitres of distilled water were added to the test tube.

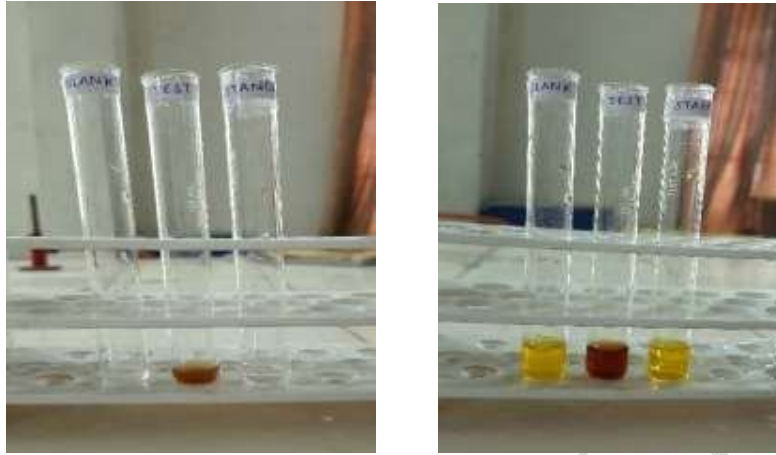


Figure 6: Blank, Test and Standard test tubes after incubation and addition of DNS reagent

Preparation of Blank Solution:

- To a test tube 500 microlitres of starch solution was added.
- Then 500 microlitres of working glucose solution was added.
- This was then incubated at 25 degrees Celsius for 3 minutes.
- DNS reagent of 1 millilitre was added to the test tube.
- This was then placed in boiling water bath for 5 minutes.
- Then 10 millilitres of distilled water were added to the test tube.



Figure 7: Blank, Test and Standard test tubes placed in boiling water bath and after that.

Preparation of Test Solution:

- To a test tube 500 microlitres of starch solution was added.
- Then 500 microlitres of working glucose solution was added.
- This was then incubated at 25 degrees Celsius for 3 minutes.
- DNS reagent of 1 millilitre was added to the test tube.
- This was placed in boiling water bath for 5 minutes.
- Then 10 millilitres of distilled water were added to the test tube.

16. Purification and Downstream Processing of Enzyme:

16.1 Protein precipitation using Ammonium Sulfate:

The addition of ammonium sulphate results in the salting in and salting out of the solution. Initially the protein is suspended in the solution. On addition of ammonium sulfate the protein solubility increases at first due to the addition of the salt. This is the salting in process. However, after certain concentrations of the added salt the salting out begins to occur where the proteins precipitate as the solubility of the salt increases in the solvent. The precipitated proteins can then be extracted by centrifugation.

Preparation of Tris buffer: Take 2.428g of Tris and add into 100 ml of distilled water. Procedure:

- 25 millilitres of the centrifuged enzyme were taken in a beaker.
- This was placed on a magnetic stirrer and the temperature was maintained at 4°C.



Figure 8: Centrifuged enzyme on magnetic stirrer and Ammonium sulfate added

- To this 18g of Ammonium sulfate was added gradually pinch by pinch and was mixed thoroughly.
- The solution was then centrifuged at 10000 rpm for 10 minutes.
- The supernatant was removed and about 500 microlitres of Tris buffer is added to it.
- This was thoroughly mixed with the pellets and was stored for further purification at 4°C.

16.2. Dialysis of Starch Hydrolysing enzyme:

Dialysis process works on the principle of diffusion and size exclusion. It is a process by which a membrane is used to allow the retention of the enzyme and then allow further diffusion of the buffer outside. This is based on the principle of diffusion in which

the enzyme remains inside the dialysis bag on the basis of the concentration gradient. This helps isolate the pure enzyme.

Procedure:

- A dialysis bag was taken and dipped in SDS solution 3-4 times.
- Then it was dipped in a boiling water bath at 100 degrees Celsius around 5-6 times.
- After this, a knot was tied at one end of the bag.
- The enzyme pellets with buffer were placed inside the bag.
- The bag was then knotted at the other end.



Figure 9: Conical flask containing Tris buffer and dialysis bag with enzyme inside.

- In a conical flask containing Tris buffer, the dialysis bag containing enzyme was dipped and maintained on a magnetic stirrer at 4 degrees Celsius for 48 hours.
- Thus, the pure enzyme was retained inside the bag while the buffer was diffused out.



Figure10: Undergoing dialysis process

The proper diffusion process took within 4-5 days and then, pure enzyme was transferred from the dialysis bag into a sterile condition in a beaker.

RESULTS AND DISCUSSION

1. Sample Collection:

Sample collected from local markets of Mohaan, Kakori, and Krishna Nagar, Lucknow.



Figure 11: Fruit peels washed and cut into small pieces.

2. Sample Preparation:

After collection, sample was chopped into small pieces, thoroughly washed, air dried and oven dried.



Figure 12: Sample being oven-dried

Then, after drying the sample was grinded to make fine powder as shown below



Figure13: Mixed Fruit Peel Powder

3. Raw material preparation:

The prepared broth was used further for the fermentation process by soaking fruit peel powder in double distilled water.



Figure14: Broth prepared for fermentation

4. Isolation of fungus (*Aspergillus niger*):

Fungus was isolated from an onion bulb containing blackgram mould disease was collected from a local vendor in Banthara, Lucknow.



Figure15: Onionbulbwithblackmouldisease

5. MicrobialCulture:

For the growth of fungus, a swab has been taken from the diseased onion and grown onPDAMediaasshownbelow.

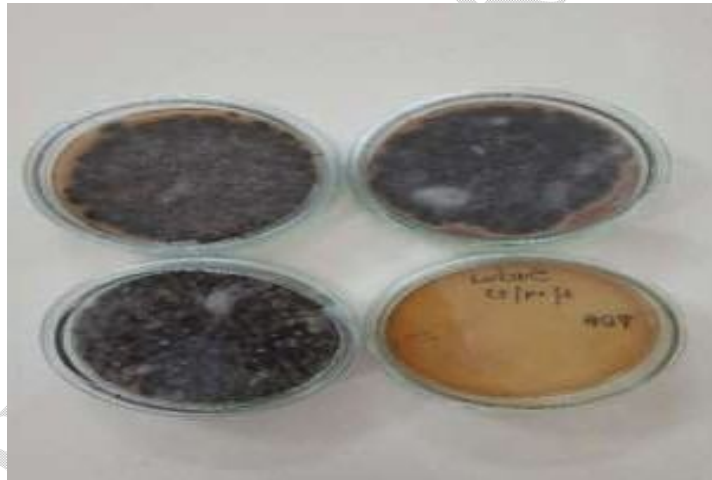


Figure 16: Plates containing solidified PDA media and fungus growth using the spreadplatemethod

6. Sub-culturingforfungalgrowth:

This is done to obtain a pure culture that will ensure that a single strain of the fungalorganism (*Aspergillus niger*)hasbeen isolated.



Figure17: Pure culture of fungus

7. Detection of isolated species (*Aspergillus niger*):

The staining technique has been used by using lactophenol blue dye for identification and *Aspergillus niger* fungus was identified as shown below.



Figure18: Stained *Aspergillus niger*

8. Suspension of the fungal Cells:

A suspension is formed using the Tween 80 solution to count the spores using a hemacytometer.



Figure19: Tween80 solution containing fungal spores

9. Counting of the fungal spores:

A hemacytometer is a suitably divided chamber that can be used to count the spores that are present in the solution.

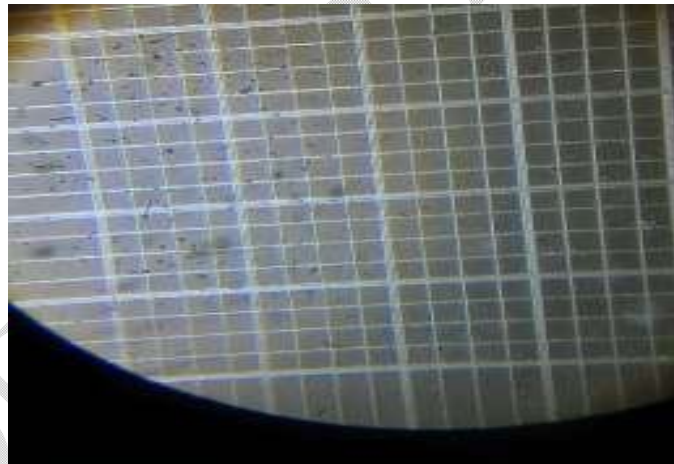


Figure20: Spores visualized on the haemocytometer using a microscope.

Result: The average number of spores estimated was: 2980 spores per millilitre

10. Establishment of suitable dilution for fermentation:

To perform fermentation for a desired volume, a suitable volume of inoculum solution is added. To create this suspension Tween 80 is utilized. According to the Clinical and Laboratory Standards Institute, an absorbance of 0.08 to 0.1 is said to be suitable for inoculation.

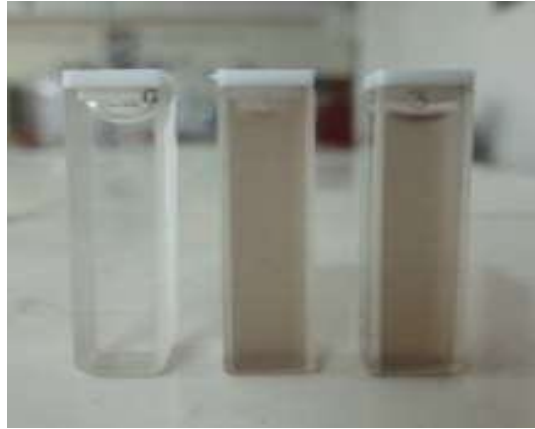


Figure21: Cuvette contain blank sample and Tween 80 suspension

| Microliters of Tween 80 suspension | Optical density at 530nm |
|------------------------------------|--------------------------|
| Blank | 0 |
| 300 | 0.079 |
| 500 | 0.164 |

Table1: Demonstrating the optical density of dilution used for fermentation

Result: Therefore, the dilution of 300 microlitres was selected for fermentation.

11. Fermentation process:

Inoculum was added in a conical flask containing fruit peel waste broth and kept for fermentation process for 5-6 days.



Figure22: Broth kept for fermentation in BOD incubator shaker

The optical density is measured at regular intervals to check the growth of the microbes. The optical density of the media was taken at 540 nanometres.



Figure 23: Cuvette containing fermentation broth

Results:

| Number of Days | Optical Density of fermentation |
|----------------|---------------------------------|
| Day 1 | 0.349 |
| Day 3 | 1.354 |
| Day 5 | 0.561 |

Table 2: Demonstrating the optical density of fermentation media

Therefore, the optical density was greater in the first two measurements but later decreased as the nutrients from the media began to deplete by Day 5.

12. Microbial Count:

The microbial count is performed on fermentation broth to estimate the amount of *Aspergillus niger* grown.

While filtering the fermentation media, the fungal mass was weighed which was wet mass.



Figure24:Wetmass

Results:

| Wetmass(g) | Drymass(g) |
|------------|------------|
| 3.34 | 0.69 |

Table3: Demonstrating the weight of fungal mass.

The microbial count is measured by subtracting the dry mass from the wet mass which is **2.65**. This can be used to quantify the number of cells generated.

13. Crude Enzyme:

The liquid fermentation broth left behind after the filtration of microbial growth was collected and considered a Crude Enzyme.



Figure25:Crudeenzyme

14. Starch assay of starch hydrolysing enzyme:

This includes the use of citrate buffer and starch solution to modulate the activity of the produced starch hydrolysing enzyme.



Figure 26: Freshly prepared Citrate buffer and Starch solution.

The resultant activity was measured by taking the optical density at 540 nanometers.

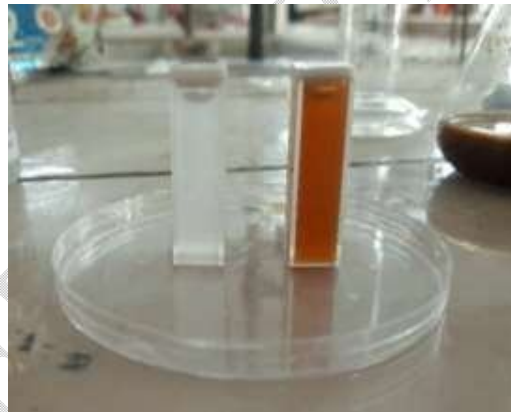


Figure 27: Cuvette containing blank and sample solutions

Results:

| Solution | Optical density at 540 nanometres |
|----------|-----------------------------------|
| Blank | 0.508 |
| Sample | 1.868 |

Table 4: Demonstrating Starch assay of crude enzyme

The increase in optical density of the cuvette containing enzyme shows the effect of the amylase enzyme on the starch solution. Hence, the absorbance

eis greater.

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15. Preparation of Stock Solution:

- 1.34g sodium citrate and 1.095g citric acid added in 100ml distilled water.
- 0.25g starch added in 25ml distilled water.
- Crude enzyme.
- 1.6g NaOH pellets added in 200ml distilled water.
- 25g glucose added in 100ml distilled water.
- Potassium sodium tartrate, NaOH solution, and 3,5-DNS solution added together to form a DNS reagent.



Figure 28: Stock Solution

16. Assay of Enzyme activity by using the DNS method:

Enzyme activity is the process used to determine the activity of enzymes by the DNS method.

The activity of amylase on starch can be measured using this method by checking the absorbance of the solution at 540 nanometres.

For this, three test tubes of blank, test, and standard were made respectively by different reagents and the optical densities were measured.



Figure29: Blank, Test, and Standard test tubes after addition of 10ml distilled water

After that all the test tubes were kept at room temperature and the optical densities were measured according to their mentioned name.

Results:

| Solution | Optical density at 540 nanometres |
|-----------------|--|
| Blank | 0.588 |
| Standard | 0.413 |
| Test | 0.330 |

Table5: Demonstrating Optical densities of different test tubes

Thus, the decreased absorbance shows the activity of the enzyme on the starch solution.

17. Purification and Downstream Processing of Enzyme:



Figure30:Pureenzyme

CONCLUSION

Based on the performed experiment, we can conclude that amylase can be produced from fruit peel waste as substrate by using submerged fermentation which was carried out for 5-6 days in a B.O.D incubator shaker machine at 37°C.

Enzyme activity was performed to know the activity of the enzyme by using the DNS method. After performing the enzyme activity by using extracted crude enzyme solution the obtained O.D. of amylase enzyme-0.504.

This project was carried out on a laboratory scale. If we want to enhance the production of these enzymes, we have to carry out all the processes at an industrial scale. As the population of the world is rapidly increasing and approaching the exhaustion of various natural resources, the production of enzymes by using mixed fruit peel offers great potential for many industries to help meet the challenges (30–32).

ETHICAL STATEMENT

Uphold the highest standards of personal behaviour, being truthful in all of our interactions and activities on the job. Be sincere in both our words and deeds.

CONFLICT OF INTEREST

The authors attest that they are free of any known financial or personal conflicts of interest that would taint the findings of this study.

INFORMED CONSENT

Using websites, review articles, and other sources to produce research content.

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