Isolation and Characterization of Bioactive Compound from Endophytic Fungus of Spoiled Fruits

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Abstract

Fungi are major source of fruit spoilage all over the world, as they can produce spores which can tolerate environmental stress and one of the major problems is the improper food storage methods. These fungi have ability to digest starch and protein by production of amylase and protease enzymes. Amylase and Protease have high industrial application in food, detergent, textile, paper, pharmaceutical and beverages industries. In this study, we isolated fungal species from spoiled pineapple and cultured it in suitable media and for enzymatic screening we screened it with media containing starch and protein and inoculated those in media for extraction of the enzymes and their activities were checked. The major challenge is to maintain all the parameters like proper media to get fungal biomass; pH and Temperature was maintained for efficient production of enzymes and its extraction. The main objective of the current study is to find a good alternative, to produce enzymes from fungal species which could be grown and cultured at a very low cost and meet the need for the enzymes for the industries.

Keywords: α-Amylase, Protease, LPCB, Endophytic fungi, Blood stain, Activity, glycine-NaOH buffer

Key Messages: This study is based on the concept of BEST from WASTE. We all know the enzyme extraction cost from fruits and the amount of extraction is less. By this study, we are trying to say that the spoiled fruits or its peels could be used by the inhabited fungus grew on them inherit the same property for the production of enzymes found in the fruit. These fungus can be exploited to meet our benefits.

Introduction

α-Amylases enzymes (E.C. 3.2.1.1) are commonly used in starch processing industries, which frequently use amylases to hydrolysis of complex polysaccharides such as starch into simple sugar which is used in food and detergent industries[1]. Amylases are of universal event, delivered by plants, creatures, and microorganisms. In any case, microbial sources are the most favored one for enormous scale creation. Today an enormous number of microbial α-amylases are promoted with applications in various modern areas. This survey centers on around the microbial amylases and their application with a biotechnological point of view [2]. Currently, amylase production was reached above 65%, hence resembling its great usage [3].

Protease is a hydrolizing catalyst that hydrolyze the peptide obligations of proteins into peptides and amino acids. Proteolytic compounds have potential applications in a wide number of modern procedures, for example, nourishment, clothing, cleanser, and pharmaceutical. Proteases from microbial sources have commanded applications in mechanical divisions [4]. Microbial proteases are among the most critical hydrolytic impetuses or catalysts and have been considered extensively. Proteases from microorganisms have pulled in a ton of thought in the latest decade because of their biotechnology potential in various mechanical techniques, for example, cleanser, material, cowhide, dairy, and pharmaceutical arrangements [5]. These extracellular enzymes can be prepared by culturing superior fungal strains by specific cultural conditions and parameters [6].

According to many studies, Aspergillus spp. are mostly found in spoiled pineapples, However, pineapple contains high levels of sugars and other nutrients and due to their low pH values make them desirable to fungi decay. Fungi can survive on pineapple because of the nutrients present such as proteins, fats, and carbohydrates that support the growth of pathogens [7]. Aspergillus spp. produce amylase which breaks down starch, present in the starch casein agar [8], and these species also produce protease enzyme which digests the proteins in Skim Milk Agar and form clear zones [9]. Only a few microorganisms including *Aspergillus* species have been reported to possess the ability to produce raw starch degrading amylase [10 Molds of the genera *Aspergillus*, *Penicillium*, and *Rhizopus* are especially significant for conveying proteases, as a couple of sorts of these genera are overall seen as secured [11]. *Aspergillus clavatus* ES1 has been as of late distinguished as a maker of an extracellular dying stable antacid protease [12]. In our work, we isolated the fungi from spoiled fruits and screen for its enzymatic activity. The purpose of the work is to find a good alternative for the production of a microbial enzyme like amylase and protease which are commercially important so that it can be economically beneficial.

Materials and methods

Collection of fruit sample.

Pineapple was collected from the local market of Vellore, Tamil Nadu, India, and kept for 5-7 days for proper spoilage.

Isolation of fungi

The spoiled part of the pineapple was taken and crushed properly by using mortar pestle. The juice of spoiled fruits was extracted by using cheesecloth.

The extract then serially diluted up to dilution 10^{-6}. The dilution of 10^-3, 10^-4, and 10^-5 was plated in czapek dox agar media by spread plate method. The plates were kept in an incubator at 27°C for 3-5 days. Pure culture technique was performed to isolate particular fungal colonies by using czapek dox agar plate, which was again incubated for 3-5 days.

Microscopic characterization

Microscopic examination by staining with Lacto Phenol Cotton Blue staining.

Screening for amylase and protease

For amylase screening, the pure culture was streaked on starch casein agar and for protease screening, the pure culture was streaked on Skim Milk Agar. The plates were kept for 3 days incubation at 27°C.

Isolation of amylase and protease enzyme

The pure culture was inoculated in the Czapek dox broth and kept in a shaker for 3-5 days. The broth was filtered by Whatman filter paper and the filtrate was stored for 2-3 weeks at low temperature.

Preparation of glycine-NaOH buffer (pH 9)

0.05 M glycine-NaOH buffer was prepared by adding 0.38 gm of glycine and 0.04 gm of NaOH. The pH was adjusted by adding 0.1 N HCL.

Preparation of 0.5% casein solution

Then 50ml of 0.5% casein solution was prepared by adding 0.25 gm of casein in 50ml of 0.05 M glycine-NaOH buffer solution, which is incubated for 30 minutes, at 45°C.

Test for amylolytic Activity

The fungal plates were flooded with gram's iodine solution (0.01 M I2-KI solutions), The amylolytic strain starts hydrolyzing the starch present in starch casein agar and observed for zone of degradation of starch which is revealed

as a clear and transparent zone. Among various colonies, the colony that exhibited the highest degradation of starch was selected for pure culture.

Identification of the isolated strain (LPCB)

Fungi was identified by observing them at 40X magnification of a light microscope after staining them with a dye such as lactophenol cotton blue (LPCB). A small portion of the fungal mat is taken on the slide and stabbed and stained with few drops of lctophenol cotton blue for 2 - 3 minutes. The excess stain is blotted out with the help of a tissue paper and a coverslip is placed over it. The slide is then observed under a microscope at 40X. [13]

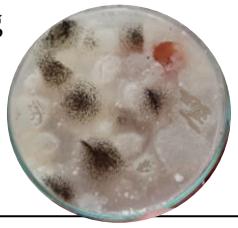


Figure1- Isolation of fungal species from spoiled pineapple

Results and Discussion

Spoiled fruits are the major source of endophytic fungi, which produce a wide range of bio-active compounds like enzymes. The extract of spoiled fruits, collected was serially diluted and plated on Czapek dox agar and incubated at 37°C for 72 hours which revealed the presence of fungi(Figure1). It was also observed that the colonies were blackish mold like structure. The isolated fungal colony was later subcultured in Starch Casein Agar. When Gram's iodine reagent was added, the colonies exhibited clear and white transparent zone around the colonies, indicating their nature of amylolysis due to starch hydrolysis(Figure5). The isolates also form clear zones in Skim Milk Agar resembling their protease activity. A fungal genus was identified by adding lactophenol blue mounting(Figure3-4).

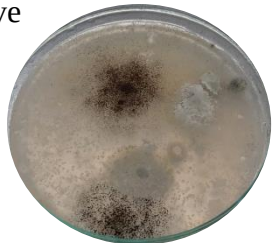


Figure2- Pure culture of fungus *Aspergillus niger*

Isolation of fungi

Two different fungal isolates were identified based on color morphology, these were obtained after spreading and were found to be black and white isolates. All the two isolates were subcultured in czapek dox agar and were used for further studies. Microscopic identification of mold like structure, sporangia, hyphae, mycelium resembles the fungal genus is *Aspergillus* (Figure3-4). According to many study *Aspergillus niger* is an endophytic fungi, mostly found in citrus fruits, as we had used spoiled pineapple we consider the species of *Aspergillus* could be *niger*.

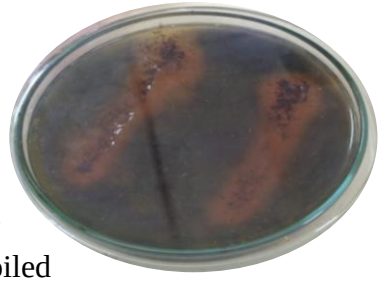


Figure5- Screening of amylase activity by using Grams iodine

Screening of Fungal Isolates for Alpha-Amylase Production

All the two fungal isolates are subjected to screening procedure and after completion of incubation period plates were flooded with iodine solution and observed for the zone of hydrolysis. The black colony showed the zone of hydrolysis visible after flooding with Gram's iodine solution (Figure5).

Screening of fungal isolates for protease production

All the fungal isolates were cultured on Skim milk agar, to see the clear zones around colonies due to the hydrolysis of proteins, hence confirmed the production of protease.

Figure 7- Cell free extract isolated from pure culture of fungi by centrifugation

Fungal staining by Lacto-phenol cotton blue

A small portion of the fungal mat is taken on the slide and stained with few drops of lactophenol cotton blue for 2 – 3 minutes. The excess stain is blotted out with the help of a tissue paper and a coverslip is placed over it. In the present investigation, a pure strain of fungi was isolated from the spoiled fruits. The fungal isolate was identified as *Aspergillums niger* (Figure3-4).

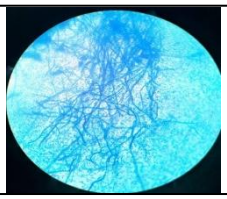


Figure3- Mycelia of fungus isolated from spoiled pineapple under microscope (40X)

Isolation of protease enzyme. (Figure 6)

Pure culture isolates were again cultured as enrichment media of cape do broth, and kept in shake for 3 to 5 days. The cultured broth was filtered with what man's filter paper, the extract was stored at a lower temperature. 1ml of casein solution and 0.5 ml of culture supernatant were added in a centrifugation tube and centrifuged at 4°C at 8000rpm for 15 minutes and the culture supernatant were collected which is the cell-free extract (Figure 6). The supernatant was incubated at 45° for 30 minutes. The reaction was terminated by adding 10% TCA solution, 50ml of 10% TCA into 50 ml of distilled water. The suspension then again centrifuged at 10000 rpm for 10 minutes for 4°C. The supernatant again collected and absorbance were measured at 660nm wavelength [14]

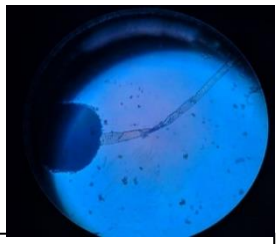


Figure4- Sporangium of fungus isolated from spoiled pineapple under microscope (40X)

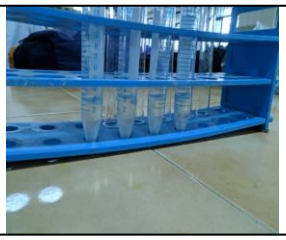


Figure 6- protease assay of enzyme isolated from fungus *Aspergillus niger*

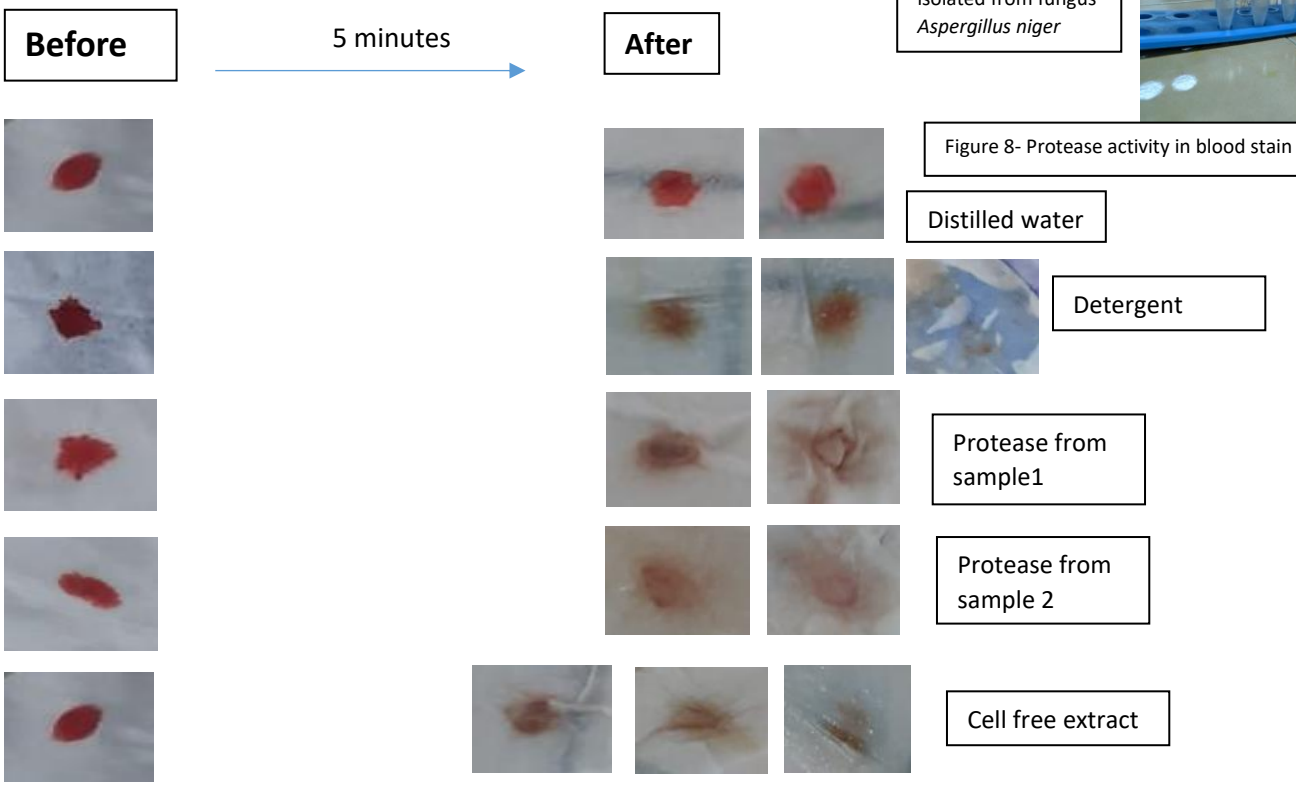


Figure 8- Protease activity in blood stain dissolving test

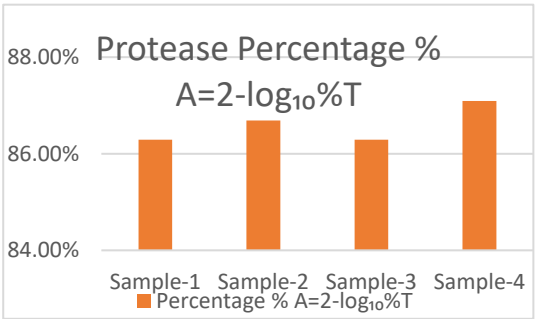
As in this application activity test, the activity of blood stain dissolution by protease isolated and cell free extract with other enzymes including protease is comparable with the standard with is detergent.

Isolation of amylase enzyme

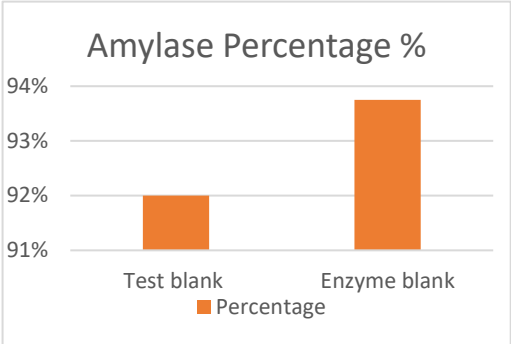
Amylase isolation from the selected strains of isolates was transmitted at 37°C for 24 h in 50 ml of 8% (w/v) of starch medium located in 250 ml flasks and placed in a shaker incubator operated at 120 rpm at 30°C. The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 minutes, the supernatant acquired was collected and used as an enzyme source [15].

Enzyme assay: Both amylase and protease activity are observed by DNSA method.

Sample	Absorbance	Percentage % A=2-log₁₀%T
Sample-1	0.064	86.29%
Sample-2	0.062	86.69%
Sample-3	0.064	86.29%
Sample-4	0.060	87.09%



Test	Absorbance	Percentage
Test blank	0.38	92%
Enzyme blank	0.28	93.75%



DISCUSSION

According to our study the enzymes which are produced by our isolates are amylase and protease. We targeted these two enzymes because these two enzymes are used widely in various industries such as food, beverages, textile, detergent, pharmaceuticals and so on [28, 29]. Generally identification of the Aspergillus species is based on the morphological characteristics of the mycelia and microscopic examinations [37]. Based on the results of cell morphology observers, these isolates belong the group of Aspergillus spp. because they have similar characteristics like globular vesicles, conidiophores, semi conidia spore which are round-shaped and the colour varies from light green to brownish green [38]. In the year 1831, a scientist named Erhard Friedrich Leuchs explained the hydrolysis of starch by saliva, because of the presence of an enzyme in saliva, "ptyalin"(old name), an amylase [17, 18]. It was named after the Ancient Greek name for saliva that is sialon. AS of now three different types of amylases are known to be exists, those are α-amylase, β- amylase and γ-Amylase.α- amylase can act on any region of substrate structure, α-amylase tends to be faster-acting than β-amylase. In animals amylases are the major digestive enzyme, and its optimum pH is 6.7–7.0 [19]. Amylases group is one of the most important enzymes used in the industries for biotechnology constituting a class of industrial enzymes having nearly 25% of the world enzyme market [20, 21]. The amylases produced from microorganisms have a very wide spectrum of industrial applications. Microbial amylases are more stable than the amylases produced from animals and plants [22]. α-Amylase has been produced by several fungal species, yeasts and bacteria. But, enzymes produced by fungal species and bacterial species have maximum applications in various industries [23]. α-Amylase is able to cleave α-1,4 glycosidic bonds present in the inner part of the amylose or amylopectin chain [24,25,26,27]. α-amylase can be produced from limited to a few species of mesophilic fungi, and attempts have been made to specify the culture conditions and to know which strains of the fungus can produce these enzymes on a commercial scale [23].Fungal sources are limited to fungus present in terrestrial environment, mostly to Aspergillus and Penicillium [30].The Aspergillus species can produce a large variety of extracellular enzymes, and amylases and proteases are the ones with most significant industrial importance [31]. *Aspergillus niger* has important capacities in the production of α-amylase and, it allows the avoidance of bacterial contamination due to its tolerance of acidity (pH < 3) [32].The fungal α-amylases and protease are more suitable than other microbial sources for production of enzymes because of their more accepted GRAS (Generally Recognized As Safe) status [23].During the screening process of amylases and proteases clear zone is formed around the colonies this is due to the iodine solution and the congo red used are unable to bind efficiently with monosaccharide, disaccharide and peptides respectively. IN 1825 pepsin, which is an aspartic protease which is present in the stomach, was one of the first enzymes discovered, characterized, and named. Pepsin was crystallized in 1930 Pepsin [33]. Particularly detail study of Aspergillus species have been done because they have the capacity to produce numerous amounts of enzymes. Many of the secreted enzymes can be produce in a large-scale submerged fermentation, which are widely used in the food and beverage industry for years [34].The protease enzyme constitutes two thirds of total enzymes used in various industries and this amount in the market is expected to increase in the coming years [35]. On the other hand, fungi are normally known as safe strains to produce extracellular enzymes, which are easier to harvest from fermentation broth [36].When we calculated in terms of percentage our isolate produce more than 85% of both the enzymes. For proteases 1% BSA was used and also a small assay in which distilled water was used and the negative control and the detergent solution was used as the positive control the results were compared with both the positive and negative controls and for amylases DNSA method was used determine the enzyme activity.In conclusion microorganisms especially fungi present in the spoiled fruits and vegetables like *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus* and Penicillum spp. can produce enzymes which are industrially and biotechnologically important.

Conclusion

This study could provide a good alternative in the production of enzymes, which could be used on a large scale. We isolated fungal species (*Aspergillus species*) from spoiled pineapple, and cultured in Czapek dox agar medium. We used different types of media but the best results were obtained from Czapek dox agar. The pure culture was done by using the same media to get black and white sporangia and then the loop of culture taken to screen for amylase and protease activity with the help of starch casein media and skim milk agar respectively. These enzymes were isolated from endophytic fungus like *Aspergillus niger*, which forms black mold like structure. Endophytic fungus is a big source of production of bioactive compounds. The endophytic fungus could be used in the production of many anti-oxidants, anti-cancerous compounds, as the cheapest alternative.

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