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Mixed-Culture Fungal Fermentation for Protease and Amylase Production from Adansonia Digitata Seed Through Solid State Technique

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Abstract: Production of amylase from the seed of A. digitata by mixed-culture fungal fermentation was examined. Six fungi were isolated from naturally fermented A. digitata seed in the laboratory and identified as Mucor racemosus, Mucor hiemalis, Penicillium citrinum, Penicillium chrysogenum, Alternaria tenuis and Rhizopus stolonifer. Spores obtained from the fungi were randomly mixed for protease and amylase production using solid-state technique and the enzyme activity was monitor for 120 hours incubation period. The highest protease and amylase activity was obtained at 72 hours (100.8 and 53.60 µmol/mg respectively) by a mixed-culture of P. citrinum, R. stolonifera, A. tenuis and M. racemosus. While the lowest activity (22.2 and 4.0 Umol/mg) was obtained at 120 and 24 hours of fermentation by a mixed-culture of A. tenuis, M. racemosus, M. hiemalis and M. racemosus respectively. The optimum pH for amylase production was pH 5.5 at 28 ± 2 oC. This study revealed that mixed-culture of these fungi especially A. tenuis and M. racemosus were able to produce amylase, hence may be considered as a source for the production of industrial amylase from A. ditata seed.

Keywords: amylase, mixed-culture, enzyme activity, A. digitata, fermentation.

Introduction

Mixed-culture fermentation refers to a process in which the inoculums always consist of two or more organisms [1]. It may also be a case of fermentation under natural or uncontrolled environment. The cultures can consist of known species to the exclusion of all others, or they may be composed of mixtures of unknown species. Mixed-cultures may be of one microbial

group that is, consists of only bacteria or fungi, and sometimes they may consist of a mixture of fungi and bacteria or fungi and yeasts or other combinations in which the components are quite unrelated. Many important biochemical processes are usually accomplished by co-culturing two or more microorganisms for better performance [2]. Among microorganisms, various types of synergistic effects have been noticed. Compared to pure culture fermentation, mixed culture fermentation has some absolute advantages and some new substances are usually produced with various desirable characteristics [3, 4, 5]. The application of pure cultures dominates in biotechnological processes but through the application of mixed cultures their combined metabolism results in considerable higher yield of ethanol, enzymes, acetic acid and enrichment of the vitamin content of fermented foods [2]. Special attention has been given to the mixed cultures used for increasing biological enzyme production, such as amylase, inulinase, xylanase, endoglucanase and β-glucosidase [1]. Solid-state fermentation involves the cultivation of microorganisms on a solid substrate, such as grains, rice and wheat bran. This method is an alternative to the production of enzymes in liquid by submerged fermentation. SSF has many advantages over submerged fermentation. These include, high volumetric productivity, relatively high concentration of product, less effluent generated and simple fermentation equipment. Fermentation with microorganisms, like bacteria, yeast and moulds is used to produce enzymes [6, 7]. Filamentous group of fungi constitutes a storehouse of economically important enzymes, alcohols, organic acids, and pharmaceuticals. Enzymes like amylases, glucoamylases, and invertase used in the biodegradation of starch, cellulase, active degrader of cellulose, are produced from various species of moulds and bacteria respectively [8]. Amylases and proteases are the major enzymes involve in biodegradation of plants and other organic molecules. Fungi secrete the enzymes to break down complex organic molecules into simpler ones, which they can easily absorb during metabolism.

Amylases are groups of enzymes that has been defined as a group of hydrolases that can specifically cleave the O-glycosidic bonds in starch. The important groups of amylases are glucoamylase and α-amylase. The alpha-amylases can be derived from various sources such as plants, animals and microorganisms due to the wide distribution in nature [9, 10]. However, fungal and bacterial amylases have predominant applications in the industrial sector. Major advantage of using fungi for the production of amylases is the economical bulk production capacity and ease of manipulation. Many species of Aspergillus and Rhizopus are used as a source of fungal α-amylase [10, 11].

Protease is a very important industrial enzyme whose production is estimated to be approximately about 60% of the total world-wide enzyme production [12]. They occupy key position with respect to their applications in both physiological and commercial products [8]. Fungal proteases have an advantage over bacterial protease as mycelium can be easily removed by filtration. Their areas of applications include industries such as detergent, food, pharmaceuticals and de-hairing of leather, tanning, dairy, baking, surgical cleaning, brewing and medical applications [13, 14, 15].

Adansonia digitata, (Linn) the baobab tree is a member of the Bombacaceae family. This deciduous tree was originally located in South Africa, Botswana, Namibia, Mozambique and Zimbabwe [16], but can be found in most countries within the African continent. African A. digitata is a

very long-lived tree with local multipurpose uses [17]. Currently, the seed has no industrial importance and it is grossly underutilized. This research sought to produce protease and amylase from the seed through mixed-culture fungal fermentation as previous effort on enzyme production has always been concentrated on mono-culture fermentation technique.

Materials and Methods

Collection and authentication of plant materials:

Adansonia digitata (Baobab) seeds were collected from the premises of University of Ilorin, Ilorin, Kwara State and were authenticated at the Herbarium unit of the Department of Plant Biology, University of Ilorin Adansonia digitata (UIH 1048).

Preparation of seed

A. digitata pods were cracked open manually and washed with clean water to remove the pulp from the seeds. They were dried at 60 0C in the oven for four hours to remove the wetness. The seeds were pulverized with an electrical grinder to rough particle sizes of about 01 to 0.01 mm in diameter and stored in air tight container for further uses.

Determination of pH of seed

The pH of the seed was determined by using a Crison micro pH meter (Model 2000). Ten grams of the seed was mashed separately into a 100 ml beaker; 40 ml of distilled water (pH 7.0) was added and the mixture was left undisturbed for 30 minutess followed by occasional stirring with a glass rod before the pH of the seeds was measured [18].

Determination of the Water Holding Capacity (WHC) of the seed

The water holding capacity of the seed was determined according to the method of [19]. This was done to determine the quantity of water required for SSF of the seed.

Isolation of organisms

Fungi were isolated from the naturally fermented naturally fermented seed through serial dilution. Culturing was done by pour plate method using Potato Dextrose Agar (PDA) into which 10% Streptomycin solution has been added to inhibit bacteria growth. The plates were incubated at room temperature (28±2 0C) for 48 hours. Visible fungal colonies were isolated and further sub-cultured to obtain pure cultures. The pure isolates were maintained on agar slant and kept in the refrigerator at 4 0C for further studies [20]; [21]; [22].

Identification of the Isolates

Morphological and microscopical analysis for the identification of the isolates were carried out and compared with literature as described by [23].

Selection of fungal isolates for mixed-culture fermentation

Six of the fungal isolated were used after they were identified as Penicillium chrysogenum, Penicilliuim citrinum, Mucor racemosus, Mucor hiemalis, Rhizopus stolonifer, and Alternaria tenuis.. Each of the organisms was used to prepare spore suspension used as starter cultures for the fermentation of seed mashes before incubation at 28 ±2 0C for 120 hours [21], selected fungi were randomly mixed to obtain six mixed cultures.

Fungal spore preparation

Fungal spore suspension of actively growing mid log phase culture of the fungal isolates were prepared according to the method described by [24]. An agar slant of four day old pure culture of each of the organisms was used. Sterile distilled water (10 ml) was added to the slant and shaken to wash the spores. The spore suspension was counted using the Neubauer counting chamber. A spore suspension of about 5x104 spore suspension/ml was used in each case for inoculation.

Preparation of inoculum

The inoculums used for the study were prepared as highlighted:

CtCh: Penicillium citrinum (5 ml containing about 5 x 104 spore/ml suspension) mixed

with Penicillium chrysogenum (5 ml containing about5 x 104spore/ml suspension);

CtSt: Penicillium citrinum (5 ml containing about 5 x 104 spore/ml suspension) mixed with Rhizopus stolonifer (5 ml containing about 5 x 104 spore/ml suspension);

HeTe: Mucor hiemalis (5 ml containing about 5 x 104spore/ml suspension) mixed with Alternaria teneus (5 ml containing about 5 x 104 spore/ml suspension);

HeRa: Mucor hiemalis (5 ml containing about 5 x 104spore/ml suspension) mixed with Mucor racemosus (5 ml containing about 5 x 104 spore/ml suspension);

StTe: Rhizopus stolonifer (5 ml containing about 5 x 104 spore/ml suspension) mixed with Alternaria teneus (5 ml containing about 5 x 104 spore/ml suspension) and

CtRa: Penicillium citrinum (5 ml containing about 5 x 104 spore/ml suspension) mixed with Mucor racemosus (5 ml containing about 5 x 104 spore/ml suspension) [24].

Fermentation

Twenty grams (20 g) of the seed was measured into 250 ml Erlenmeyer flasks and 20 ml of water was added and sterilized in the autoclave at 121 0C for 15minutes. The sterile samples were mixed and stirred properly until uniform mashes were obtained in each case. Two millilitres (2 ml) of the spore suspension was used as fermentation starter to inoculate each of the samples in the fermentors. The mixtures were allowed to ferment for 120 hours at 28±2 0C [21]; [22].

Enzyme extraction

One gram of the fermented substrate was extracted with 100 ml of distilled water on a rotary shaker at 200 rpm for 30 minutes at 28±2, the mixture was filtered with several (16) layers of cheese cloth and the filterate was used as the crude enzyme extract for the assay.

Protease assay

Protease activity was determined using spectrophotometric method [25]. Enzyme solution (2 ml) was incubated with 2 ml of 1.0% (w/v) casein in phosphate buffer (50mM, pH 7.0) at 37 0C for 30 minutes. The mixture was filtered with Whatman No 1 filter paper. To 2 ml of the filterate was added 2ml of Biurret reagent was added to each tube and mixed thoroughly. (Biurret reagent was prepared by mixing 0.5 ml of 1% cupric sulphate with 0.5 ml of 2% sodium potassium tartrate, followed by the addition of 50 ml of 2% sodium carbonate in 0.1 N NaOH). The mixture was then allowed to incubate at room temperature for 10 minutes prior to the addition of 0.2 ml of 1.0 N Folin-Ciocalteu's reagent. The resulting solution was incubated for 30 min at room temperature to develop the blue colour and the absorbance was read at 650 nm using a tyrosine standard. A separate blank was set up as standard. One unit of enzyme activity was defined as the amount of enzyme required to release one µg of tyrosine per min with Bovine Egg Albumin (BEA) as standard under the assay condition.

Amylase assay

Amylase activity was determined according to [26]; [27]. The reaction mixture contained 2 ml of crude enzyme and 2 ml of 0.1M phosphate buffer (pH 7.0) containing 1% (w/v) of soluble starch. The mixture was incubated at 37 0C for 30 minutes. After 30 minutes of incubation, then filtered, to the filtrate was added 2 ml of DNSA solution containing 1 g of DNSA dissolved in 20 ml of 1 M NaOH, to which 30 g of sodium potassium tartarate and water were added to make it 100 ml. The amount of reducing sugar released into the mixture after enzyme reaction was determined by reading the absorbance at 540 nm using the UV visible spectrophotometer.

Amylase activity was defined as the amount of enzyme that released 1µm of the reducing sugar (glucose) per minute under standard assay condition.

Results

Enzyme activities

The enzyme activities of protease and amylase are presented in Figures 1 and 2 respectively. The peak of activities occurred at the 72 hours of fermentation for both proteases and amylases generally. The maximum protease activity occurred at 72 hours of fermentation as 100 Umol/mg in substrate fermented with mixed-culture of P. citrinum and M. racemosus while the minimum was 22.2 Umol/mg at 120 hours. The highest amylase activity was 53.6 Umol/mg in substrate fermented with mixed-culture of A. tenuis and M. racemosus while the lowest activity was 4.0 Umol/mg at 24 hour. Protease and amylase activity generally reduced after 72 hours of incubation.

pH

The pH range obtained during fermentation was found to fall within 5.4 to 7.6. The highest activities of protease and amylase was observed at pH 7.6 after which the pH began to decrease.

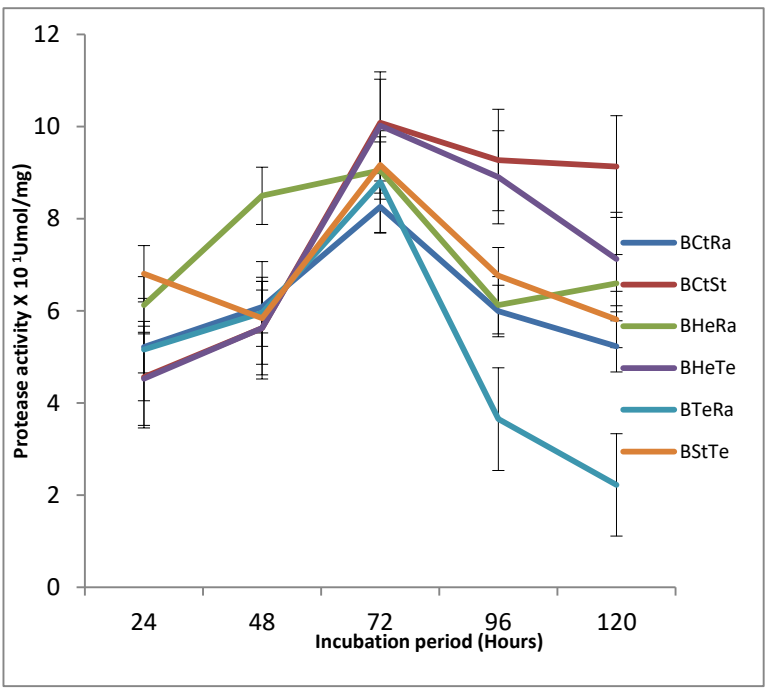

Figure 1: Protease activity during mixed-culture fungal fermentation of the seed of A. digitata Data are mean of two replicates ± SEM.

BCtRa= A. digitata seed fermented with mixed-culture P. citrinum and M. racemosus; BHeTe= A. digitata seed fermented with M. hiemalis and A. tenuis; BHeRa= A. digitata seed fermented with M. hiemalis and M. racemosus; BStTe= A. digitata seed fermented with R. stolonifer and A. tenuis; BCtSt= A. digitata seed fermented with P. citrinum and R. stolonifer; BTeRa= A. digitata fermented with A. tenuis and M. racemosus.

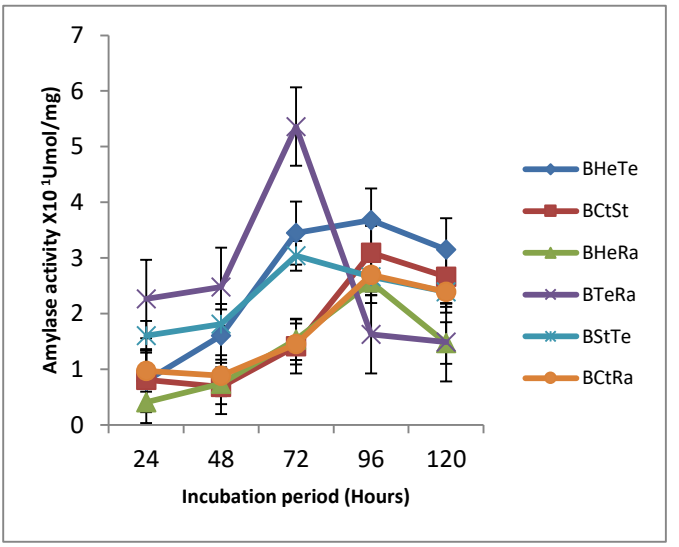

Figure 2: Amylase activity during mixed-culture fungal fermentation of the seed of A. digitata Data are mean of two replicates ± SEM.

BCtRa=A. digitata seed fermented with mixed-culture P. citrinum and M. racemosus; BHeTe= A. digitata seed fermented with M. hiemalis and A. tenuis; BHeRa= A. digitata seed fermented with M. hiemalis and M. racemosus; BStTe= A. digitata seed fermented with R. stolonifer and A. tenuis; BCtSt= A. digitata seed fermented with P. citrinum and R. stolonifer; BTeRa= A. digitata fermented with A.tenuis and M. racemosus.

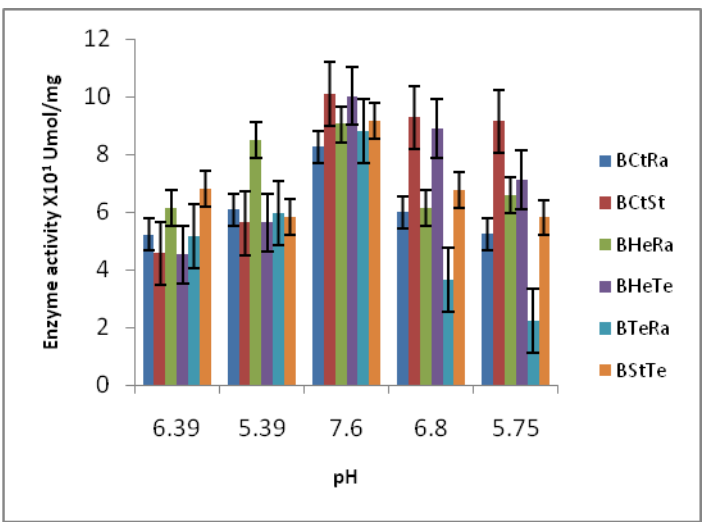

Figure 3: Changes in pH during fermentation
Data are mean of two replicates ± SEM.

BCtRa= A. digitata seed fermented with mixed-culture P. citrinum and M. racemosus; BHeTe= A. digitata seed fermented with M. hiemalis and A. tenuis; BHeRa= A. digitata seed fermented with M. hiemalis and M. racemosus; BStTe= A. digitata seed fermented with R. stolonifer and A. tenuis; BCtSt= A. digitata seed fermented with P. citrinum and R. stolonifer; BTeRa= A. digitata fermented with A.tenuis and M. racemosus.

Discussion

Protease and amylase activity increased steadily during the fermentation of the seed from 24 hours. Activity was found to increase steadily as incubation period increased. The maximum protease activity was recorded in substrate fermented with a mixed-culture of P. citrinum and M. racemosus while the highest amylase activity was recorded in substrate fermented with mixed-culture of A. tenuis and M. racemosus. Other mixed-culture also recorded various amount of enzyme activities. Generally the activities followed a similar trend. The peak of enzyme production was within the late exponential to early stationary phase of growth, beyond which the activity dropped (72 to 96 hours of fermentation). This may be due to depletion in nutrients and accumulation of toxic metabolites/wastes as a result of the increase in the population of the fungi. The incubation time for achieving the maximum enzyme level is governed by the characteristics of the culture and is based on growth rate and enzyme production. Maximum accumulation of amylase has been said to occur during stationary phase and further increase in incubation period beyond this decreased the production of amylase. This might be due to the deficiency of nutrients, accumulation of toxic substances and proteolysis of amylase as suggested by many workers [28]; [29]; [30]. The activities of enzymes achieved in this research were higher than those observed when Aspergillus species (a known enzyme producing organism) were used in pure culture for fermentation [31].

Microbial fermentation of organic compounds is usually accomplished through the enzymatic breakdown of complexes. The metabolic activities of fungi that result in fermentation and its subsequent effect is often through the secretion of enzyme into the substrate upon which they are growing. Fungi are saprophytes which normally release extra-cellular enzymes into their surroundings. The enzymes are usually organic catalysts that aid the breaking down of the organic complexes which are later absorbed by the fungi for growth and synthesis of various products. [31] recorded a similar result during a research carried out to produce enzyme.

The incubation time for achieving maximum enzyme yield has been reported to be governed by the characteristics of the culture and depended on growth rate and enzyme production [32];[33]. The findings in this study indicated that enzyme was secreted early in active growth phase and reached maximum towards the end of exponential growth phase. Time course has been reported to play a very crucial role in fungal metabolic activity and growth. The incubation time necessary for optimal biosynthesis varied between different enzymes produced from one substrate [34]; [24]; [35]; [36].

The decrease in enzyme production after 72 hours of incubation period could also be due to the inactivation of protease by other constituent enzymes. This suggested that the production of amylases and proteases by these microorganisms was fully correlated with their growth.

Enzyme production by microbial strains depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell

growth and fermentation systems product production [37]. In this research, the pH of the medium ranges between weakly acidic to weakly alkaline condition. Maximum activities of amylase and protease were recorded at pH around neutral value (7.0). The range of pH from 5-9 has been reported for maximum amylase and protease activity [24];[37]; [36].

Recommendation and conclusion

In view of potential applications of amylase and protease enzymes and economy of production, attempt could be made to optimize culture parameters for their production from these two seeds.

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