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Comparative analysis on immobilized cells of *Aspergillus oryzae* and *Bacillus cereus* in production of amylase by solid state and submerged fermentation

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ABSTRACT

Alpha amylase (α-amylase) enzyme is used as a thinning agent in the starch hydrolysis and it is widely used in the food processing, paper and textile industries. The intention of the present study was to analyse the α-amylase activity in solid state fermentation and submerged fermentation using immobilized cells of Aspergillus oryzae (A. oryzae) and Bacillus cereus (B. cereus), respectively. The enzyme activity was measured at different time intervals. Substrates used in fermentation were rice bran, paddy husk, raw rice and brown rice. Rice bran was noted to be the best substrate marking an average of 49.4 ppm/g for 120 h in solid state fermentation by A. oryzae followed by raw rice showing 43.5, brown rice 40.2 ppm/g and paddy husk 7.1 ppm/g correspondingly. Similarly, rice bran was found to be a superior substrate giving an average amylase activity of 132.6 ppm/g followed by brown rice with 105.9 ppm/g, raw rice with 98.0 ppm/g and paddy husk with 15.0 ppm/g among all substrates after immobilization of the enzyme. The present findings that amylase production is higher in solid state fermentation by A. oryzae with rice bran as the substrate.

Keywords: Solid state fermentation, Submerged fermentation, Immobilization, *A. oryzae*, *B. cereus*

INTRODUCTION

Amylases, also known as ptyalin, are enzymes of plant, animal and microbial origin which break down starch or glycogen. They are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents [1]. The starch was hydrolyzed by α-amylase into a variety of products such as glucose and maltose or malto-oligosaccharide or mixed malto-oligosaccharide [2]. They are employed in industries like food processing, detergents, textiles, paper etc., for different purpose such as glucose and maltose forming α-amylases which find application in alcohol fermentation, sugar syrup formulation and malto-oligosaccharide [3,4]. The α-amylase enzyme is acting as a thinning agent in starch hydrolysis process and is widely applied in the industries for food processing, paper and textile [5,6].

The major advantage for the production of microbial α-amylase is the economic bulk production capacity and easy to prepare genetic manipulation of microorganisms to obtain the desired enzyme. Many enzyme preparations such as proteases, lipases, xylanases, pullulanases, pentosanases, cellulases, glucose oxidases, lipoxygenases etc. have been alternative to other enzyme substituted but none have α-amylases [7,8].

Inexpensive agriculture and agro-industrial residues represent one of the most energy-rich sources serving as a substrate in solid state fermentation. These residues are the best reservoirs of fixed carbon in nature. Solid substrate supplies not only the nutrients to culture but also serves as an anchorage for microbial cells [9]. There is not much study on the utilization of sugarcane bagasse hydrolysate for the production of α-amylase from bacterial species in

submerged or solid state fermentation [10]. Certain bacterial sp are well known to produce α-amylase from media consisting of agro-residues such as wheat bran, cheese, whey and rice husk in solid state fermentation [11]. Wheat bran is a rich source of carbon and nitrogen thus supplementation of other nitrogen sources in the medium does not show significant increase in enzyme yield [12]. The amount of nitrogen source plays a very critical role in the production of α-amylase. Wheat bran was found to be the best substrate for glucoamylase production by *A. niger* [13].

Agro-residues are generally considered the best substrates for fermentation processes. Agro-residues are degraded by microbial strains may improve the substrate value as animal feed [14]. Amylase production and physiochemical parameter optimization using wheat bran has been extensively studied by submerged fermentation and solid state fermentation [15]. Paddy husk along with other nutrients or rice bran is considered as a support for the fungal growth during glucoamylase production [16]. *Bacillus* spp. are major sources of industrial enzymes and one of the most widely used species for the bulk production of α-amylase [17].

With the ever-increasing applications of amylases, the advantages of immobilized enzyme over its soluble counterpart arise from their improved stability and easy separation from the reaction media, leading to decrease in production cost. The commercially available enzymes are efficient as reactants, maximizing catalytic velocity and enhancement of the operational lifetime [18]. To improve their economic value in food, medical, pharmaceutical, industrial and technological processes soluble enzymes are usually immobilized on to a solid support. The enzyme is immobilized on to a solid supports, either organic or inorganic, is a very effective way to increase their stability and operational lifetime.

The support material used for enzyme immobilization is very important although it is difficult to predict in advance which support will be most suitable for a particular enzyme. The support must be insoluble in water and should have a high capacity to bind with the enzyme and be mechanically stable [19]. Amylase has been immobilized to collagen [20], silica carriers using glutaraldehyde or titanium chloride [21] and glass support [22]. The intention of the present study was to analyse the α-amylase activity in solid state fermentation and submerged fermentation using *A. oryzae* and *B. cereus* and enzyme immobilization.

MATERIALS AND METHODS

Microorganism: *A. oryzae* was selected as the fungal strain for fermentation due to its high amylase activity and significant starch degrading capability. It was isolated from spoiled grapes and grown on potato dextrose agar medium. *B. cereus* was obtained from rotten potato and grown on nutrient agar medium containing 2% w/v soluble starch. The culture slants were incubated at 30^{o}C for 7 days (d).

Inoculum preparation: To the 7 d old nutrient agar and potato dextrose agar slant cultures, 10 ml of 0.1% Tween 80 solution was added and the spores were scrapped by a sterile inoculation loop. 2 milliliter (ml) culture suspension was inoculated in a sterilized medium (100ml in each flask) containing soluble starch 5 g, yeast extract 2 g, KH_2PO_4 1g, $MgSO_47H_2O$ 0.5 g with 1000 ml distilled water [23]. The flasks were incubated at 35^{o}C and 120 rpm.

Solid state fermentation: Substrates used for solid state fermentation were rice bran, paddy husk, raw rice and brown rice. 5 grams (g) of each substrate was washed thoroughly in running water and subjected to bleaching operation by immersing in hot water (75-80^{o}C) for 20 min followed by oven drying at 45^{o}C [23]. Further, they were minced in a grinder and sterilized at 121^{o}C at 15 psi pressure for 15 min. After sterilization the substrates were stored at 4^{o}C for further use. After cooling, the substrates were transferred to 250ml sterile water previously inoculated with *A. oryzae*. The flasks were incubated in a shaker incubator at 30°C and 80 rpm for 72 h.

Submerged fermentation: *B. cereus* was freshly inoculated to nutrient broth and incubated in a shaker at 30°C. Medium for bacterial amylase production by submerged fermentation consisted of 6g bacteriological peptone, 0.5g $MgSO_47H_2O$, 0.5g KCl and 1g starch in 1000 ml distilled water. The media was prepared and distributed in 30 to 50 ml volumes in 100ml conical flasks and sterilized by autoclaving at 121°C for 15 min. After cooling about 1ml of the inoculum was added to the flask and incubated at 30°C in a shaker incubator for 72 h at 80 rpm.

Extraction of enzyme from *A. oryzae* and *B.* cereus: For the solid state fermentation, the solid substrates were mixed thoroughly with 50 ml acetate buffer (pH 6) containing 0.1% Tween 80 surfactant. The contents were subjected to shaking at 200 rpm for 2 h at 50^{o}C. The filtrate was extracted by passing the slurry through muslin cloth. The crude α-amylase was obtained by further filtering through Whatman No. 1 filter paper. Similarly, for the submerged fermentation by *B. cereus* the culture media were poured into centrifuge tubes and centrifuged at 5000 rpm for 20 min. The supernatant which were the crude enzyme extract were collected for the enzyme activity assay.

Determination of enzyme activity: 1 ml of crude enzyme was pipetted into a test tube. To this 1ml of 1% soluble starch was added with citrate phosphate buffer (pH 6.5). This was incubated in a water bath at 40°C for 30 min. A blank was set up consisting of 2 ml of the enzyme extract that was boiled for 20 min as boiling inactivates the enzymes. Further, this was added to the starch solution and treated with the same reagent as the experimental tubes. The reaction was stopped by adding 2ml of dinitrosalicylic reagent and was boiled for 5 min. It was cooled and 20ml of distilled water was added. The enzyme activity was determined at 540 nm.

Immobilization methods: The α-amylase enzyme at stationary growth phase was harvested by centrifugation process. After homogenization, 4% sodium alginate solution was added and the contents were mixed well by continuous shaking. Amylase containing sodium alginate was extracted drop wise through sterile syringes into a calcium chloride solution containing beaker. Sodium was replaced by calcium ions and fine beads of calcium alginate gel were formed. The immobilized beads for amylase activity were obtained.

RESULTS AND DISCUSSION

A comparative study was conducted in amylase production between *A. oryzae* and *B. cereus*. *A. oryzae* was inoculated in different substrates such as brown rice, paddy husk, raw rice and rice bran in solid state fermentation. *B. cereus* was inoculated in different time intervals of 2, 4, 6, 8, and 10 h in submerged fermentation.

For the production of amylase, *A. oryzae* and *B. cereus* were employed in solid state and submerged fermentation respectively. The amylase activity was found to be its highest reaching 21.6 and 36.2 ppm/g in brown rice at 24 and 48 h of solid state fermentation respectively by *A. oryzae* which was significantly superior over other substrates (Fig. 1).

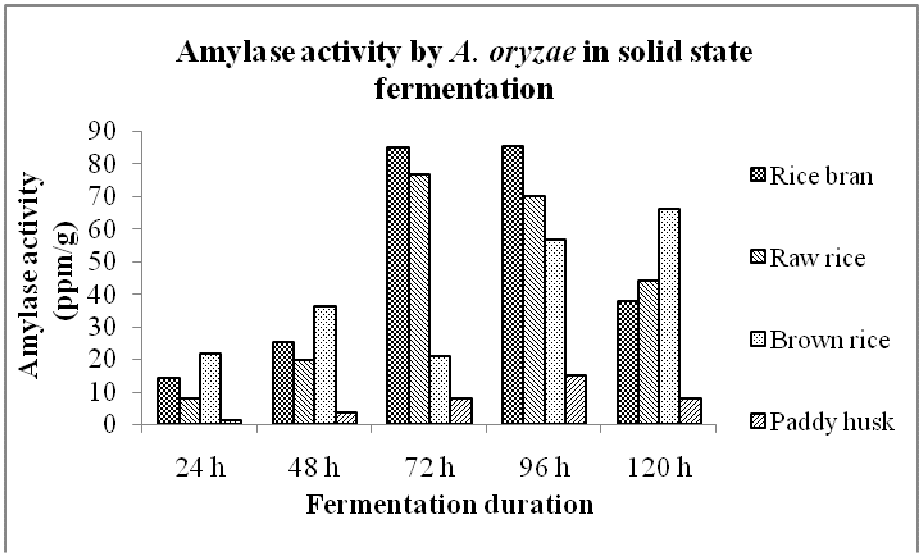

Fig. 1 The level of enzyme activity by *A. oryzae* on exposure to different substrates in solid state fermentation

At 72 h, enzyme activity was highest in rice bran reaching 84.8 ppm/g with raw rice presenting its highest enzyme activity of 76.5 ppm/g throughout its fermentation. Amylase activity reached 85.2 ppm/g in rice bran at 96 h which was recorded the highest amongst all substrates. Similarly around the same time duration, paddy husk recorded its highest amylase activity of 15.1 ppm/g in its fermentation. At 120 h brown rice activity was found to be highest at 66.0 ppm/g. Overall paddy husk recovered the lowest amylase levels throughout the fermentation as compared with other substrates. Rice bran was noted to be the best substrate marking an average of 49.4 ppm/g until 120 h of fermentation followed by raw rice showing 43.5 and brown rice 40.2 ppm/g correspondingly. On the other hand, paddy husk can be explained as inferior among the substrates with an average of 7.1 ppm/g in amylase activity.
After 24 h of fermentation the enzyme activity was identified by immobilization of beads (Fig. 2).

Upto 48 h, the amylase activity was found to be highest in raw rice representing 27.0 and 27.8 ppm/g at 24 and 48 h respectively. The amylase activity in rice bran and brown rice did not vary much with that of raw rice until 48 h. Amylase activity was highest reaching 164.6 and 209.4 and 236.7 ppm/g in rice bran at 72, 96 and 120 h of fermentation respectively. Paddy husk recovered highest activity of 18.1 ppm/g at 120 h throughout its fermentation period. Rice bran was found to be a superior substrate giving an average amylase activity of 132.6 ppm/g followed

by brown rice with 105.9 ppm/g and raw rice with 98.0 ppm/g. Again, paddy husk measured lowest in amylase activity of 15.0 ppm/g among all substrates.

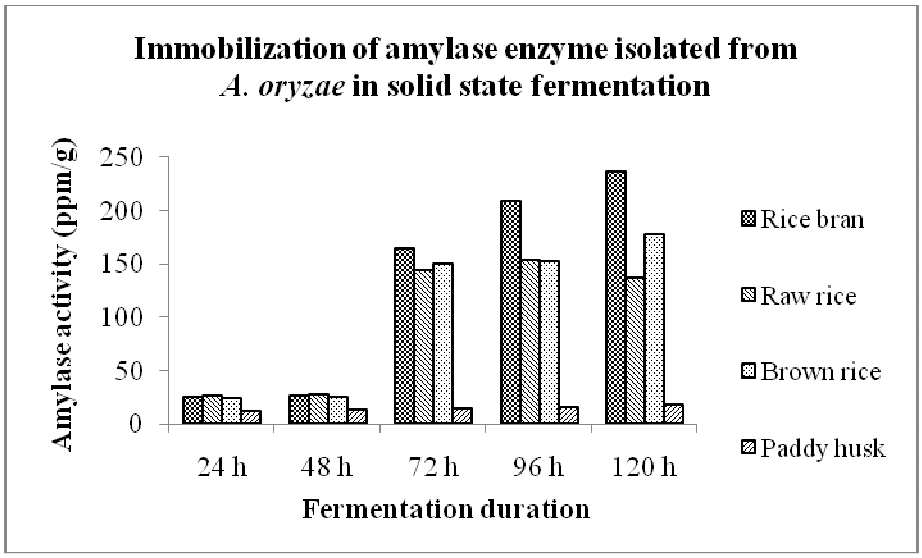

Fig. 2 Immobilization of amylase enzyme isolated from *A. oryzae* in solid state fermentation

Asgher et al [24] reported *Bacillus subtilis* JS-2004 strain was cultured in liquid media supplemented with waste potato starch to produce α-amylase. Maximum enzyme production 72 U/mL was achieved within 48 h at pH 7.0 and 50 °C. The α-amylase obtained from *Bacillus* sp. KR-8104 in solid state fermentation was studied using a substrate such as wheat bran. The maximum enzyme production of 140 U/g dry fermented substrate was recovered fromwheat bran and supplemented with 1% (w/w) NH_4NO_3 and 1% (w/w) lactose after 48 h incubation at 37 °C [25]. The results of fermentation showed that the isolated *Aspergillus Oryzae* strain S2 attained a production yield of 350 U/ ml after 92 h using the optimized medium for α-amylase production [26]. The *Aspergillus oryzae* var. viridis CBS 819.72 was produce 148 U/ml of amylase under optimized culture conditions [27]. However in this present study, amylase produced by *B. cereus* was the highest of 24.4 ppm/ml at 6 h in submerged fermentation and the lowest in 2 h with 4.5 ppm/ml of amylase (Fig. 3).

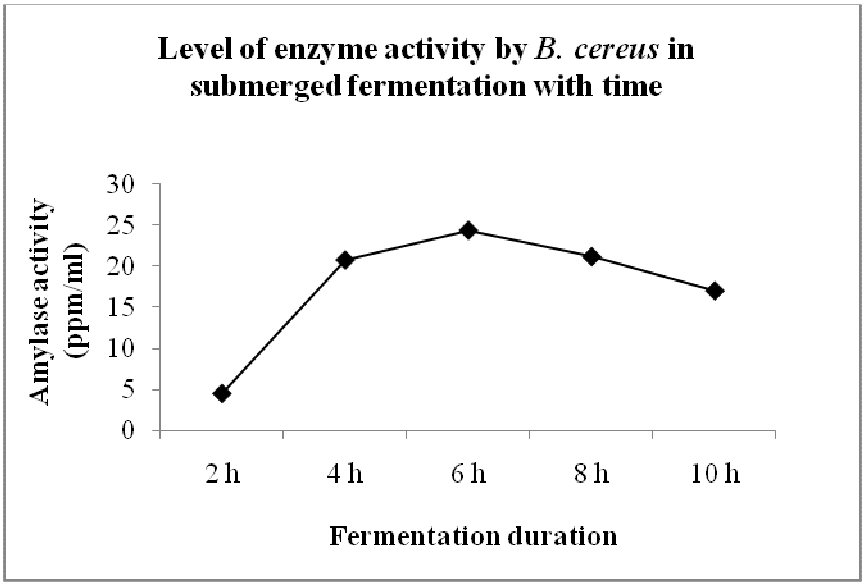

Fig. 3 The level of enzyme activity by *B. cereus* on exposure to different incubation periods in submerged fermentation

With the immobilization of amylase, the activity was found to be almost similar upto 4 h of fermentation with 24.1 and 24.8 ppm/ml at 2 and 4 h respectively (Fig. 4).

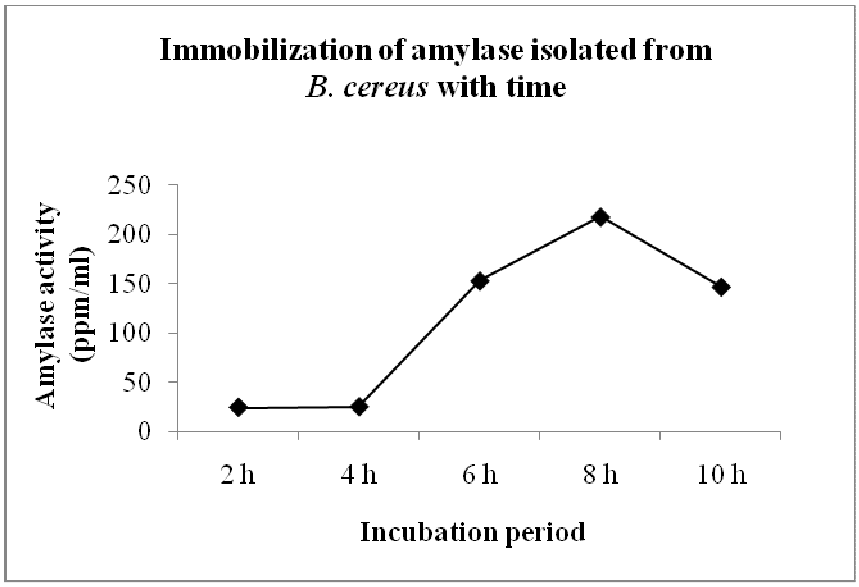

Fig. 4 Immobilization of amylase enzyme isolated from *B. cereus* at different incubation periods

The highest value recorded was 217.8 ppm/ml at 8 h of fermentation. The α-amylase production by solid state fermentation using coconut oil cake has been reported [14]. Fungal cultures have been reported to give optimum enzyme production at pH 5 using various substrates [28]. Bacterial cells secreted the enzyme in nutrient broth indicating to be constitutively expressed while amylase was reported to be inductive in other hemophilic bacteria [29].

The inoculation of *A. niger* to different substrates in solid state fermentation gave no product of $CaCl_2$. This was found related to the study by Gangadharan et al. [30] where similar results among the variables screened, substrate concentration, incubation period and $CaCl_2$ concentration were identified as most significant variables influencing α-amylase production. Generally, rice bran was found to be a good substrate for amylase production and paddy husk gave unsatisfactory results in this study. Arasaratnam et al. [16] reported glucoamylase production by *A. niger* using rice bran and paddy husk as alternative substrate to wheat bran. Paddy husk is reported to enhance the nutrient utilization when mixed with the substrate like rice bran, corn cobs, soy flour and soy meal powder by *A. niger* CFTRI 1105 during solid state fermentation increasing glucoamylase production. Maximum glucoamylase production using wheat bran has been reported in the presence of fructose as carbon source. The presence of peptone in fermentation media is found to enhance the enzyme production with urea as a nitrogen supplement in wheat bran [31].

CONCLUSION

The *A. oryzae* was isolated from spoiled grapes and grown on potato dextrose agar medium and *B. cereus* was obtained from rotten potato and grown on nutrient agar medium containing 2% w/v soluble starch. According to the results obtained it can be asserted that *A. oryzae* is suitable support to produce amylase under solid state fermentation. Rice bran was the best substrate giving an average amylase activity in solid state fermentation by *A. oryzae* followed by raw rice showing 43.5 ppm/g and brown rice 40.2 ppm/g. With the present findings conclude that amylase production is higher in solid state fermentation by *A. oryzae* with rice bran as the substrate.

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