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Optimization of Cultural Conditions for Solid State Fermentation of Amylase Production by *Aspergillus* species

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Abstract

Amylase is an amylolytic enzyme used in food industry which is generally produced by *Aspergillus* spp. under solid state fermentation. The present study is concerned with the isolation, screening and selection of suitable strains of *Aspergillus* spp. and optimization of cultural conditions for the biosynthesis of amylase. Rice and wheat brans were used as substrates which are readily available inexpensive raw materials for amylase production. From 85 samples of rice and wheat grains, 55 colonies obtained on potato dextrose agar (PDA) were suspected to be *Aspergillus oryzae* and only 35 colonies possessed the morphological characteristics similar to that of *A. oryzae* indicating the isolates were most likely the strains of *A. oryzae*. Of all the fungal isolates of *Aspergillus* spps., Asp.31 gave maximum production of amylase (720.782 $IUgds^{-1}$) in solid state fermentation media. This strain was selected as a parental strain for optimization for cultural conditions. The obtained data were analyzed using SPSS-11.5 program. Of all the substrates (rice bran, wheat bran and their mixture), rice bran was the best for producing amylase of highest activity 611.614 $IUgds^{-1}$.The highest enzyme activity of 698.749 $IUgds^{-1}$ was observed at 50% initial moisture level of the substrate. The optimum temperature was 25ºC for producing the crude amylase enzyme with amylase activity of 577.757 $IUgds^{-1}$.

Key words: amylase activity, *Aspergillus* spp., crude amylase enzyme, solid state fermentation

Introduction

Amylolytic enzymes are those enzymes, which catalyze the hydrolysis of alpha 1, 4 and/ or alpha 1, 6 linkages in starch and related compounds. They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved (Teka 2006). The microbial amylases meet industrial demands with a large number of them available commercially and have almost completely replaced chemical hydrolysis of starch in starch processing industry (Patel *et al*. 2005). Solid state fermentation (SSF) constitutes an interesting alternative to most popular Submerged State Fermentation since the metabolites so produced are concentrated and purification procedures are less costly (Ellaiah *et al*. 2002).

Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, *Aspergilli* are particularly interesting due to their easy cultivation, and high production of extracellular enzymes with potential industrial exploitation (Morya & Yadav 2009). *Aspergillus oryzae* has an efficient system for secretion of proteins and is extensively used to produce industrial enzymes (Sivaramakrishnan *et al*. 2007). *A. oryzae* has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. á-amylase (de Souza & de Oliveira 2010). Fungi are commonly used in SSF, due to their relatively high tolerance to low water activities, their high potential to excrete hydrolytic enzymes and their morphology (Rahardjo, 2005).

Brans milled from cereal grains are particularly rich in dietary fiber and essential fatty acids and contain significant quantities of starch, protein, vitamins and dietary minerals (Wikipedia, 2011). Food and agricultural wastes can serve as substrates for the production of various fermented products and enzymes (Singh *et al*. 2009).

Methodology

Sample collection

Samples of rice and wheat grains were collected from different places of Kathmandu valley, Pokhara, Biratnagar, Dolakha, Syangja and Chitwan. Rice and wheat brans were collected from Kathmandu valley.

Mold flora isolation

Samples of rice and wheat grains that were assumed to be contaminated with *A. oryzae* were collected. The International Seed Testing Association Techniques (ISTA) especially (Agar plate method) was used to detect *A. oryzae* (Elbashiti *et al*., 2010). Each sample kernels were surface disinfected with 2% sodium hypochlorite (NaOCl) solution and washed with distilled water for 3 times. Subsequently, the seeds were aseptically dried on sterile blotting paper and plated on PDA medium in order to isolate the associated mycoflora. The plates were incubated at 30ºC for 7 days. After seven days, the developing fungal colonies were isolated. Isolated spores (assumed to be *A. oryzae*) were identified through macroscopic and microscopic observations and sub-cultured on PDA medium to obtain pure cultures of the isolates and maintained on PDA slants for further identification, incubated at 30ºC for 7 days and stored at 4ºC and sub cultured fortnightly (Maharjan, 2009).

Identification of *A. oryzae*

The isolates were inoculated on CYA (Czapex Yeast Agar) medium and incubated at 30ºC for 7 days. CYA was prepared by adding yeast extract in CDA. After incubation period, all the plates were observed for macroscopic characteristics such as colony diameter, colony color, colony reverse, colony texture, and nature of spores (Elbashiti *et al*. 2010).

The microscopic characteristics were observed by preparing slides by Cellophane Tape Method (Aneja 2008).

Screening of non-toxigenic *Aspergillus* spp. on *Aspergillus* differential medium

Aspergillus differential medium (ADM) was used to identify aflatoxin producing and non-producing strains of *A. oryzae*. An inoculating needle was sterilized and point inoculation was performed on ADM. Inoculated ADM plates were incubated at 30ºC for 48 hrs and observed for development of bright yellow orange pigments (Bothast & Fennell, 1974, Salkin & Jordon, 1975).

Screening of potent amylolytic mycoflora

Preliminary screening for amylase production from *Aspergillus* isolates were carried out by Starch Agar (SA) plate assay (Morya & Yadav 2009). The final constituents of media was 2.0g soluble starch, 5.0g peptone, 3g beef extract and 15.0g agar dissolved in 1.0 liter distilled water, warmed and autoclaved at 121ºC for 15 minutes (Hi-media). SA media plate was point

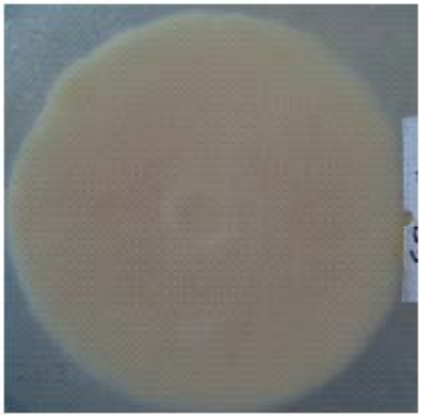

Fig. 1. Colony reverse of Aspergillus spp. Isolated from rice grain

Fig. 2. Colony surface of *Aspergillus* spp. isolated from rice grain

Microscopic characteristics

The microscopic characteristics of the isolates as observed were compared with identification key for *A. oryzae* and were found to comply with the given characteristics.

Conidial head- radiate to loosly columnar; Conidiophore- Relatively thin walls, definitely roughened throughout all or most of their length; Vesicle- Subspherical, less commonly flasked shaped, sterigmata covering the entire surface or upper three fourth; Conidiogenous Cell- Uniseriate and biseriate; Metulae or phialides covering the entire surface or the upper three-fourth of the vesicle; Conidia- (Sub) spherical to ovoidal, smooth walled to roughened Hyphae- Septate.

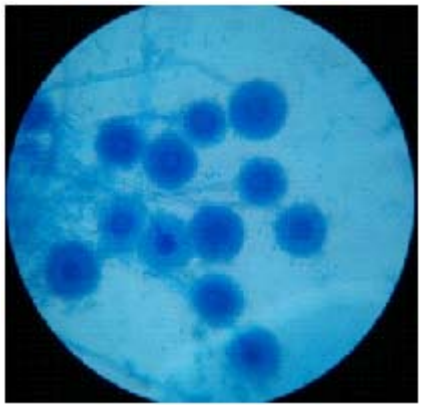

Fig. 3. Microscopic feature of *Aspergillus* spp. isolated from rice grains

Screening of *A. oryzae*

Isolates were grown on ADM to screen non-toxigenic strain. The development of bright yellow orange colonies indicates toxigenic strain whereas absence of bright yellow orange pigmentation indicates the strain to be non toxigenic. Similarly amylolytic activity of isolates was determined by performing starch hydrolysis test on SA plates and the zone of hydrolysis formed by each isolates were compared. Strains which showed maximum zone of starch hydrolysis were selected for enzyme production.

Selection of fungal strains for enzyme production

All the isolates suspected to be *A. oryzae* were compared and three strains with high amylolytic activity were selected for enzyme production. Three isolates of *Aspergillus* spp. Asp.19, Asp.14 and Asp.31 with zone of hydrolysis of 1.3, 1.5 and 1.3 cm respectively were selected.

Fig. 4. Zone of hydrolysis on starch agar

Solid state fermentation

On the basis of cultural and morphological characteristics the isolate Asp.19, Asp.14, Asp.31 and Asp.33 were found to belong to the genus *Aspergillus* and closely related to the species *A.oryzae* while compared to standard description. SSF was adopted for enzyme production using rice bran, wheat bran and their mixture as substrate, employing three strains of *Aspergillus*, Asp.19, Asp.14 and Asp.31 and fermentation was carried out at different temperature (25°C, 30°C and 40°C) and different moisture content of substrate (50%, 60% and 70%) maintaining initial pH of substrate between 6-7.

Determination of enzyme activity

Different culture conditions greatly affect on the production of amylase (Cherry *et al.* 2004). Farid and Shata 2011 reported that the enzyme production was affected by strain type, incubation periods, and level of moisture content and carbon source supplementation. Laboratory experiment was carried out to optimize culture conditions for enzyme production by fungal isolates.

Enzyme activity was determined for these different strains in different substrate with different moisture content at different temperature. All the experiments for different studies explained below in figure and text were carried out in triplicates and values were averaged. One unit (IU) of amylase is defined as the number of

micromoles of reducing sugars (glucose equivalent) released per 10 minutes by the total amount of enzyme under the assay conditions and enzyme activity is expressed in terms of IU per gram dry fermented substrate (IU gds^{-1}). Weight of dry fermented substrate is determined by drying fermented residue obtained after filtration of crude enzyme extract in hot air oven at 80º C for 16 hrs and weighing the dry fermented substrate without filter paper.

Effect of substrate on enzyme production

Three kinds of substrate rice bran (RB), wheat bran (WB) and mixture of rice and wheat bran (RB_WB) were inoculated with isolated strain Asp.14 and incubated at 30ºC with initial moisture content of 60% and pH of substrate between 6 to 7 for 120 hrs. The crude enzymes extracted from fermented mash were assayed for amylase by DNS method. The result obtained is depicted below in Fig. 5.

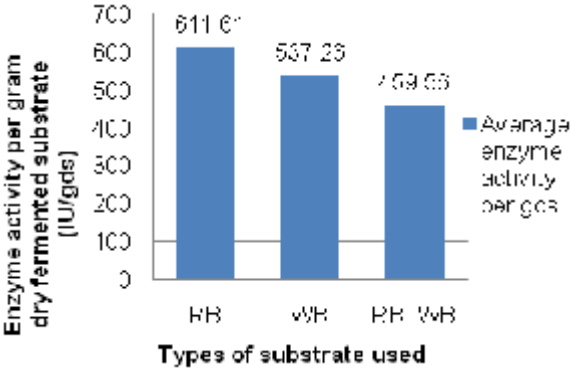

Fig. 5. Average enzyme activity per gram dry fermented substrate with respect to types of substrate

Crude enzyme extract extracted from fermented matter using rice bran as substrate gave amylase enzyme activity of 611.614 IU gds^{-1} while in case of substrate using wheat bran and mixture of rice and wheat bran in the ratio of 1:1 gave enzyme activity of 537.258 and 459.563 IU gds^{-1}. Figure 5 reveals the enzyme activity to be highest in case of rice bran followed by wheat bran and lastly mixture of rice and wheat bran (1:1) with least enzyme activity. The result was similar to the result obtained by Alva *et al*. (2007), and Kumar and Duhan (2011). Highest activity in rice bran may be due to its high carbohydrate contents and suitable texture (Kumar and Duhan, 2011).

Effect of strain on enzyme production

Three different isolates Asp.19, Asp.14 and Asp.31 were inoculated separately in single type of substrate i.e. rice bran (RB) and incubated at 30ºC maintaining moisture content to 60% and pH between 6 and 7. Crude enzyme were extracted from fermented matter after 120 hrs and assayed for amylase enzyme activity.

Amylase activity of 549.046 IU gds^{-1} was obtained from assay and calculation in case of substrate inoculated with Asp.19 and it was 563.687 IU gds^{-1} and 720.782 IU gds^{-1} for Asp.14 and Asp.31 respectively. The result obtained is outlined in Fig. 6.

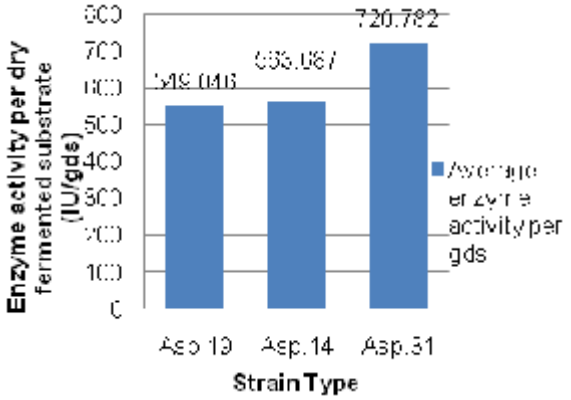

Fig. 6. Average enzyme activity per gds for different types of strains of *Aspergillus* spp.

Figure 6 shows that enzyme activity of crude enzyme obtained by the strain 31 was highest followed by strain 14 and strain 19 respectively in decreasing order. It clearly demonstrates the highest potency of Asp.31 to produce Amylase enzyme in comparison to Asp.14 and Asp.19. It might be because its potency to grow on solid form of substrate such as rice bran or wheat bran is high compared to others.

Effect of moisture content on enzyme production

Single strain of *Aspergillus* (Asp.31) was inoculated on rice bran (RB) and incubated at 30ºC maintaining different moisture content (50%, 60% and 70%) of substrate. Crude enzyme were extracted from fermented matter after 120hrs and assayed for amylase enzyme activity. The result obtained is presented in Fig. 7.

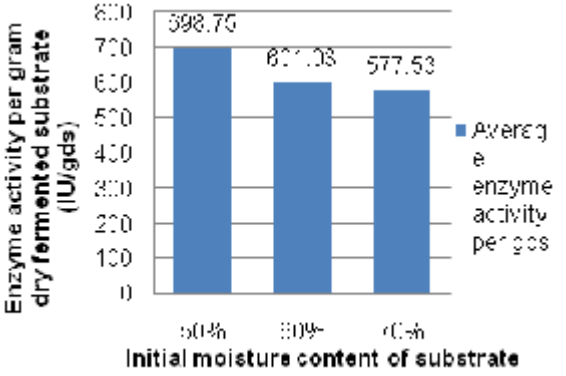

Fig. 7. Average enzyme activity per gram dry fermented substrate with respect to different initial moisture content of substrate

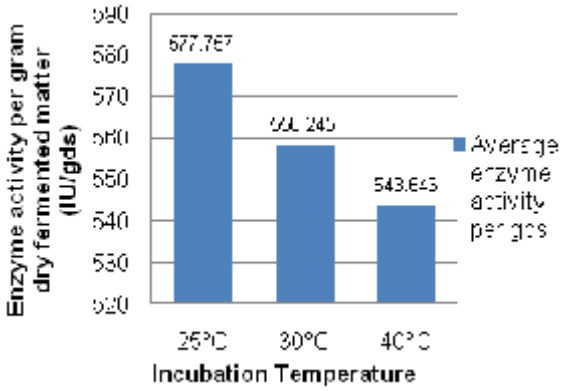

Fig. 8. Average enzyme activity per gram dry fermented substrate with respect to incubation temperature

Crude enzyme extract possessing amylase enzyme activity of 698.749, 601.033 and 577.529 IU gds^{-1} were obtained from substrate with initial moisture content of 50%, 60% and 70% respectively. Figure 7 reveals that enzyme activity of crude enzyme was highest in substrate with moisture content 50%. Least enzyme activity was obtained at 70% moisture content of substrate. This result was found to comply with the research finding of Chutmanop *et al*. (2008) that was the optimum initial moisture level for highest enzyme production was about 50%. According to Chutmanop *et al*. (2008), substrate moistened at this level afforded a high protease activity. Moisture levels much above 50% reduced enzyme production as the substrate became waterlogged. In contrast, a low moisture level reduces water activity to levels that are not conducive to supporting good fungal growth (Chutmanop *et al*. 2008). But from most reports of other researchers in literature, the optimum initial moisture content was found to vary. This kind of variation might be due to difference in substrate types, fungal isolates, etc. The adverse effect of catabolic repression in SSF system is related with moisture content- high moisture content leads to high catabolic repression resulting into reduced enzyme production.

Effect of temperature on enzyme production

Rice bran was inoculated with single type of isolated strain Asp.31 and incubated at 30ºC with initial moisture content 60% with pH of substrate between 6 and 7. The crude enzymes extracted from fermented matter were assayed for amylase by DNS method. Fig. 8 given below represents the result obtained.

From figure 8 it is clear that the amylase activity of crude enzyme extracted was high with 577.757 IU gds^{-1} in case of substrate incubated at 25ºC and lowest at 40ºC which was 543.646 IU gds^{-1}. It was 558.245 IU gds^{-1} in case of fermented matter incubated at 30ºC. The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic.

pH of substrate and fermentation time

In this study initial pH of the substrate was found out to range between 6 and 7. The pH of substrate was not changed as it requires addition of chemicals such as acid (HCl) or alkali which may degrade or deteriorate quality of amylase or difficulties might occur during purification. According to Van der Maarel *et al.* (2002) lowering pH of substrate involves the addition of acid (HCl), which could decrease the quality of the product and hence incurs additional cost for later purification.

Fermentation time

It has been reported that maximum enzyme production by *Aspergillus* species was obtained after an incubation period of 120 h. Farid & Shata 2011, Sidkey *et al*. 2010, Bhattacharya *et al*. 2011 Zambare 2010. In the present study also incubation period was set up to 120 hrs following most research reports.

Purification of amylase enzyme

Organic solvents cause precipitation of protein largely by changing the solubility of the protein with water. The reaction is carried out at low temperature to

prevent denaturation. Crude enzyme was further purified by acetone precipitation at different percentage and salting out method by use of ammonium sulfate of different concentration. Purified forms of amylase obtained were assayed for amylase activity. The data are presented in Table 1.

Table 1. Purification of amylase enzyme

Test sample	Enzyme activity (IU ml^{-1})
Crude enzyme	982.4561
35% acetone	5380.12
50% acetone	11228.07
30% ammonium sulfate	6081.87

The use of 35 % of acetone gave enzyme activity of 5380IU ml^{-1} and 50% acetone gave 11228.07 IU ml^{-1} amylase enzyme activities. Amylase enzyme activity was found to be gradually increasing by the use of acetone at 30% and 50% concentration. Amylase enzyme activity of crude enzyme was 982.4561 IU ml^{-1}. Enzyme with 6081.87 IU ml^{-1} amylase enzyme activity was obtained after 30% ammonium sulfate precipitation. This decrease in enzyme activity might be due to loss in amylase activity during enzyme purification and also may be because ammonium sulfate salt was not of enzyme grade

From the present study, starch hydrolysis test and DNS test Asp.31 was identified to be the most potent fungal isolate. Rice bran was found to be most suitable substrate and Asp.31 to be most potent isolate while 50% moisture content and 25ºC temperature were found to be optimum for maximum amylase production. The enzyme was purified by acetone and ammonium sulfate precipitation. Amylase activity was found to increase after purification of enzyme by acetone and ammonium sulfate.

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