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Optimization of α-amylase and glucoamylase production in solid state fermentation of deoiled rice bran (DRB) by *Rhizopus oryzae*

Harjeet Kaur*, Arora M., Bhatia S. and Alam M.S.

Department of Microbiology, College of Basic Science and Humanities, Punjab Agricultural University, Ludhiana-141004, Punjab, India

*Corresponding Author E-mail: panesarpreet92@gmail.com

ABSTRACT

The present study is concerned with the optimization of cultural conditions for the production of fungal enzymes from deoiled rice bran in solid state fermentation by isolated strain of Rhizopu soryzae MTCC no.1987 (GRAS status). The process optimization for production of α -amylase and glucoamylase was carried out in 250 ml Erlenmeyer flask. Different cultural conditions such as temperature (20 to 40^0 C) moisture content (5 to 25ml/5g of substrate), pH (4 to 6), inoculum size (1 x 10^5 to1 x 10^8 spores/ml) and incubation period (3 to 6 days)were optimized to obtain maximum activity of α -amylase and glucoamylase. Under all the optimized cultural conditions: temperature 30^0 C, 67% moisture content (10ml/5g of substrate), pH 5.5, inoculum 1 x 10^7 spores/ml and incubation period of 5 days, yielding an average amylase and glucoamylase activity of 2.08 IU/ml and 0.27 IU/ml respectively.

Key words: *Deoiled rice bran, Solid state fermentation, α–amylase, glucoamylase, Rhizopu soryzae*

INTRODUCTION

Amylases are a group of hydrolases that can specifically cleave the O-glycosidic bonds in starch. Two important groups of amylases are glucoamylase and α-amylase. Glucoamylase (exo-1,4-α-D-glucanglucanohydrolase, E.C. 3.2.1.3) degrade both amylose and amylopectin by hydrolyzing both α-1,4 and α-1,6 glucosidic links of starch and produce glucose[13]. Hence glucoamylase can convert starch completely to glucose. Now a days, glucoamylase is one of the most important enzymes in food industries[5,40,41], as it is used for the production of glucose and fructose syrup from liquefied starch[12,27,28]. It is also employed in baking, juice, beverage pharmaceuticals, and many fermented foodstuffs industries for commercial production[16,32,34], in some cases textile, leather and detergents industries. Whereas α-amylases (endo-1,4-α-D-glucanglucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units inside the linear amylose chain[2,7,31]. Alpha-amylases are widely distributed in nature and can be derived from various sources such as plants, animals and microorganisms[31,3]. Nowadays, spectrum of applications of α- amylase is also extending in many other areas such as analytical chemistry, clinical and medicinal diagnosis e.g. diagnosis of acute inflammation of pancreas, macroamylasemia, perforated pelvic ulcer and mumps[2,9,26,29]. However, fungal and bacterial amylases have predominant applications in the industrial sector.

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Major advantages 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[31]. Theglucoamylase enzyme was reported to produce by many fungi like *Aspergillus awamori, A. saitoi, A. oryzae*, *Rhizopus* sp, *Mucor* sp, *Penicillium* sp., and Yeast[36,44]. Among these, *Rhizopus* spp. are considered good producers of amylolytic enzyme[10,19]. Usually amylase production from fungi has been carried out using well defined chemical media by submerged fermentation and solid state fermentation[25]. The economics of enzyme production using inexpensive raw materials can make an industrial enzyme process competitive[10]. For the microbial α-amylase production, two types of fermentation methods are mainly used i.e. submerged and solid state. However, in recent year SSF has emerged as a well-developed biotechnological tool for the production of enzymes[6]. Use of suitable low cost fermentation medium for production of alpha amylase using agricultural by-products has been reported[15].

On the basis of the importance of glucoamylase and α-amylases, the present study was aimed for the process optimization for α -amylase and glucoamylase production from fungal specie i.e. *Rhizopus oryzae* MTCC-1987 using deoiled rice bran as a solid substrate fermentation.

MATERIALS AND METHODS

Substrate

The substrate deoiled rice bran (DRB) was procured from Ricela Health Foods Limited, Dhuri, Punjab and was used as a substrate for α-amylases and glucoamylase production in solid state fermentation.

Microorganism and culture maintenance

Rhizopus oryzae (MTCC no. 1987) was procured from MTCC (Microbial Type Culture Collection), Institute of Microbial Technology, Chandigarh, Punjab. The culture is Generally Recognized as Safe (GRAS)[3,21,23]. The culture was maintained by sub-culturing on potato-Dextrose Agar (PDA) slants (pH 5.6). The slants were grown at 30^0c for 5 days and stored at 4^0c in a refrigerator.

Mineral medium forα-amylases and glucoamylase production

The composition of mineral medium was given by Singh *et al*[39]. Mineral medium used for enzyme production composed of soluble starch (5g/l), yeast extract (2g/l), potassium dihydrogen phosphate (1g/l) and magnesium sulphate (0.5g/l). Components of medium were dissolved in 1000 ml and then the medium was autoclaved at 1.1 kg/cm^2 for 20 minutes.

Inoculum preparation

A spore inoculum was prepared by adding 10-15 ml of sterile Tween 80 (0.8%) to each slants having the fungal cultures and shaken vigorously. One ml of the inoculum (1x 10^7 spores/ml) from the fungal slant was used per flask to carry out solid state fermentation of deoiled rice bran.

Solid state fermentation

5g of deoiled rice bran amended with 10ml of mineral medium was taken in 250 ml cotton plugged Erlenmeyer flask, mixed homogenously and sterilized at 121^0c for 15 min in an autoclave. Thereafter, the flask material was cooled at room temperature and inoculated with 1ml spore suspension of *Rhizopu soryzae.* The flasks were then incubated at 30^0c for 5 days.

Optimization of cultural conditions

Various process parameters were optimized for maximal enzyme production.

The effect of moisture level on enzyme production was tested by varying the substrate to mineral medium ratio (w/v) in the range of 1:1, 1:2, 1:3, 1:4 and 1:5 (w/v). The flask (250ml Erlenmeyer flask) containing 5g of substrate and 5ml, 10 ml, 15ml, 20ml and 25ml of medium respectively were inoculated with 1ml of spore suspension (1 x 10^7 spores/ml) and incubated for 5 days at 30^0 C in incubator. Moisture was provided by medium itself at pH 5.5.

The effect of initial pH on enzyme production was investigated by adjusting the initial pH of mineral medium to 4.0, 4.5, 5.0, 5.5 and 6.0. The flasks containing 5g of substrate and 10 ml of sterile mineral medium was inoculated 1ml of spore suspension (1 x 10^7 spores/ml) and incubated for 5 days at 30^0 C in an incubator.

The effect of incubation temperature on enzyme production was examined by incubating the inoculated flasks containing 5g of substrate with 10 ml of mineral medium of pH 5.5 at different temperatures ranging from 20^0 C to 40^0 C with 5^0 C interval i.e. 20^0 C, 25^0 C, 30^0 C, 35^0 C and 40^0 C for five days in a BOD incubator.

The effect of inoculum size based on the number of spores was examined using a spore concentration of 1×10^5 spores/ml, 1×10^6 spores/ml, 1×10^7 spores/ml and 1×10^8 spores/ml. Tween-80[20]was used for making the spore suspension. The flasks having the sterile basal medium were inoculated for 5 days (120 h) and then the partial purified enzymes were used for the determination of the enzyme were used for determination of the enzyme activities.

Flasks containing 5 g of substrate were inoculated with 1ml (1×10^7 spores/ml) of spore suspension and were incubated at 30^0 C in a BOD incubator. The enzymes were extracted and assayed after 3, 4, 5 and 6 days interval.

Enzyme extraction

After the specified incubation period, the flasks were taken out and brought to room temperature. The product was recovered from the substrate by shaking it for 30 min in shaking incubator (250 rpm) with 0.1M citrate buffer at a solid to moistening agent ratio of 1:10. The extract was then, filtered through filter paper to obtain a clear filtrate. The filtrate was then centrifuged at 5000 rpm for 20 minutes. The supernatant obtained was again filtered through filter paper so as to obtain a cell free supernatant which was used as a source of crude enzyme[20]. The α-amylase and glucoamylase activity were estimated by spectrophotometric method.

Enzyme assays

The activities of α-amylase and glucoamylase enzyme were expressed in International Units (IU). One IU is defined as one μmol of glucose (for α-amylase and glucoamylase activity) equivalents released per minute per ml under the assay conditions by using glucose standard curve[38]. Appropriate dilution factors were used during the estimation of enzyme activity. The α-amylase and glucoamylase activity was determined by according to the method reported by Miller[24].

Alpha amylase activity

The activity of α-amylase was measured by incubating 1% of soluble starch in 0.1 M citrate buffer of pH 5.0 at 45°C for 30 min. The enzyme was assayed by using one ml of diluted enzyme solution (culture filtrate) and adding 1 ml of buffered solution. This mix was incubated for 30 min at 45°C. The enzyme reaction was stopped by the addition of 3 ml of 3, 5- dinitrosalicylic acid reagent. Two ml of buffer (0.1M citrate buffer) was used as reference blank. All the tubes containing 3, 5-DNS treated reaction products were heated for 15 minutes in boiling water bath. One ml of 40% solution of Rochelle salt was added to each tube prior to cooling to room temperature so as to maintain the color. The final volume in each case was made to 7 ml by adding distilled water. Absorbance was measured at 575 nm using UV-Visible spectrophotometer and compared with standard curve using 0.10 to 1.0 mg of glucose/ml.

Glucoamylase activity

Glucoamylase activity was measured by incubating 1% maltose in 0.1 M citrate buffer of pH 5.0 at 45°C for 30 min. The enzyme was assayed by using one ml of diluted enzyme solution (culture filtrate) and adding 1 ml of buffered solution. This mix was incubated for 30 min at 45°C. The enzyme reaction was stopped by the addition of 3 ml of 3, 5- dinitrosalicylic acid reagent. Two ml of buffer (0.1M citrate buffer) was used as reference blank. All the tubes containing 3, 5-DNS treated reaction products were heated for 15 minutes in boiling water bath. One ml of 40% solution of Rochelle salt was added to each tube prior to cooling to room temperature so as to maintain the color. The final volume in each case was made to 7 ml by adding distilled water. Absorbance was measured at 575 nm using UV-Visible spectrophotometer and compared with standard curve using 0.10 to 1.0 mg of glucose/ml.

RESULTSAND DISCUSSION

Optimization of different cultural conditions for maximum α -amylase and glucoamylase activity

Different cultural conditions viz., moisture, pH, temperature, inoculum concentration and incubation period were optimized for maximum alpha-amylase and glucoamylase activity.

Effect of moisture content on α-amylase and glucoamylase activity

Varying amount of mineral medium was added to the substrate, to study the effect of moisture content on enzyme production. The results showed significant ($p < 0.05$) increase in the alpha-amylase (2.09 IU/ml) and glucoamylase (0.29 IU/ml) activities with increase in mineral medium level from 5 to 10 ml/ 5g substrate, but with further increase in moisture content up to 25 ml/5g substrate led to decrease in enzyme activity (Table 3.1). Hence, 10ml medium/ 5 g substrate i.e. 67% was regarded as the optimum moisture level.

Moisture content of the substrate influenced degradation by fungal species[35]. Cruz *et al*[11] reported solid substrate fermentation of bagasse with 60% moisture, by *Aspergillus niger* and *Rhizopus nigricans.* Hokkao Kakobi[1] composted solid potato starch waste containing 60-70% water, mixed with soil, rice, wheat straw etc. to 50-65 % water in a tank equipped with an aeration device. Zadrazil and Brunnert[45]found that addition of defined amount of water to the substrate altered the gas-phase and thus gas exchange. As the water content increases, the gas-phase is reduced and gas exchange is thus increasingly impeded, hence substrate suspension conditions become anaerobic. On the other hand, at low water content, the growth conditions of the fungi are also sub-optimal because the water tension is high and the degree of substrate swelling is low. Therefore, the conditions for solid state fermentation were optimal generally in the intermediate range of water content.

Table 3.1 Effect of moisture content on α -amylase and glucoamylase activity

Enzyme activity (IU/ml)		
Moisture content (ml)	**Alpha-amylase**	**Glucoamylase**
5	1.97±0.01	0.21±0.02
10	2.09±0.05	0.29±0.04
15	1.92±0.02	0.26±0.08
20	1.86±0.01	0.23±0.01
25	1.80±0.01	0.20±0.01

Substrate-5 g, Temperature 30^0 C, pH 5.5, Incubation time- 5days, Inoculum concentration- 1 x 10^7 spores/ml
P (<0.05) Values are mean of triplicate samples± Standard Error (n=2)

Effect of pH on α -amylase and glucoamylase activity

Hydrogen ion concentration (pH) plays an important role in the various physiological processes of the microorganism. The effect of pH on alpha-amylase and glucoamylase activities of *Rhizopus oryzae* was studied by varying the pH from 4 to 6. The results indicated that with increase in pH value from 4 to 5.5, the activities of alpha-amylase and glucoamylase enzymes reached to the maximum 2.07 and 0.28 IU/ml respectively, followed by a gradual decrease thereafter (Table 3.2). Optimum pH for both amylase and glucoamylase activity was 5.5. The change in pH from optimum to extreme levels results in inactivation of the enzyme that hinder saccharification of the substrate[38]. Solid state fermentation of *Rhizopus oryzae* for amylase production using agro-industrial residues was studied by Ferreira *et al*[14], who reported highest amylase production of 6.01 U/ml of fermented wheat bran at optimum pH 5.0. Similarly, fermentation of wheat bran for the production of glucoamylase enzyme by *Aspergillus oryzae* reported maximum activity (4.65 U/ml) at pH 5.0[46].

Table 3.2 Effect of pH on α -amylase and glucoamylase activity

Enzyme activity (IU/ml)		
pH	**Alpha-amylase**	**Glucoamylase**
4.0	1.87±0.01	0.20±0.03
4.5	1.91±0.02	0.22±0.02
5.0	1.95±0.03	0.25±0.01
5.5	2.07±0.01	0.28±0.02
6.0	1.90±0.07	0.23±0.03

Medium-10 ml mineral medium, Substrate-5 g, Temperature 30^0 C, Incubation time- 5days,
Inoculum concentration- 1 x 10^7 spores/ml P (<0.05) Values are mean of triplicate samples ± Standard Error (n=2)

Effect of incubation temperature on α -amylase and glucoamylase activity

Incubation temperature not only influences the growth of microorganisms, but also their biological activities. The results depicted in Table 3.3 show that the optimum temperature for alpha-amylase and glucoamylase activities was found to be 30^0 C (2.07 IU/ml and 0.27 IU/ml respectively). It was observed that at 20^0 C enzyme activities were low and showed a gradual increase with the increase in temperature to 30^0 C. Further increase in temperature led to acceleration of the denaturation induced by higher physiological temperature. It is widely known that at high temperatures enzymatic activity can be destroyed because enzymes are proteinaceous molecules[18]. In a similar finding by Kupski *et al*[22], optimum temperature was 30^0 C for solid state fermentation of rice bran using *Rhizopus oryzae.*

Table 3.3 Effect of incubation temperature on α -amylase and glucoamylase activity

Enzyme activity (IU/ml)		
Temperature (^{0} C)	**Alpha-amylase**	**Glucoamylase**
20	1.80±0.10	0.20±0.03
25	1.93±0.06	0.25±0.01
30	2.07±0.02	0.27±0.03
35	1.91±0.01	0.23±0.03
40	1.89±0.01	0.21±0.02

Medium-10 ml mineral medium, Substrate-5 g, pH 5.5, Incubation time- 5days,
Inoculum concentration- 1 x 10^7 spores/ml P (<0.05) Values are mean of triplicate samples± Standard Error (n=2)

Effect of inoculum concentration on α-amylase and glucoamylase activity

Proper amount of inoculum is essential for an efficient solid state fermentation. A spore suspension was prepared and each flask was inoculated with spore suspension (1 ml) of 1 x 10^5 spores/ml, 1 x 10^6 spores/ml, 1 x 10^7 spores/ml and 1 x 10^8 spores/ml respectively. The results (Table 3.4) showed that with the increase in inoculum size from 1x 10^5 to 1x10^7 spores/ml, there was significant ($p < 0.05$) increase in enzyme activity from 1.83 to 2.08 IU/ml of alpha-amylase and 0.21 to 0.28 IU/ml of glucoamylase respectively. Thus, inoculum size of 1×10^7 spores/ml was optimum for maximum alpha-amylase and glucoamylase activities. Further increase of inoculum to 1 x 10^8 spores/ml led to a decrease in enzyme production. A higher inoculum size may increase moisture content and lead to a decrease in growth and enzyme production. A lower inoculum size may require a longer time for fermentation to form the desired product[4].Soccol*et al*[42]used cassava as substrate for the synthesis of alpha-amylase and glucoamylase by *Rhizopus oryzae* in solid state fermentation. Maximum alpha-amylase and glucoamylase activity obtained on cooked cassava was with a spore suspension of 2×10^9 spores/ml.

Table 3.4 Effect of inoculum concentration on α -amylase and glucoamylase activity

Enzyme activity (IU/ml)		
Inoculum concentration	**Alpha amylase**	**Glucoamylase**
1 x 10^5	1.83±0.01	0.21±0.02
1 x 10^6	1.94±0.02	0.25±0.01
1 x 10^7	2.08±0.02	0.28±0.02
1 x 10^8	1.90±0.01	0.23±0.03

Medium-10 ml mineral medium, Substrate-5 g, Temperature 30^0 C, pH 5.5, Incubation time- 5days,
P (<0.05) Values are mean of triplicate samples± Standard Error (n=2)

Effect of incubation period on α -amylase and glucoamylase activity

Incubation period plays an important role in substrate utilization and its protein enrichment for enzyme production. The effect of incubation period was evaluated by checking enzyme activities after 3, 4, 5 and 6 days of incubation at 30^0 C. The alpha-amylase and glucoamylase activities were determined after every 24h of incubation in order to determine the optimum incubation period for maximum production of extracellular enzymes. The maximum yield of alpha amylase (2.10 IU/ml) and glucoamylase (0.29 IU/ml) was observed on the fifth day of incubation (Table 3.5). Maximum accumulation of α-amylase occurs during stationary phase. Further increase in incubation period decreased the production of α-amylase.

It might be due to the deficiency of nutrients, accumulation of toxic substances and proteolysis of α-amylase[8,37]. Abu *et al*[1] reported maximum α-amylase production by *Aspergillus niger* after an incubation period of 96 h. Similarly, Ferreira *et al*[14] reported that the amylase production was highest (3.861 IU/ml) on 4th day of incubation period at 35^0C, when wheat bran was fermented in solid state using *Rhizopus oryzae*.

Table 3.5 Effect of incubation period on α -amylase and glucoamylase activity

Enzyme activity (IU/ml)		
Incubation period (days)	**Alpha amylase**	**Glucoamylase**
3	1.83±0.01	0.22±0.03
4	1.96±0.01	0.24±0.01
5	2.10±0.11	0.29±0.01
6	1.88±0.02	0.25±0.08

Medium-10 ml mineral medium, Substrate-5 g, Temperature 30^0 C, pH 5.5,
Inoculum concentration- 1 x 10^7 spores/ml P (<0.05) Values are mean of triplicate samples± Standard Error (n=2)

CONCLUSION

It was demonstrated that *Rhizopus oryzae* has potential for production of fungal enzymes α-amylase and glucoamylase. Various parameters viz. temperature, pH, initial moisture, incubation time and inoculum concentration were studied to optimize the conditions to carry out solid state fermentation of deoiled rice bran by *Rhizopus oryzae.* Under all the optimized cultural conditions: temperature 30^0 C, 67% moisture content (10ml/5g of substrate), pH 5.5, inoculum 1 x 10^7 spores/ml and incubation period of 5 days, yielding an average amylase and glucoamylase activity of 2.08 IU/ml and 0.27 IU/ml respectively. Hence from the present study we conclude that solid state fermentation of the deoiled rice bran is an economical method for enzyme production.

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