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Optimization of process parameters for α-amylase production under solid-state fermentation by A*spergillus awamori* MTCC 9997

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An alpha amylase producing fungal strain was isolated from spoiled food and identified as *Aspergillus awamori*. Production of extracellular α-amylase by *Aspergillus awamori* was studied in solid-state fermentation. Twelve different agro-residues such as wheat bran, maize bran, corn bran, millet bran, rice bran, green gram bran, black gram bran, cassava peel powder, cotton seed oil cake, coconut oil cake, sesame oil cake and groundnut oil cake were screened for α-amylase production using *Aspergillus awamori* MTCC 9997. Among them, cassava peel powder was found to be the best substrate for α-amylase production. The physical and chemical parameters that influence the production were optimized. Maximum α-amylase production was obtained at pH 6 after 96h of fermentation at 40°C. An inoculum level of 10% (volume per mass) was found to be optimum for α-amylase production. Among different carbon and nitrogen sources supplemented, starch and beef extract at 2% concentration enhanced α-amylase production considerably. Calcium chloride at a concentration of 0.8 % was found to stimulate α-amylase production.

Keywords: α-Amylase, Agro-residues, *Aspergillus awamori*, Solid-state fermentation

Introduction

Amylases (1,4-α-D-glucan-4-glucanohydrolase, E.C.3.2.1.1) are extracellular endo-enzymes that randomly hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units[1]. Amylases also play a significant role in starch, detergent, beverage, and textile industries and its commercial production from microorganisms represent 25–33% of the world enzyme market[2]. The major advantages of using microorganisms for production of α-amylases are in economical bulk production capacity. Most commercial α-amylases are derived from the *Aspergillus* genera[3].α-Amylase produced by solid-state fermentation dominates over submerged fermentation in aspects such as better yield, morphology and high stability[4]. Many agro-industrial by-products have been screened as low cost solid substrates for microbial production of α-amylase in solid-state fermentation[5].In the present study, the strain employed is a newly isolated strain of *Aspergillus awamori* MTCC 9997. There are only a few reports on α-amylase production in solid-state fermentation by *Aspergillus awamori*. Hence, the aim of the present study was to identify an effective agro-residue as the substrate for the production of α-amylase and to optimize various factors that influence α-amylase production by *Aspergillus awamori* through solid-state fermentation.

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Materials and Methods

Screening and maintenance of α-amylase producing fungi

A total of 186 fungal isolates from 22 different sources including field soils, waste water discharged soils, effluents and spoiled food sources were screened for α-amylase production on starch agar plates at 28ºC. Amylolytic isolates were selected by flooding the starch agar plates with Gram's iodine solution to visualize starch hydrolysis. Isolates having a higher ratio of clearing zone to colony size were grown in liquid broth and the amount of amylase production was determined from culture filtrate. The fungal isolate, which showed maximum activity, was selected for further study. The selected fungal isolate was grown on rose bengal chloramphenicol agar slants and subcultured periodically after every 15 days and stored at 4ºC.

Preparation of inoculum

A volume of 7ml of sterile distilled water was transferred to a sporulated (7-day-old) rose bengal

chloramphenicol agar slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and the suspension was used as inoculum.

Solid-state fermentation

Five grams of the dried wheat bran was taken in 100 ml Erlenmeyer flask and moistened with 10ml of sterile distilled water. The flask was then autoclaved at 121ºC (15lb) for 15 minutes. After cooling, the flask was inoculated with 1 % inoculum and incubated at 28ºC for 5 days.

Enzyme extraction

The enzyme from the fermented fungal bran was extracted twice with 50 ml of 10 mM phosphate buffer (pH 7.0). Extraction was done by soaking the fermented solids with phosphate buffer for 30 minutes at 30ºC on a rotary shaker at 150 rpm. The slurry was squeezed through a damp cheese cloth. The extracts were pooled and centrifuged at 4ºC for 15 minutes at 5000 rpm to separate small wheat bran particles, cells and spores. The brown clear supernatant was used as the source of α-amylase.

α-Amylase activity

α-Amylase activity was determined by DNS method[6]. One unit of α-amylase activity is defined as the number of μmol of maltose liberated by one ml of enzyme solution per minute. Amylase production was expressed as units per gram of dry substrate.

Screening of agro-residues as substrates for Solid-state fermentation

In an attempt to choose a potential substrate for α-amylase production, various agro-residues like wheat bran, maize bran, corn bran, millet bran, rice bran, greengram bran, blackgram bran, cassava peel powder, cotton seed oil cake, coconut oil cake, sesame oil cake and groundnut oil cake were screened. The fermentation was carried out by taking 5 g each of the selected substrate separately in a 250 ml Erlenmeyer flask, to which 10 ml of distilled water was added. The flasks were inoculated with the fungal inoculum (1 %) and incubated at 28ºC for 96 h. α-Amylase activity was determined in the cell free supernatant.

Effect of process parameters on α-amylase production

Various process parameters influencing α-amylase production were optimized. Various inoculum levels (2, 4, 6, 8, 10 and 12 % by volume per mass) and incubation time (24, 48, 72, 96 and 120 h) were tried to study their effect on enzyme production. To optimize the initial pH of the basal medium, the pH of the moistening agent was varied from 4 to 10 with one unit interval using 0.1N HCl and 0.1N NaOH. An inoculum level of 10 per cent and moisture ratio of 1:2 was employed. The fermentation was carried out at 28ºC for 96 h. After the incubation period, the enzyme was extracted and assayed. The fermentation was carried out at 30, 40, 50,60,70,80 and 90ºC to study their effect on α-amylase production. Carbon sources (0.01 g/g dry substrate) such as starch, maltose, lactose, sucrose, glucose and fructose and nitrogen sources (0.01g/g dry substrate) as yeast extract, peptone, tryptone, casein, beef extract, urea, ammonium sulphate, ammonium chloride and sodium nitrate were supplemented as individual components to the production media to check their effect on enzyme production. Varying concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) of the best inducers were also studied. Influence of different metal salts on α-amylase production was studied by incubating the culture medium with various metal salts, namely, calcium chloride, magnesium sulphate, ferric chloride, manganese sulphate, copper sulphate, mercuric chloride, zinc sulphate, silver chloride, sodium chloride, potassium chloride, lead nitrate and lithium sulphate each at a concentration of 1mM which was mixed with the moistening agent individually. Varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0%) of the best inducer were also studied.

Results and Discussion

A fungal isolate from spoiled cooked rice showed maximum α-amylase activity (44.82 U/ml) and was selected as the potent producer of α-amylase. Based on the colony morphology and characters, the selected fungal strain was identified as *Aspergillus* sp. and further confirmed as *Aspergillus awamori* at the Institute of Microbial Technology (IMTECH), Chandigarh, India and deposited in their culture collection with the accession number MTCC 9997. The physical support and the energy required for a fungus to grow and produce the desired metabolite is primarily provided by a substrate. Among the various inexpensive agro-residues tested, cassava peel powder proved to be the most suitable substrate (Table 1) as indicated by the maximum visible growth of *Aspergillus awamori* on the surface of the substrate and significant yield of α-amylase (31.64 U/gds) as compared to other substrates. This is possibly due to its most suitable particle size and consistency required for anchorage. The fermentation profile of an

organism is usually affected by the initial inoculum concentration. There was a gradual increase in the α-amylase production with increase in inoculum size and significant ($p<0.05$) increase was noticed at 10 per cent v/w (34.20 U/gds) when compared with other inoculum levels. Increase in inoculum level above 10 per cent was found to decline the enzyme production. This might be due to higher concentration of inoculum resulting in increased competition for carbon source and nutrients, which might lead to exhaustion of nutrients. Also the free excess liquid present in an unabsorbed form would have given rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate and might lead to a decrease in growth and enzyme production[7]. Lower inoculum level would have resulted in a lesser number of cells in the production medium. These require a longer time to grow to optimum number to utilize the substrate and form the desired product[8]. The results of the present study are in concurrence with Ellaiah *et al*[9] and Kalaiarasi and Parvatham[10] who reported 10 per cent as optimum inoculum concentration for the production of amylase by *Aspergillus niger* A3 and *Bacillus cereus* respectively.Maximum α-amylase production was observed at 96 h of incubation (Fig.1). When the fermentation period was prolonged after 96 h, there was a decline in enzyme production. This might be due to less moisture content and decomposition of amylase by the interaction with other components in the medium[11]. pH is one of the important factors that determine the growth and enzyme secretion of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium[11]. The optimum pH for α-amylase production by *Aspergillus awamori* was found to be significantly high ($p<0.05$) at pH 6 (42.13 U/gds) when compared to other pH. α-Amylase production by *Aspergillus awamori* increased significantly ($p<0.05$) and reached maximum (33.63 U/gds) at 40°C. Above 40°C, there was a significant decline in α-amylase yield (Fig. 2). This might be due to evaporation of moisture in the substrate.Among the different carbon supplements examined, starch at 2 per cent concentration promoted high enzyme titre (54.65 U/gds). Glucose and fructose supplementation resulted in the repression of enzyme production. Previous studies have also shown that starch is the best supplement for fungal species *Aspergillus oryzae*[13] and *Aspergillus niger* JGI 24[14]. α-Amylase production was significantly ($p< 0.05$) increased when cassava peel powder was supplemented with beef extract at 2.0 per cent concentration (63.84 U/gds) when compared to other nitrogen supplements. Beef extract as nitrogen supplement at 1.0 per cent level increased α-amylase production with *Aspergillus niger* JGI 24[13]. Supplementation of salts of certain metal ions is found to influence the growth of microorganisms and thereby stimulate or inhibit enzyme production. Among the metal ions supplemented, $CaCl_2$ at 0.8%

Table 1—α-Amylase production by *Aspergillus awamori* on selected agro-residues

Agro-residues	α-Amylase activity (U/gds)
Wheat bran	28.95±1.31
Maize bran	16.52±0.88
Corn bran	11.50±1.91
Millet bran	15.41±0.97
Rice bran	20.68±2.57
Greengram bran	16.81±0.66
Blackgram bran	8.87±0.89
Cassava peel powder	31.64±0.81
Cotton seed oil cake	11.62±1.50
Coconut oil cake	19.30±0.44
Sesame oil cake	11.78±0.41
Groundnut oil cake	21.00±0.48

U/gds – Units per gram of dry substrate
Values are mean of three replicates ± SD

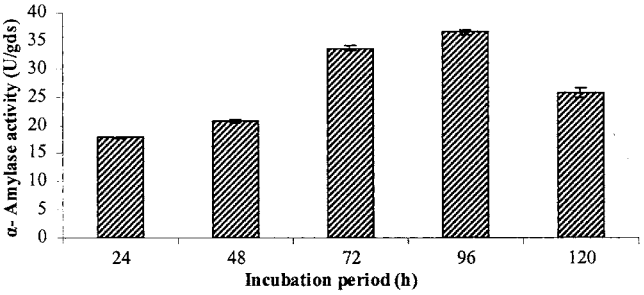

Fig. 1—Effect of incubation period on α- amylase production by *Aspergillus awamori* under SSF using cassava peel powder as substrate

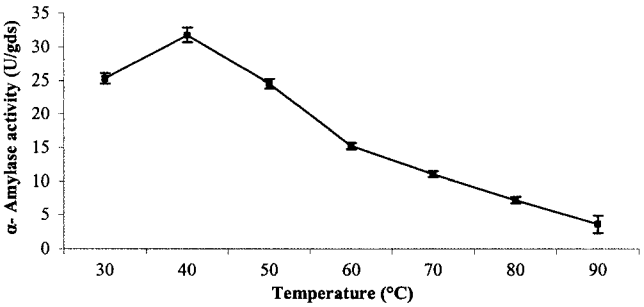

Fig. 2—Effect of temperature on α-amylase production

significantly ($p<0.05$) increased α-amylase production (78.97 U/gds) when compared with other metal salts. $MgSO_4$, $LiSO_4$, NaCl, $MnSO_4$, $FeCl_2$ and KCl stimulated α-amylase production, whereas $AgCl_2$, $PbNO_3$, $HgCl_2$, $CuSO_4$ and $ZnSO_4$ had negative effect on amylase production.

Conclusions

The present study describes the suitability of the laboratory isolate *Aspergillus awamori* for the commercial exploitation using simple, less expensive and economically feasible substrate, cassava peel powder with the supplementation of simple nutrients like starch and beef extract. The maximum productivity of α-amylase was achieved by optimized process parameters such as 10% inoculum concentration, incubation time of 96 h, incubation temperature at 40°C and pH 6.

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