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Alpha amylase production by *Aspergillus oryzae* employing solid-state fermentation

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This study presents production of α-amylase by *Aspergillus oryzae* in solid-state fermentation using 14 agro-industrial wastes as substrate. Enzyme production was growth associated and maximum titers (15095 U/gds) were obtained after 72 h when incubated at 30°C on wheat bran (initial moisture content, 60%; initial medium pH, 5). Enzyme titers increased significantly when the solid medium was supplemented with additional N (sodium nitrate) and C (starch) sources.

Keywords*:* Agro-industrial substrate, Alpha amylase, *Aspergillus oryzae* var *brunneus*, Solid state fermentation

Introduction

Alpha amylase (EC 3.2.1.1) is an extra cellular enzyme, which catalyzes hydrolysis of internal α-1,4-O-glycosidic bonds in starch and related polysaccharides liberating α-anomeric sugars and limit dextrins[1]. Fungal and bacterial amylases are widely used for the commercial applications in food processing industries[2]. Fungal amylases particularly from *Aspergillus* species, find various applications in antistaling (baking industry), haze clarification in fruit juices and alcoholic beverages, glucose and maltose syrup production and other food products[3]. These amylases have a high efficiency in saccharification of starch when compared to bacterial α-amylases[4]. *A. oryzae* has an efficient system for secretion of proteins and is extensively used to produce industrial enzymes[5].

Solid-state fermentation (SSF) is widely established for the production of enzymes by filamentous fungi[6-8]. Morphology and physiology of these molds enable them to penetrate and colonize various solid substrates[9]. SSF utilizes various agro-industrial wastes as substrate that acts both as physical support and source of nutrients[10]. Food and agricultural wastes can serve as substrates for the production of various fermented products and enzymes[11]. SSF offers advantages such as high volumetric productivity, better product recovery and product characteristics, low capital investment, reduced levels of catabolite repression, value addition of agricultural industrial wastes reducing pollution problems and less effluent generation[12].

This study screens a variety of easily available and inexpensive agro-industrial substrates for the production of α-amylase using *A. oryzae* var *brunneus* under SSF.

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Materials and Methods

Microorganism and its Maintenance

A. oryzae var *brunneus* was propagated on potato dextrose agar (PDA) medium (Hi-media, Mumbai, India). Slants were grown at 30°C for 7 days and stored at 4°C, and sub-cultured fortnightly.

Preparation of Inoculum

To the 7 days old culture slants, 10 ml of 0.1% Tween-80 solution was added and the spores were dislodged using an inoculation needle under sterile conditions. Spores in the solution were collected in a sterile flask and the suspension was diluted appropriately for the required spore density. Viable spore density was determined by

the serial dilution of the spore suspension and spread plating.

Solid-state Fermentation

Dry substrate (5 g) taken into an Erlenmeyer flask (250 ml) was added with 2 ml of salt solution containing ((KH_2PO_4 2, NaCl 1, $MgSO_4$, $7H_2O$ 1 g/l and distilled water) to obtain an initial moisture level of 60%, unless specified otherwise. Contents of flasks were mixed and autoclaved at 121°C for 20 min. Spore suspension (1 ml; density, 1×107 spores/ml) was used as the inoculum. Inoculated flasks were incubated at 30°C for 72 h. Substrates obtained from local markets were coconut oil cake (COC), groundnut oil cake (GOC), sesame oil cake (SOC), wheat bran (WB), spent brewing grain (SBG), cassava bagasse (CB), jackfruit seed powder (JSP), tamarind seed powder (TSP), rice bran (RB), palm kernel cake (PKC), olive oil cake (OOC), mustard oil cake (MOC), cotton seed oil cake (CSOC) and rice husk (RH). Combinations (1:1) of significant substrates obtained, supporting alpha amylase production were further screened.

Enzyme Extraction

Crude enzyme was extracted by mixing a known quantity of the fermented matter in 0.1% Tween-80 solution on a rotary shaker at 180 rpm for 60 min. The mixture was centrifuged at 8000×g at 4°C for 10 min. Supernatant was collected and used for enzyme assay. Dry matter of the samples was determined by drying them in a hot air oven at 80°C for 16 h.

Optimization of Cultural Conditions

Various physical and chemical parameters such as fermentation period (24, 48, 72, 96, 120 and 144 h), initial moisture content (45, 50, 55, 60, 65 and 70%), effect of inorganic (0.25M) N (ammonium nitrate, ammonium chloride, ammonium phosphate, ammonium sulphate and sodium nitrate) and organic N (1% w/w) sources (beef extract, corn steep solids, malt extract, peptone, soybean meal, tryptone and yeast extract) and effects of temperature (20, 25, 30, 40 and 45°C) and pH (3, 4, 5, 6, 7, 8 and 9) were studied. The pH of moistening agent (distilled water) were adjusted as per requirement and used for preparing the solid media (pH, 3-9). Different concentrations of the best nitrogen source were incorporated into the medium. The effect of supplementation of additional carbon sources [soluble starch, maltose, glycerol, lactose and sucrose (1%, w/w)] was studied; the optimal concentration of the best source for induction was also studied. All the experiments were conducted in triplicate and values were averaged.

Analytical Methods

Enzyme Assay

Alpha amylase activity was determined[13]. Reaction mixture contained: 1% soluble starch, 1.25; 0.1M acetate buffer (pH 5.0), 0.5; and appropriately diluted crude enzyme extract, 0.25 ml. After 10 min of incubation at 50°C, liberated reducing sugars (glucose equivalents) were estimated by 3,5-dinitrosalicylic acid (DNS) method of Miller[14]. The colour developed was read at 510 nm using a Shimazdu UV-160A spectrophotometer. Glucose was used as the standard. Blank contained: 0.1M acetate buffer (pH 5.0), 0.75; and 1% starch solution, 1.25 ml. One unit (IU) of α-amylase is defined as the amount of enzyme releasing one μmol glucose equivalent per minute under the assay conditions and enzyme activity is expressed in terms of IU per gram dry fermented substrate (U/gds).

Biomass Estimation

Fungal biomass estimation was done by determining N-acetyl glucosamine released by acid hydrolysis of chitin present in cell wall of the fungus. Glucosamine liberated from chitin by acid hydrolysis was mixed with acetyl acetone reagent (1 ml) and incubated in a boiling water bath for 20 min. After cooling, ethanol (6 ml) was added, followed by the addition of Ehrlich's reagent (1 ml) and incubated at 65°C for 10 min. After cooling, optical density was taken at 535 nm against the reagent blank. Glucosamine (Sigma) was used as the standard[15]. Biomass is expressed in terms of milligram of N-acetyl glucosamine released per gram of dry fermented substrate (mg/gds).

Results and Discussion

Evaluation of Agro-industrial Residues as Substrates for SSF

Among 14 substrates screened (Fig. 1), WB gave highest enzyme production (9065 U/gds), which was almost two times higher than that produced by other substrates. WB has been a highly reported substrate producing promising results, among the various agro-industrial substrates used[16-18]. Widespread suitability of WB may be due to the presence of sufficient nutrients and its ability to remain loose even in moist conditions, thus providing a large surface area[19]. Oil cakes [COC (4521 U/gds), GOC (6074 U/gds), and SOC (4581 U/gds)] also yielded significant enzyme yields. Enzyme

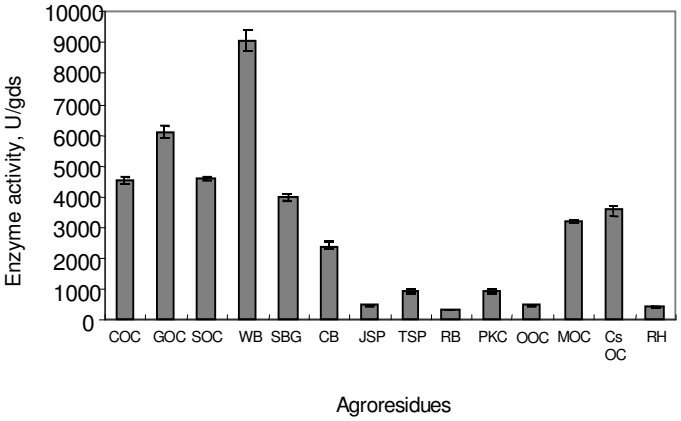
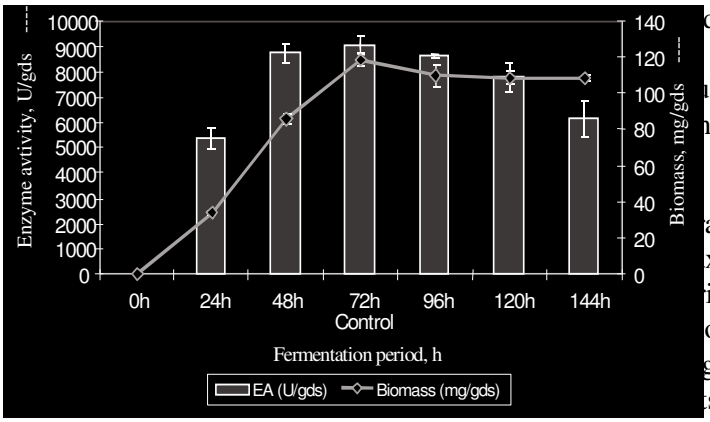


Fig. 1—Screening of agro-industrial residues for the production of α-amylase using *Aspergillus oryzae var brunneus*: COC, coconut oil cake; GOC, groundnut oil cake; SOC, sesame oil cake; WB, wheat bran; SBG, spent brewing grain; CB, cassava bagasse; JSP, jackfruit seed powder; TSP, tamarind seed powder; RB, rice bran; PKC, palm kernel cake; OOC, olive oil cake; MOC, mustard oil cake; CSOC, cotton seed oil cake; and RH, rice husk

production was lower on substrates such as JSP, TSP, RB, OOC and RH. Substrate combinations of COC, GOC, SOC and WB at the ratio 1:1 (w/w) showed that combinations of oil cakes with WB resulted in the



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adually ximum ing the o drop, glucose s in the medium formed during stationary phase[20,21]. *A. oryzae* var *brunneus* was a fast growing fungus with a log phase (up to 72 h), followed by a stationary phase in SSF (Fig. 2). The α-amylase production pattern was associated with the growth phase of the fungus. Association of growth and enzyme synthesis has also been reported by Carlsen *et al*[22].

Effect of Moisture

Alpha amylase production increased with an increase in initial moisture content (Fig. 3) with a maximum at initial moisture of 60%. In most of the cases, 40-70% moisture requirements have been reported for maximum

Fig. 2—Effect of fermentation period on α-amylase production using *Aspergillus oryzae var brunneus*

Table 1—Screening of substrate combinations (1:1) for the production of α-amylase using *Aspergillus oryzae* var *brunneus*

Substrate mixtures(1:1)	Enzyme activity U/gds
COC + GOC	2633 ± 59
GOS +SOC	3882 ± 166
SOC+WB	8235 ± 309
COC+WB	6418 ± 188
GOC+WB	7870 ± 209
COC+SOC	3231 ± 83

growth and substrate utilization[23]. Although the fungal growth occurred at a lower moisture level (45%), it was associated with early sporulation and a significant reduction (32%) in the enzyme yield. This could be due to the non-availability of nutrients as lower moisture content has been known to reduce the solubility of nutrients of the substrate, a lower degree of swelling and high water tension affecting microbial activity. Drastic reduction in enzyme titres occurred at initial moisture content (70%). This was because high moisture level decreased porosity of particles, developed stickiness of substrate resulting in agglomeration, and reduced gas volume and gaseous diffusion resulting in low oxygen transfer[24,25].

Effect of Nitrogen Sources

None of the supplied organic nitrogen sources showed any positive effect on the enzyme production, although all of them promoted good fungal growth (Table 2). Some of the organic nitrogen sources (peptone and yeast extract) resulted in substantial reduction of enzyme yield as excess complex nitrogen turned out to have an adverse

Table 2—Effect of nitrogen sources on α-amylase production using *Aspergillus oryzae* var *brunneus*

Inorganic nitrogen sources, 0.25M	Enzyme activity, U/gds	Organic nitrogen sources, 1% w/w	Enzyme activity U/gds
Control (without any nitrogen source)	9139 ± 256	Beef extract	8275 ± 207
Ammonium nitrate	10033 ± 332	Corn steep solids	7811 ± 263
Ammonium chloride	5436 ± 158	Malt extract	8230 ± 163
Sodium nitrate	10079 ± 358	Peptone	3468 ± 97
Ammonium phosphate	5670 ± 159	Soybean meal	5433 ± 99
Ammonium sulphate	3867 ± 77	Tryptone	5807 ± 190
		Yeast extract	2176 ± 64

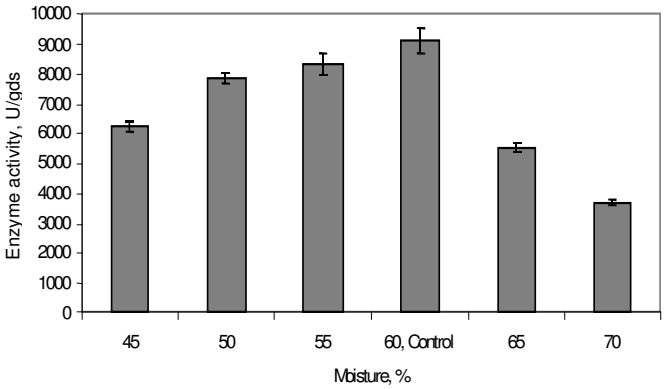

Fig. 3—Effect of moisture on α-amylase production using *Aspergillus oryzae var brunneus*

effect on enzyme synthesis[26]. Inorganic nitrogen additives (ammonium chloride, ammonium phosphate and ammonium sulphate) also exerted negative effect on the microbial activity and resulted in lower enzyme titres. Jin *et al*[27] have reported the insignificant effect of ammonium sulphate and ammonium carbonate on α-amylase production by *A. oryzae*. Supplementation of ammonium nitrate and sodium nitrate (0.25 M) increased enzyme yields marginally. Since both these supplements gave similar enzyme titres, sodium nitrate (0.25 M), which gave higher specific activity (data not given) was selected for further optimization studies. The optimal level of sodium nitrate was observed to be 0.45 M, which resulted in enhancement (30%) in enzyme production in comparison to medium lacking nitrogen source (Fig. 4).

Effect of Inducers

Although lactose[26], glycerol[27] and sucrose have been reported to produce significant induction of enzyme in bacteria, no such effect was observed in enzyme yields by *A. oryzae* (Fig. 5). Alpha amylase synthesis by

Fig. 4—Effect of different concentration of sodium nitrate on α-amylase production using *Aspergillus oryzae var brunneus*

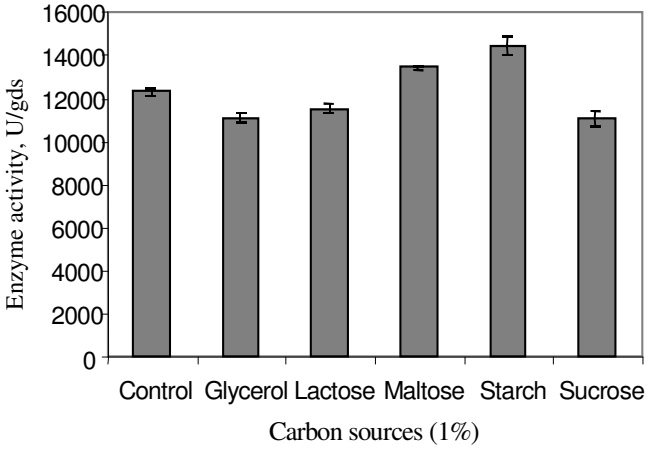

Fig. 5—Effect of inducers on α-amylase production using *Aspergillus oryzae var brunneus*

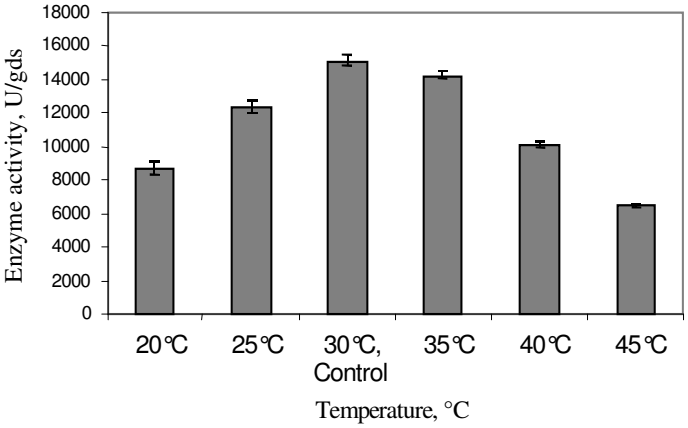

Fig. 6—Effect of temperature on α-amylase production using *Aspergillus oryzae var brunneus*

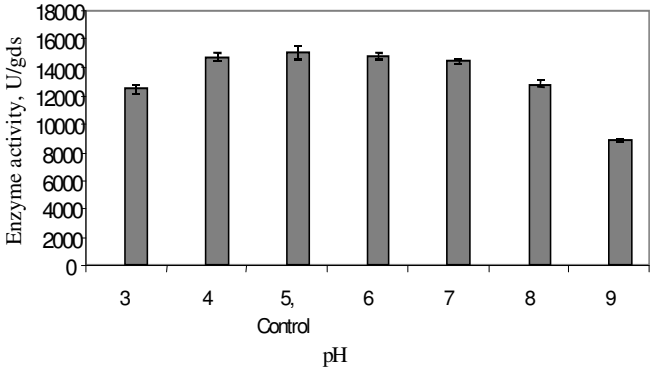

Fig. 7—Effect of pH on α-amylase production using *Aspergillus oryzae var brunneus*

A. oryzae was induced by maltose and starch, which was also reported by Carlsen *et al*[21]. Starch, which gave higher enzyme titres when compared to maltose, was selected as inducer and its various concentrations (0.5-2.5%) were tested for α-amylase production. Maximum enzyme activity was obtained when 2% starch was supplemented to the medium (15016 IU/gds). At 2.5% concentration, enzyme activity marginally decreased to 14967 IU/gds (results not shown).

Effect of Temperature and pH

Enzyme synthesis occurred between 20-45°C with an optimum at 30°C (Fig. 6). A decrease in enzyme titres was observed when temperature range fell outside the mesophilic range. Similar results have also been previously reported for *A. oryzae* by Jin *et al*[28], and Francis *et al*[29]. Each organism possesses a characteristic pH range for its growth and activity with an optimum value in between the range[30]. The pH of culture mainly changes due to the microbial metabolic activities[23]. Enzyme synthesis occurred at the pH range 3-9 (Fig. 7) and optimal enzyme titres were obtained at an initial pH of 5 (control).

Conclusions

Wheat bran possessed good efficiency as a substrate for high yields of α-amylase under SSF because of its high carbohydrate content, suitable texture with significant buffering capacity. Optimal conditions and suitable supplements provided for fermentation resulted in an increase (65 %) in enzyme yields by *A. oryzae var brunneus* indicating excellent capacity of fungal strain in α-amylase production under SSF.

Acknowledgement

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