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Coconut oil cake—a potential raw material for the production of α-amylase

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Abstract

Solid-state fermentation (SSF) was carried out using coconut oil cake (COC) as substrate for the production of α-amylase using a fungal culture of *Aspergillus oryzae*. Raw COC supported the growth of the culture, resulting in the production of 1372 U/gds α-amylase in 24 h. Process optimization using a single parameter mode showed enhanced enzyme titre, which was maximum (1827 U/gds) when SSF was carried out at 30 °C for 72 h using a substrate with 68% initial moisture. Supplementation with glucose and starch further enhanced enzyme titre, which was maximum (1911 U/gds) with 0.5% starch. However, maltose inhibited the enzyme production. Studies on the effect of addition of external organic and inorganic nitrogenous compounds further showed a positive impact on enzyme synthesis by the culture. Increase of 1.7-fold in the enzyme activity (3388 U/gds) was obtained when peptone at 1% concentration was added to the fermentation medium. The enzyme production was growth-related, the activity being the maximum when the fungal biomass was at its peak at 72 h.

Use of COC as raw material for enzyme synthesis could be of great commercial significance. To the best of our knowledge this is the first report on α-amylase production using COC in SSF.

Keywords: α-Amylase; Coconut oil cake; Solid-state fermentation; Process optimization

1. Introduction

The extensive application of amylases in the food industry such as baking, brewing, preparation of digestive aids, production of chocolate cakes, moist cakes, fruit juices, starch syrups, etc., has paved a way for their large-scale commercial production. α-Amylases (endo-1,4-α-D-glucan glucohydrolase EC 3.2.1.1) are extra-cellular endo enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Generally the production has been carried out using submerged fermentation (SmF); however, solid-state fermentation (SSF) systems appear promising due to the natural potential and advantages they offer (Pandey et al., 1999). Agro-industrial residues, which are generally used as substrates for SSF offer potential advantages for the filamentous fungi, which are generally capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium. Application of these agro-industrial residues in bioprocesses also solves pollution problems, which their disposal may otherwise cause (Pandey et al., 2000a,b,c). With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new areas have opened for their utilization as raw materials for the production of value added fine products (Pandey et al., 2001). In addition, the microbial degradation of these residues by GRAS (generally regarded as safe) strain may improve the substrate value as animal feed.

Coconut oil cake (COC) is a byproduct obtained after oil extraction from dried copra. COC is generally fed to animals and finds no other application. It contains starch, soluble sugars, soluble proteins, lipids and trace amounts of nitrogen. SSF has been tried using COC for

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different applications in bioconversion processes. Selvakumar et al. (1998) studied the production of glucoamylase by *Aspergillus niger* under SSF. Phytase, a feed enzyme, has been produced on COC by *Rhizopus oligosporus* (Sabu et al., 2002). *Candida rugosa* was cultivated on COC for lipase production by Benjamin and Pandey (1996). COC served as a good substrate, which not only provided nutrients but also good surface area for proper growth and aeration (Benjamin and Pandey, 1997, 1998).

Aspergillus oryzae has been used in the production of sake, miso, soy sauce, shoyu, amazake, shouchu, mirin (Manabe et al., 1984) and the enzymatic mixture sold under the name of Takadiastase and Polyzime. Morphology and physiology of *A. oryzae* were studied by Carlsen et al. (1996a), while Ozawa et al. (1995) studied the production of kojic acid from *A. oryzae* var. *oryzae* IFO-30103 by the membrane-surface liquid culture method. Sphor et al. (1998) have discussed the growth-associated production of α-amylase in a recombinant strain of *A. oryzae*. Francis et al. (2003) used spent brewing grains (SBG) in SSF for the production of α-amylase using response surface methodology. It has been common to supplement the fermentation media to get enhanced α-amylase activity, such as addition of Tween-80 (Arnesen et al., 1998), mercuric chloride, mercuric iodide, (Sukul et al., 2002), calcium ions (Nirmala and Muralikrishna, 2003), etc.

The objective of this work was to investigate the potential of coconut oil cake as substrate for the production of α-amylase using a GRAS strain of *A. oryzae*. To the best of our knowledge this is the first report on α-amylase production using COC.

2. Methods

2.1. Microorganism, maintenance of culture

A fungal strain of *A. oryzae* (IFO-30103) was used in this study, and was maintained on potato-dextrose-agar (PDA) (Hi-media, Mumbai) medium. The slants were grown at 30 °C for seven days and stored at 4 °C. These were sub-cultured fortnightly.

2.1.1. Viable spore count

The total viable spore number on a PDA slant was determined by colony count technique. The spores were suspended in 10 ml of distilled water with 0.1% Tween-80, using a sterile transfer needle, and diluted serially. One milliliter of spore suspension was poured onto sterile Petri-plates, containing sterile PDA medium and spread uniformly. The inoculated Petri-plates were incubated at 30 °C for 48 h. A plate that developed between 6 and 300 colonies was selected for counting. The spore density was calculated as the count multiplied by the dilution factor.

2.1.2. Preparation of inoculum

Ten milliliter of distilled water containing 0.1% Tween-80 was transferred to a fully sporulated (7 days old) PDA slant culture. The spores were dislodged using the inoculation needle under aseptic conditions and the suspension, with appropriate dilution was used as inoculum.

2.2. Substrate preparation

Coconut oil cake (COC) was used as substrate and was obtained from a local coconut oil manufacturing mill in Trivandrum. Five grams of dry COC was taken into a 250 ml Erlenmeyer flask and to this a salt solution (2 ml) containing (g/l) KH_2PO_4 2 g, NH_4NO_3 5 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 1 g and distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121 °C for 20 min.

2.3. Solid-state fermentation

SSF was carried out to study the effect of various physico-chemical parameters required for the optimum production of α-amylase by *A. oryzae*. Incubation time (24, 48, 72, 96, 120 h), incubation temperature (20, 30, 35, 40, 45 °C), initial moisture content of the substrate (66%, 67%, 68%, 69%, 73%), inoculum size (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 ml) were the parameters optimized. Studies were also performed to evaluate the influence of different carbon sources (glucose, maltose, starch, sucrose, lactose at 2% w/v) and nitrogen sources (peptone, urea, corn-steep solid, sodium nitrate, ammonium sulphate, ammonium nitrate at 1% w/v) when added to the fermentation medium. An experiment was also performed to evaluate the effect of addition of starch (soluble starch, Qualigens, Mumbai). The concentrations used were 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%. Unless otherwise mentioned, SSF was carried out with 2 ml spore suspension (6×10^8 spores/ml) on COC with the initial moisture content adjusted to 66%, and incubated at 30 °C. Each experiment was done in three sets.

2.3.1. Dry weight determination

Dry weight of the samples was determined by drying them in a hot air oven at 80 °C for 24 h.

2.3.2. Enzyme extraction

Crude enzyme was extracted by mixing a known quantity of fermented matter with distilled water containing 0.1% Tween-80 on a rotary shaker (180*g*) for 1 h. The suspension was then centrifuged at 7000*g* at 4 °C for 10 min and the supernatant was used for enzyme assay.

2.3.2.1. α-Amylase assay. α-Amylase activity was determined as Okolo et al. (1995). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50 °C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method of Miller (1959). The colour developed was read at 575 nm using a Shimadzu UV-160A spectrophotometer. Glucose was used as the standard. The blank contained 0.5 ml of 0.1 M acetate buffer (pH 5.0), 1.25 ml of 1% starch solution and 0.25 ml of distilled water. One unit (IU) of α-amylase is defined as the amount of enzyme releasing 1 μmol glucose equivalent per minute under the assay conditions.

2.3.3. Biomass estimation

The fungal biomass estimation was carried out by determining the *N*-acetyl glucosamine released by the acid hydrolysis of the chitin, present in the cell wall of the fungi (Sakurai et al., 1977). Glucosamine released from the chitin by the acid hydrolysis was mixed with 1 ml acetyl acetone reagent and incubated in a boiling water bath for 20 min. After cooling ethanol (6 ml) was added followed by the addition of 1 ml of Ehrlich reagent and incubated at 65 °C for 10 min. After cooling the optical density at 530 nm was taken against the reagent blank. Glucosamine (Sigma) was used as the standard.

3. Results and discussion

Results obtained showed that coconut oil cake served as a good substrate, enabling the growth of *A. oryzae*, which produced a considerable amount of the enzyme. The physico-chemical parameters influenced the production of the enzyme. The results reported are the average of three values and standard deviation was less than 5%.

3.1. Effect of incubation time

SSF was carried out with 2 ml spore suspension (6×10^8 spores/ml) on COC with the initial moisture content adjusted to 66% at 30 °C. The enzyme assays carried out with the extract collected from the fermented samples revealed a growth-related production of α-amylase. After 24 h of incubation, 1372 U/gds of the enzyme was produced, which exponentially increased to 1752 U/gds after 72 h. Incubation beyond 72 h was undesirable as this resulted in decreased enzyme yields (Fig. 1). The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium (Ramesh and Lonsane, 1987). It could have been also be due to the

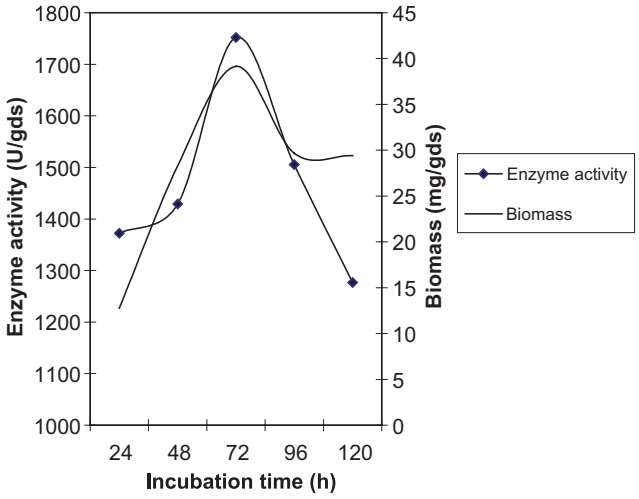

Fig. 1. Effect of incubation period on growth and enzyme production by *A. oryzae* in SSF (–◆–) α-amylase (—) biomass.

fact that the microorganism was on its exponential phase during the third day of fermentation and resulted in the maximum production of enzyme. At the later stage, when nutrients were depleted, it reached its stationary phase and could have started producing secondary metabolites, resulting in a lower yield of enzyme.

Biomass estimated was related to the enzyme producing capability of the fungal strain. During the first 24 h of fermentation, the microorganism was at its log phase, which extended up to further 48 h. Interestingly, after 72 h, there was a slight decline in the growth curve, followed by a period when growth did not increase, which indicated that the fungus was its stationary phase from 72 to 120 h. Enzyme production was maximum when the fungus was at its peak log phase. Sphor et al. (1998) have reported on the growth-associated production of α-amylase in a recombinant *A. oryzae*. Carlsen et al. (1996b) have also observed that the specific α-amylase production was closely coupled to the growth of the fungus with a low α-amylase production rate at low specific growth rates, suggesting that growth of the mycelium is crucial for high production of extra-cellular protein.

3.2. Effect of incubation temperature

SSF was carried out with 2 ml spore suspension (6×10^8 spores/ml) on COC with the initial moisture content adjusted to 66% for 72 h to evaluate the effect of incubation temperatures on the enzyme production. Variation of the temperature brought about a change in metabolic pattern of the microorganism; it exhibited its best enzyme production in the mesophilic range (data not shown). Temperature is one of the important factors, which strongly affect the SSF process (Pandey, 1992; Pandey et al., 2000). It has been reported that

during microbial cultivation in SSF, the temperature of the fermenting bed increased, which exerted harmful effects on the microbial activity (Pandey, 1990). In the present study, 30 °C proved to be the best temperature for the enzyme synthesis. Incubation at higher temperature affected the fungus harmfully, which reflected on the enzyme synthesis, which was almost 3-fold less at 45 °C than at 30 °C (results not shown). During SSF, a large amount of heat is generated which is directly proportional to the metabolic activities of microorganism (Pandey, 1990).

3.3. Effect of initial moisture content of substrate

Variation in initial moisture content of substrate showed that the enzyme synthesis was related to the availability of moisture. Substrate moisture is a crucial factor in SSF and its importance for enzyme production has been well established. With the initial moisture content of 66%, α-amylase yield was 1332 U/gds, which considerably increased with increase in moisture content. The maximum yield was at 68% (1827 U/gds) (results not shown). Higher moisture level decreases porosity, promotes development of stickiness, increases the chances of contamination (Lonsane et al., 1985).

3.4. Effect of inoculum size

Lower inoculum size required longer time for the cells to multiply to sufficient number to utilize the substrate and produce the desired product. An increase in the number of spores in inoculum would ensure a rapid proliferation and biomass synthesis. A balance between the proliferating biomass and available nutrient would yield an optimum at which the enzyme synthesis would be maximum. This was evident as the strain showed increased enzyme production with the increase in inoculum size from the lowest value of 0.5 ml and showed maximum enzyme activity (1857 U/gds) at 2 ml inoculum (6×10^8 spores/ml). However, further increase in the inoculum size resulted in decreased enzyme synthesis, indicating that limitation of nutrients occurred due to the increased microbial activity (results not shown).

3.5. Effect of supplementation of COC with carbon sources

The supplementation of COC with the different carbon sources; starch, glucose, sucrose, maltose, lactose at 2% (w/v) concentration showed marginal increased production of the enzyme with starch (1887 U/gds) and glucose (1878 U/gds) in comparison to 1852 U/gds in control (Fig. 2). Agger et al. (2002) have reported that starch was the best inducer for α-amylase production in TA1 strain of *Aspergillus nidulans*, which was comparable with only glucose. As shown in Fig. 2, addition of

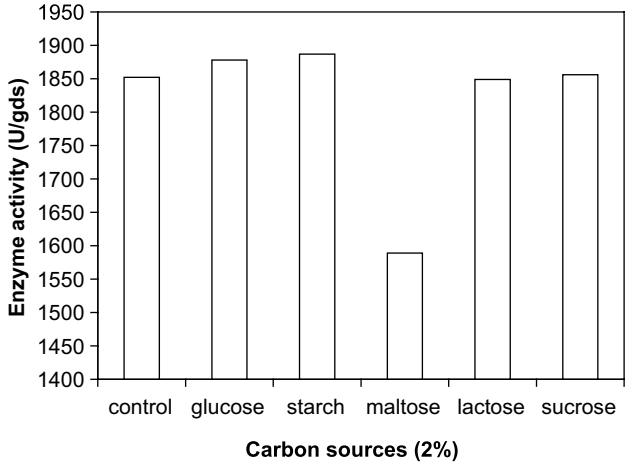

Fig. 2. Effect of carbon sources on α-amylase production by *A. oryzae* in SSF.

maltose to COC resulted in decreased enzyme activities. Yoo et al. (1988) also documented that maltose was found to have no induction on the excretion of α-amylase. Since carbon source represents the energetic source that is available for the growth of the microorganism, it could be that the enzyme production was growth associated and the presence of starch in the medium stimulated the increased production of the enzyme. Other two compounds, i.e., sucrose and lactose did not exert any impact on the culture's activity of enzyme synthesis.

Since supplementation of starch resulted in marginally increased activities in comparison to glucose, SSF was carried out with different concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%) of starch at 30 °C for 72 h with the inoculum size of 2 ml. Results showed that 0.5% of starch was most suitable, which yielded 1911 U/gds enzyme. Higher concentrations of starch resulted in the inhibition of enzyme synthesis (data not shown).

3.6. Effect of supplementation of nitrogen source

Studies on supplementation of inorganic and organic nitrogen sources such as peptone, corn steep solid, urea, sodium nitrate, ammonium sulphate, ammonium nitrate at 1% concentration to the coconut oil cake showed a mixed trend on enzyme production (Fig. 3). Among the organic sources, supplementation of peptone showed a 3-fold increase in the enzyme activity (3388 U/gds), which proved to be the best among all the nitrogen sources. Ammonium salts also enhanced the enzyme activity relatively. Sodium nitrate showed a negative influence, showing a steep decrease in α-amylase activity (513.6 U/gds) as shown in Fig. 3. Pederson and Nielsen (2000) also reported that nitrate was inferior to ammonia in α-amylase production.

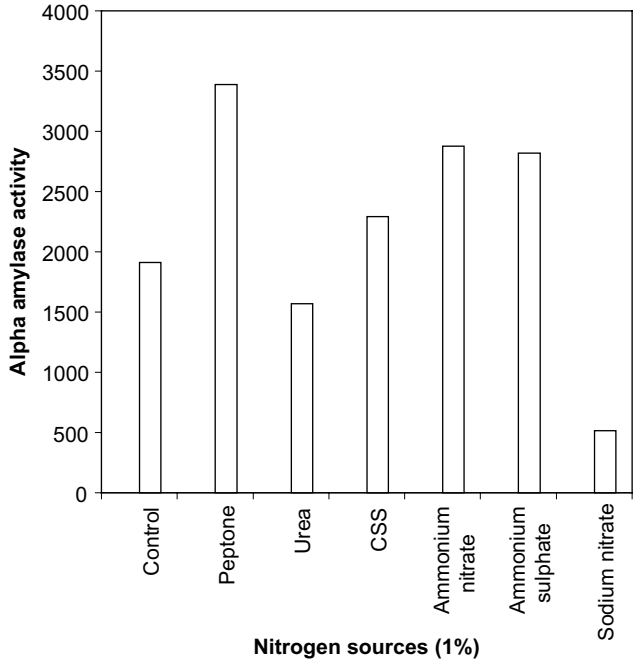

Fig. 3. Effect of nitrogen sources on α-amylase production by *A. oryzae* in SSF.

4. Conclusions

These studies showed that COC could be a good substrate for α-amylase synthesis by fungal culture of *A. oryzae*. Evidently COC provided necessary nutrients for the microorganism to grow and synthesize the enzyme. Supplementation with 0.5% starch and 1% peptone to the substrate positively enhanced the enzyme synthesis producing 3388 U/gds, proving COC a promising substrate for its production.

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