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Biosynthesis of Lipase by *Rhizopus oligosporous* ISU^{UV}-16 using Agricultural By-products as Substrate

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Abstract: The present study is concerned with the biosynthesis of lipase by *Rhizopus oligosporous* ISU^{UV}-16 through solid-state fermentation. The optimal substrate for the biosynthesis of lipase was almond meal at the level of 10 g per 250 ml Erlenmeyer flask. The optimal lipase production was obtained when incubated at 30°C, for 48 h. Maximal lipase activity of 54 Ug^{-1} was reached at 1 % level of inoculum. Water was used as diluent for enzyme extraction from fermented mash. Substrate to diluent ratio was kept at 1:0.7.

Key words: Lipases, agricultural by-products, fermentation and *Rhizopus oligosporous*

Introduction

Lipases are a kind of esterases characterized by the unique ability to act upon emulsified substrate and hydrolyze glycerides to fatty acid and glycerol (Bigey *et al.*, 2003). Lipases have received considerable research interest because of their efficient use in transesterification of triacylglycerols, resolution of racemic mixtures and the synthesis of ester and peptide bonds (Hsu *et al.*, 2002). Lipases are hydrolytic enzymes produced by microorganisms as well as by plants and animal tissue. Fungi such as *Aspergillus, Rhizopus, Mucor, Gcotrichum, Penicillium* and *Candida* are potential sources of lipases production (Toide *et al.*, 1998). Lipase can be produced by submerged fermentation and solid-state fermentation. Christen *et al.* (1998) compared the yield of lipase obtained from solid-state and submerged fermentation and it was concluded that solid-state fermentation gave maximum lipase production. Among different fungi *Rhizopus oligosporous*, showed higher productivity of lipases as reported by Toshihiko *et al.* (1989). Different substrates have been used for the production of lipase in solid-state fermentation. Benjamin and Pandey (1997) reported the production of extracellular lipase by *Candida relgosa* on coconut cake as substrate to obtain maximum yield. Bhusnan *et al.* (1994) reported the production of lipase from an alkalophilic yeast species by solid-state fermentation using rice and wheat as an alternative cheep solid substrate. Korn and Fujio (1997) reported the best production of *Rhizopus oligosporous* at 48 hours and 30°C and used soybean meal as solid substrate. Ushio *et al.* (1996) optimized the inoculum size for maximum lipase production. The present study is concerned with the biosynthesis of lipase by *Rhizopus oligosporous* using agricultural by product as substrates.

Materials and Methods

Organism and Culture Maintenance: *Rhizopus oligosporous* ISU^{UV}-16, a UV treated mutant was used in present study. The culture was maintained on agar malt medium.

Substrates used: Different agricultural by products were used in the present study such as almond meal, sunflower meal, wheat bran, rice husk and soyabean meal.

Fermentation Technique: Production of fungal lipase was studied by solid-state fermentation technique (Korn and Fujio, 1997). Ten grams of substrate with 7 ml of diluent was added in 250 ml cotton wool plugged conical flask.The flasks were autoclaved at 15 lb $inch^{-2}$ pressure (121°C) for 15 minutes and cooled at room temperature. One ml of the spore suspension (2.7x 10^6 spores $m^{-1}l$) was aseptically transferred to each flask and flasks were then placed in an incubator at 30 ± 2°C for 48 hours. The flasks were run parallel in triplicates.

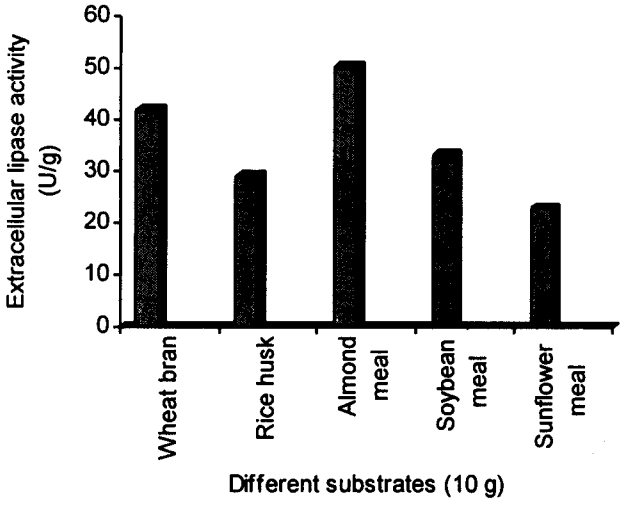
Assay Protocol

Enzyme Extraction: After 48 hours 100 ml of phosphate buffer (pH 7.0) was added to each flask. The flasks were rotated at the rotary shaker at 200 rpm for one hour at 30°C. After one hour the ingredients of the flask were filtered and filtrate was used for estimation of lipase activity.

Lipase Activity: A unit lipase is defined as "the amount of enzyme, which release one micromole fatty acid per minute under specified assay conditions". Lipase activity Ug^{-1} in the fermented meal was determined titrimetrically on the basis of olive oil hydrolysis (Kundu and Pal, 1970). One ml of culture supernatant was added to the reaction mixture containing 10% homogenized olive oil in 10% gum acacia, 0.6% $CaCl_2$ solution and phosphate buffer (pH 7.0). Liberated fatty acid was titrated against 0.1N NaOH using phenolphthalein as indicator. The end point was pink color.

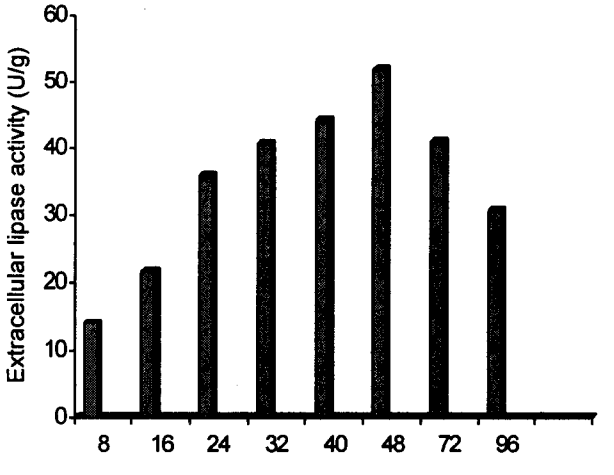
Results

Selection of substrates: For the production of lipase by *Rhizopus oligosporous* ISU^{UV}-16, different agricultural by-products were evaluated (Fig. 1). The maximum production (50.2 Ug^{-1}) of lipase was found when Almond meal was used for fermentation. The other substrates such as wheat bran, rice husk, soybean meal and sunflower meal gave 41.79 Ug^{-1}, 29.03 Ug^{-1}, 33 Ug^{-1} and 23 Ug^{-1}, respectively. Almond meal, which gave maximum production of lipase, was selected for further studies.



Temperature 30°C, Incubation period 48 hours

Fig. 1: Selection of Substrate for the production of lipase by *Rhizopus oligosporous* ISU^{UV}-16

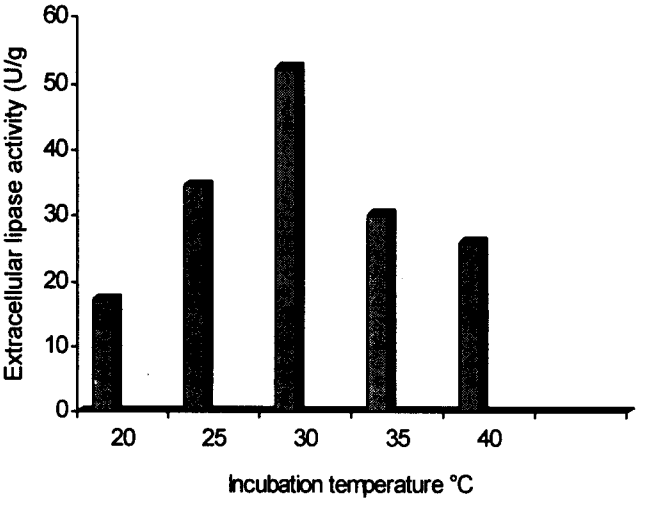


Substrate Almond meal (10 g), Temperature 30°C

Fig. 2: Incubation time for the production of lipase by *Rhizopus oligosporous* ISU^{UV}-16

Effect of Incubation Period: *Rhizopus oligosporous* ISU^{UV}-16 wass studied under different incubation period for the production of lipase (Fig. 2). The samples were incubated at different time intervals as 8, 16, 24, 32, 40, 48, 72 and 92 hours. After 24 hours incubation, enzyme was 32.72 Ug^{-1} because it was the starting point for enzyme synthesis. The maximum production of lipase was obtained at 48 hours (51.96). After 48 hours there was a gradual decrease in lipase production. Incubation period of 48 hours was optimized for further studies.

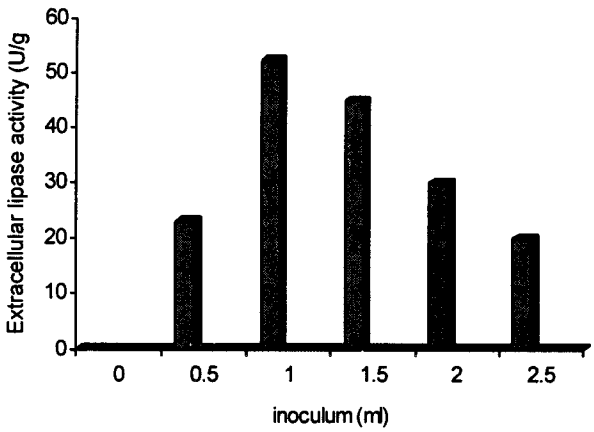
Effect of Temperature: Lipase activity at different incubation temperature (20–40°C) by *Rhizopus oligosporous* ISU^{UV}-16 was studied (Fig. 3). The fermentation medium was incubated at 20, 25, 30, 35 and 40°C. Maximum amount of lipase enzyme was reached (52.14 Ug^{-1}) when flasks were incubated at 30°C. By further increase or decrease in temperature. The enzyme activity was decreased, 17.20, 34.6, 41.40 and 25.8 Ug^{-1} at 20, 25, 35 and 40°C, respectively, it may be because lipase is sensitive to temperature. Thus incubation temperature of 30 ± 2°C was optimized for lipase production by solid-state fermentation.



Substrate Almond meal (10 g), Incubation time 48 h.

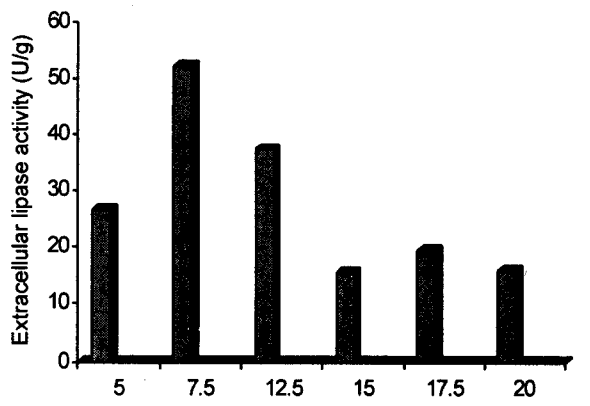
Fig. 3: Incubation temperature for the production of lipase by *Rhizopus oligosporous* ISU^{UV}-16

Effect of Size of Inoculum: Size of inoculum has great influence on the production of lipase. The production of lipase by *Rhizopus oligosporous* ISU^{UV}-16 is greatly effected by change in inoculum size. The size of inoculum was ranged from 0.5–2.5 ml (Fig. 4). Maximum lipase production was obtained(52.17 Ug^{-1}) when 10 g of substrate was inoculated with 1.0 ml of



Substrate Almond meal (10 g), Incubation time 48 h, Incubation temperature 30°C

Fig. 4: Inoculum size for the production of lipase by *Rhizopus oligosporous* ISU^{UV}-16



Substrate Almond meal (10 g), Incubation time 48 h, Incubation temperature 30°C, inoculum size 1.0 ml/10 g substrate

Fig.5 : Diluent amount for the production of lipase by *Rhizopus oligosporous* ISU^{UV}-16.

inoculum. As the size of inoculum increased the lipase production gradually decreased. Hence 1.0 ml of inoculum was optimized for maximum lipase activity.

Effect of different Substrate to Diluent Ratio: Different amount of substrate to diluent ratio has greater influence on the lipase activity by *Rhizopus oligosporous* ISU^{UV}-16. Maximum lipase activity (52.18 Ug^{-1}) was observed when 10 g of substrate were moistened with 7.5 ml of distilled water (Fig. 5).

Discussion

Different agricultural by-products were used as substrate and tested with regards to their effect on the lipase production. Almond meal gave significantly highest enzyme activity (50.20 Ug^{-1}), as compared to other substrates. Almond meal contained gum, asparagin, sucrose and 20% protein (Wallis, 1985). Thus, it was found to be the best source of carbon and nitrogen. Other substrates may not fulfill the nutritional needs of the organism. Hou and Johnston (1992) also used different agricultural by-products such as wheat bran, rice husk etc for the production of extracellular lipase. Incubation period also affects the lipase production. The maximum production of lipase was obtained (51.96 Ug^{-1}) when flasks were incubated for 48 hours. With the increase of incubation period there was gradual decrease in lipase production. It might be due to the exhaustion of nutrients in substrate, which resulted in the inactivation of enzyme. This finding is in accordance with Martinez *et al.*, 1993 and Korn and Fujio, 1997.

Temperature also plays an important role in the metabolic processes of an organism. Increasing temperature increased the rate of all physiological processes but beyond certain limits it started decreasing. A range of 20°C to 40°C was employed in the present study. Maximum lipase activity (52.14 Ug^{-1}) was achieved at 30°C. Thus, the incubation temperature of 30 ± 2°C was optimum for lipase production by solid-state fermentation. Decrease in lipase production can be associated to either decrease in fungal growth or inactive nature of enzyme itself (Lui and Chi, 1997).

The number of fungal spores in inoculum has great influence on the production of lipase. Highest yield (52.17 Ug^{-1}) at 1.0 ml of inoculum size may be due to adequate amount of mycelium produced, which synthesizes optimum level of enzyme. As the amount of mycelium increased, it consumed majority of the substrate for growth purpose, hence enzyme synthesis decreased, Ushio *et al.*, 1996 also optimized 1.0 ml of inoculum for maximum lipase production.

The concentration of substrate moistening is also an important factor. Ten gram of substrate was moistened with different volumes of diluent. *Rhizopus oligosporous* ISU^{UV}-16 exhibited maximum lipolytic activity (52.18 Ug^{-1}) when substrate to diluents ratio was 1:07. As the ratio was changed, the production of enzyme was decreased. With the increase of moisture content there was decrease in enzyme formation. It may be due to the reduction in oxygen supply for the fungus. Similar type of work has also been reported by Benjamin and Pandey (1997), in which they used 70% moisture content in coconut cake and the yield of enzyme was 25.0 Ug^{-1} without addition of nutrients to substrate.

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