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OPTIMIZATION OF CULTURAL CONDITIONS FOR THE PRODUCTION OF LIPASE BY SUBMERGED CULTURE OF *RHIZOPUS OLIGOSPOROUS* T^{UV} -31

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Abstract

The present study is concerned with the optimization of cultural conditions for the production of lipases by *Rhizopus oligosporous* $T^{UV}31$. Studies were carried out in 250 ml Erlenmeyer flasks by submerged fermentation. A medium M 5 containing peptone (30 g/l), glucose (10 g/l), KH_2PO_4 (2 g/l), $NaNO_3$ (0.5 g/l), KCl (0.5 g/l), $MgSO_4.7H_2O$ (0.5 g/l) was found suitable for optimum yield of lipase. The production of the enzymes reached maximum, 48 hours after inoculation at 30°C. The optimum inoculum size was 1.0 ml (4.63 X 10^7 spores) of fermentation medium. Maximum extracellular and intracellular lipase activities were found to be 2.79 ± 0.15 U/ml and 1.66 ± 0.04 U/g.

Introduction

Lipases (Triacylglycerol acylhydrolases; EC 3.1.1.3) are hydrolytic enzymes extensively used in the hydrolysis and synthesis of esters formed from glycerol and long chain fatty acids. Fungi like *Aspergillus, Rhizopus, Mucor, Geotrichum, Penicillium and Candida* are potential sources of lipases production (Elibol & Ozer, 2001; Mayordoma *et al.,* 2000; Chahinian *et al.*, 2000). Microbial lipases are both extracellular and intracellular which are produced by various fungi (Haq *et al.*, 1998; Pastou *et al.*, 2000). *Rhizopus oligosporous* showed higher productivity of lipase as reported by Iftikhar & Hussain (2002). Microbial lipases are produced mostly by submerged culture (Ito *et al.,* 2001). Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. Lipase production is influenced by the culture pH, the growth temperature and the dissolved oxygen concentration (Elibol & Ozer, 2001).

The amount of mycelia also affects the production of enzyme. Ushio *et al.*, (1996) optimized the inoculum size for the maximum lipase production. Martinez *et al.*, (1993) optimized the conditions to improve the production of extracellular lipase by *Rhizopus delemar*. Lipase activity at 48 hours was 12-fold greater. Korn & Fujio (1997) reported the best production of lipase from *Rhizopus oligosporous* at 48 hours and 30°C where soybean meal was used as solid substrate. Essamiri *et al.,* (1998) studied lipase production by *Rhizopus oryzae* and reported that lipase was most active at pH 8.5 and at 30°C optimum temperature.

Lipases are extremely versatile enzymes showing many interesting properties for industrial applications. They are currently applied in detergent formulations for the removal of fatty stains (Cardenas *et al.,* 2001). Lipases can be applied in fur processing (Rezanka, 1991). The hydrolytic behavior of lipases is applied in oil and fat hydrolysis (Chatterjee *et al.*, 2001). Lipases are used in bakery products for enhancing taste and

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aromatic properties of milk, butter cheese and yogurt (Undurraga *et.al.*, 2001). All these commercial applications make it a potential subject of study. Experiments were carried out for the optimization of cultural conditions for the production of Lipases by submerged culture of *Rhizopus oligosporous* T^{uv}-31.

Materials and Methods

Organism: A UV mutant strain of *Rhizopus oligosporous* T^{uv}-31 obtained from Biotechnology Laboratory, Government College University, Lahore was used.

Culture maintenance: The cultures were maintained on the PDA (potato dextrose agar) 4% (w/v) slants and revived after every month. The culture grown on PDA in screw capped bottle (Mac Conkey bottle) were maintained under liquid paraffin. The cultures were stored in a refrigerator at 4°C.

Fermentation technique

Inoculum preparation: A 5-7 days old culture of *Rhizopus oligosporous* (T^{uv} 31) was used and the spore suspension was prepared in sterilized 0.005% Monoxol.O.T. (Di-Octyl ester of sodium sulphosuccinic acid). One ml spore suspension contained 4.63 X 10^7 spores. Haemocytometer slide was used for counting the spores (Clark *et al.*, 1958).

Fermentation Procedure: Production of fungal lipase was studied by submerged fermentation technique using 250 ml Erlenmeyer flasks. Twenty ml of fermentation medium containing (g/L) Peptone, 30.0; Glucose, 10.0; (Dipotassium hydrogen phosphate) K_2HPO_4, 2.0; (Sodium Nitrate) $NaNO_3$, 0.5; (Potassium Chloride) KCl, 0.5; (Magnesium Sulphate 7-Hydrate) $MgSO_4.7H_2O$, 0.5 at pH 7 was transferred to each cotton wool plugged Erlenmeyer flask. The flasks were sterilized in an autoclave at 15 lb/inch^2 pressure on 121°C for 15 min., and cooled at room temperature. One ml of inoculum was aseptically transferred to each flask. Flasks were then placed on a rotary incubator shaker (Model: GLSC 051.HR.196-11) at 30°C for 48 hours. The agitation rate was kept at 200 rpm (revolution per minute).

Analytical Methods

Dry cell mass determination: To determine the fungal biomass the mycelium was filtered through filter paper (Wattmann 40). It was then washed with distilled water. The washed mycelium was dried at 105 ± 2 ° C to constant mass. It was placed in a dessicator to bring it at room temperature and then mass was determined (Gotleib,1984).

For extracellular lipase assay: After 48 hours the contents of the flasks were centrifuged for 15 min., at the 5000 rpm and supernatant was assayed for extracellular lipase activity.

For intracellular lipase assay: Half of the mycelial mass was then ground with sand using 10 ml of phosphate buffer pH 7 as extractant. The contents were centrifuged at 5000 rpm for 10 min., in order to settle down the sand and supernatant was assayed for intracellular lipase activity.

Assay procedure: Lipase activity in the synthetic media was determined titrimatrically on the basis of olive oil hydrolysis by the modified method of Kundu & Pal (1970). One ml of supernatants for extracellular and intracellular activity were added to assay substrate, containing 10 ml of 10% (v/v) homogenized olive oil in 10% (w/v) gum acacia, 2.0 ml of 0.6% $CaCl_2$ solution and 5 ml of phosphate buffer (pH 7.0). The enzyme substrate mixture was incubated on rotary shaker with 150 rpm at 30°C for one hour. Twenty ml of alcohol: acetone (1:1) mixture was added to the reaction mixture. Liberated fatty acids were titrated with 0.1N NaOH using phenolphthalein as an indicator. The end point was pink colour.

Lipase unit: A lipase unit is defined as "the amount of enzyme which releases one micro mole fatty acid per minute under specified assay conditions".

Statistical study: Statistical analysis was carried out according to Snedecor & Cochran (1980).

The following are the composition of the media M1 to M7 used.

M1 = Starch, 20.0 g/l; NH_4Cl, 5.0 g/l; $MgSO_4.7H_2O$, 0.25 g/l; K_2HPO_4, 0.5 g/l; $CaCO_3$, 5.0 g/l; Yeast Extract, 4.0 g/l, pH:7.

M2 = Glucose, 2.5 g/l; Peptone, 20.0 g/l; NH_4Cl, 1.0 g/l; KH_2PO_4, 1.5 g/l; $MgSO_4.7H_2O$, 0.12 g/l; $FeSO_4.7H_2O$, 0.01 g/l; $ZnSO_4$, 0.01 g/l; $MnSO_4$, 0.01 g/l, pH: 5.8.

M3 = Peptone, 20.0 g/l; Dextrose, 10.0 g/l; Yeast Extract, 5.0 g/l; NaCl, 5.0 g/l, pH: 7.0

M4 = Olive oil, 10.0 g/l; Sucrose, 10.0 g/l; NH_4NO_3, 1.0 g/l; KH_2PO_4, 2.0 g/l; $MgSO_4.7H_2O$, 0.4 g/l; $FeSO_4.7H_2O$,1.0 g/l, pH: 7.0

M5 = Peptone, 30.0 g/l; Glucose, 10.0 g/l; K_2HPO_4, 2.0 g/l; $NaNO_3$, 0.5 g/l; KCl, 0.5 g/l; $MgSO_4.7H_2O$, 0.5 g/l, pH:7.0

M6 = Olive oil, 20.0 g/l; Yeast extract, 1.0 g/l; $NH_4(SO_4)_2$,0.5 g/l; K_2HPO_4, 0.25 g/l; $MgSO_4.7H_2O$, 5.0 g/l; $CaCO_3$,5.0 g/l, pH:7.0

M7 = Olive oil, 20.0 g/l; $NH_4(SO_4)_2$,4.5 g/l; Peptone,5.0 g/l; Urea, 2.0 g/l, pH 4.5.

Results and Discussion

Selection of synthetic media: The data of Fig. 1 shows the production of lipases by *Rhizopus oligosporous* T^{uv} 31 on different synthetic media. Of all media examined, medium M5 gave maximum amount of extracellular 2.55 ± 0.2 U/ml and intracellular activity 0.99 ± 0.15 U/g. It may be due to the optimum amount of nutrients it provides to the fungus. Although M3 gave the maximum amount of dry mycelial mass 3.94 ± 0.20 g but showed poor lipase activity.

Effect of incubation period: Incubation period also affected the lipase production. The maximum extracellular (2.69 ± 0.3 U/ml) and intracellular (1.49 ± 0.15 U/g) production of lipase was obtained when flasks were incubated for 48 hours (Fig. 2) there after a gradual decrease in lipase production was observed. It might be due to the exhaustion of nutrients in substrate, which resulted in the increased mycelial mass with inactivation of enzyme. This finding is in accordance with Martinez *et al.*, (1993); Korn & Fujio, (1997). The maximum mycelial mass (1.09 ± 0.02) was found after 120 hrs of incubation period.

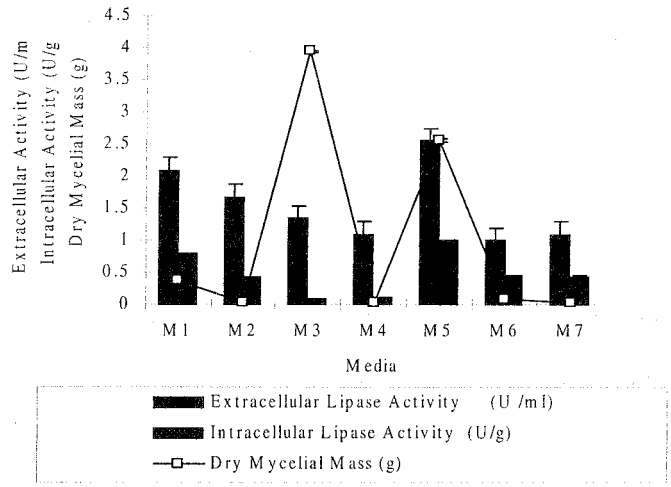

Fig. 1. Selection of synthetic medium for the production of lipase by mutant strain of *Rhizopus oligosporous* $T^{uv}31$.

Each value is an average of three replicates. ± denotes standard deviation among replicates.
Temperature = 30°C
Incubation period = 48 hours.
pH = 7

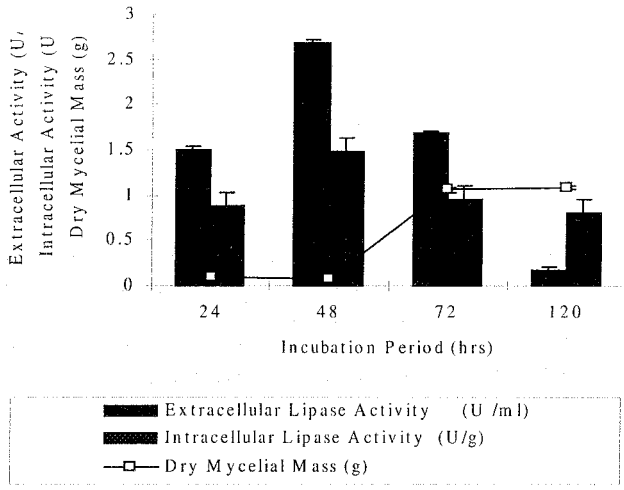

Fig. 2. Effect of incubation period on the production of lipase by mutant strain of *Rhizopus oligosporous* T^{uv}-31.

Each value is an average of three replicates. ± denotes standard deviation among replicates.
Temperature = 30°C
pH = 7

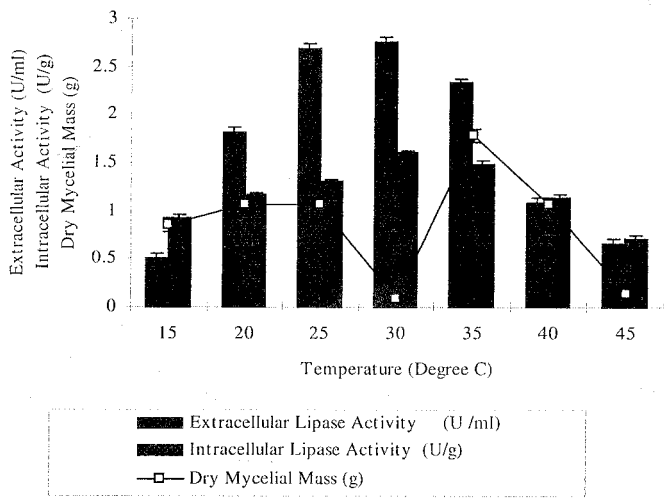

Fig. 3. Effect of temperature on the production of lipase by mutant strain of *Rhizopus oligosporous* T^{uv}-31.

Each value is an average of three replicates. ± denotes standard deviation among replicates.
Incubation period = 48 hours.
Temp. =30oC
pH = 7

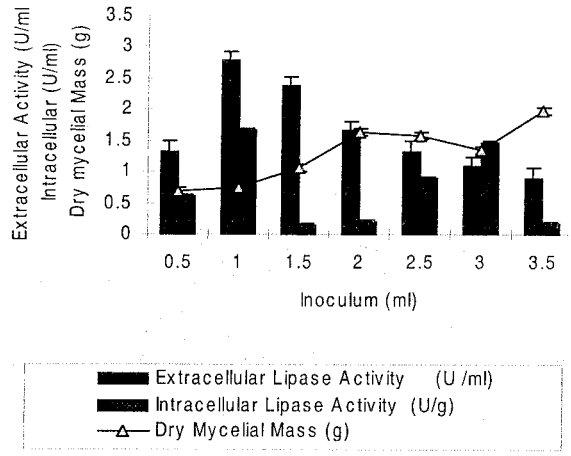

Fig. 4. Effect of inoculum size on the production of lipase by mutant strain of *Rhizopus oligosporous* T^{uv}-31.

Each value is an average of three replicates. ± denotes standard deviation among replicates.
Temperature = 30°C
Incubation period = 48 hours.
pH = 7

Effect of temperature: Temperature also played an important role in the metabolic processes. Increasing temperature increased the rate of all physiological processes but beyond certain limits it started decreasing. A range of 20°C to 45°C was employed in the present study. Maximum extracellular (2.76 ± 0.05 U/ml) and intracellular (1.6 ± 0.03 U/g) lipase activity was observed at 30°C (Fig. 3), while dry cell mass (1.78 ± 0.07 g) was found to be maximum at 35°C. Thus the incubation temperature of 30 ± 2°C was found optimum for lipase production by submerged fermentation. Decrease in lipase production can be associated to either decrease in fungal growth or inactive nature of enzyme itself. Similar results have been reported by Lui *et al.*, (1995).

Effect of inoculum size: The number of fungal cells in inoculum had great influence on the production of lipase. The size of inoculum ranged from 0.5-2.5 ml with an interval of 0.5ml for the production of lipase by mutant strain of *Rhizopus oligosporous* T^{uv}-31. Highest extracellular (2.79 ± 0.15 U/ml) and intracellular (1.66 ± 0.04 U/g) activities was found at 1.0 ml of inoculum size which may be due to adequate amount of mycelium produced, which synthesized optimum level of enzyme (Fig. 4). At 1.0 ml of inoculum the dry mycelial mass was found to be 0.76 ± 0.06 g. As the amount of mycelium increased, it consumed majority of the substrate for growth purpose, hence enzyme synthesis decreased with corresponding increase in mycelial mass. Ushio *et al.*, (1996) also observed 1.0 ml of inoculum for maximum lipase production.

The present study indicated that mutant strain *Rhizopus oligosporous* T^{uv} 31 showed optimum lipase production at 30°C after 48 hrs of incubation with 1 ml of inoculum at pH 7. The highest extracellular (2.79 ± 0.15 U/ml, intracellular units (1.66 ± 0.04 U/g) and dry cell mass (1.78 ± 0.07 g) obtained in study are higher than any previous work done in Biotechnology Laboratory Govt. College University Lahore.

We can further improve the production of lipases substantially by addition of carbon, nitrogen and phosphorous salts into the media and by the mutation of the strain with NTG.

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