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A comparative study on alkaline lipase production by a newly isolated *Aspergillus fumigatus* MTCC 9657 in submerged and solid-state fermentation using economically and industrially feasible substrate

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Abstract: A comparative study of solid-state fermentation (SSF) and submerged fermentation (SmF) for the production of alkaline lipase from a new isolate, *Aspergillus fumigatus* MTCC 9657, using defatted rice bran (DFRB) as a substrate was studied. Different process parameters, such as incubation period, initial pH and incubation temperature, were optimised to achieve the maximum yield of the alkaline lipase. Maximum enzyme production (8.13 U/mL) was obtained by day 7 of incubation in SSF compared to day 4 in SmF at an optimum pH of 8.5 and ambient temperature of 30 °C. Lipase produced by SSF was stable over a period of 15 days, whereas lipase production in SmF decreased by day 5. Lipase production in SSF with DFRB using sterile water as a moisture source exhibited more activity (577.5 U/70 mL) than that supplemented with mineral medium. Compared to the cost of culture medium, the solid-state substrate DFRB is inexpensive, and therefore this process is industrially and economically feasible. These results confirm the interesting potential of SSF in DFRB without any additional nutritional supplement.

Key words: Defatted rice bran, *Aspergillus fumigatus* MTCC 9657, alkaline lipase, SSF

Introduction

Lipases are hydrolytic enzymes that catalyse the cleavage of ester bonds in triglycerides, producing glycerol and free fatty acids. Fungal and bacterial lipases, such as most other types of industrial enzymes, can be obtained either by submerged fermentation (SmF) or solid-state fermentation (SSF). The technique of SSF involves the growth and metabolism of micro-organisms on moist solids without any free flowing water. SSF has been reassessed in recent years, especially because of the possibility of using low-cost agro-industrial residues with lower amounts of water, which consequently releases negligible or considerably lower quantities of effluent, thus reducing pollution concerns (1,2). Current trends in SSF have focused on the application of SSF for the production of value-added products such as biologically active secondary metabolites, including enzymes. The lipases produced by microbes such as bacteria, fungi and yeast are most suitable for industrial applications because of their ease of production and relatively inexpensive fermentation techniques. Lipases from filamentous fungi are preferably used for industrial applications due to the feasibility of obtaining them in high concentrations by SSF processes (3). However, the requirement of a lipid carbon source remains essential for enzyme production. The utilization of by-products and

waste from food and industrial sources has several advantages over SmF, such as superior productivity, simpler techniques, reduced energy requirements, improved product recovery and reduced production costs since they supply the micro-organisms with some nutritive substances (4). The use of cheap raw materials would diminish the operating costs of the process. Moreover, the total capital investment required for lipase production has been reported to be significantly lower in SSF than in SmF (5). SSF has some limitations, such as the limited choice of micro-organisms capable of growth under reduced moisture conditions, as well as the control and monitoring of parameters such as temperature, pH, humidity and air flow (6,7). There have been many reports on lipase production by SSF using solid substrates by different species of Aspergillus. However, no reports are available on alkaline lipase production by SSF using *A. fumigatus*.

The objective of this study was the comparison between SSF and SmF on lipase production by *A. fumigatus* MTCC 9657 and the optimisation of the temperature and pH conditions to obtain higher lipase activity.

Materials and methods

Micro-organism and maintenance of culture

A fungal strain isolated from aged and crude rice bran oil was used for the study. Sabouraud dextrose agar slants were used for the maintenance of fungal culture. Fully sporulated slants were stored at 4 °C and subcultured once every 3 weeks.

Substrate

Defatted rice bran (DFRB) was steamed and extruded for oil extraction and heated to 70 °C for desolvatisation. DFRB obtained as a waste after oil extraction was the substrate used in this study.

Production of lipase by SSF

SSF was carried out in 250 mL conical flasks. A 10 g portion of DFRB was mixed in different combinations 1) DFRB + mineral salt medium 2) DFRB + pH 8.5 Tris-HCl buffer and 3) DFRB + sterile distilled water. A final moisture ratio of 2:1 w/v was achieved. The above mixture was inoculated with 10% of a 24-h spore suspension of *A. fumigatus* MTCC 9657 and incubated at 30 °C.

Lipase extraction

Enzyme extraction was carried out by adding 10 mL of 0.2 M Tris-HCl buffer of pH 8.5 to each flask and agitating it in the orbital shaker at 120 rpm for 1 h. The particulate matter was filtered through a muslin cloth and centrifuged at 10,000 rpm for 20 min. The clear supernatant was used for lipase assay. Total protein concentration was estimated by the method of Lowry et al. (8).

Production of lipase by SmF

Media used for SmF consisted of peptone (2 g/100 mL), yeast extract (0.5 g/100 mL), NaCl (0.5 g/100 mL), $Na_2 CO_3$ (0.025 g/100 mL) and olive oil (1 g/100 mL). Cultivation was carried out in 250 mL Erlenmeyer flasks. The medium (100 mL) was inoculated with 2% inoculum. Fermentation was carried out for 7 days in an orbital shaker (120 rpm) at 30 °C. Samples were collected at 24-h intervals and centrifuged at 10,000 rpm for 10 min. The filtrate was considered as a crude enzymatic extract and was used for enzyme assay.

Lipase assay

Lipase activity was determined by incubating a reaction mixture containing 5 mL of olive oil emulsion, 5 mL of 0.1 M Tris-HCl buffer at pH 8.5, and 1.0 mL of the culture filtrate at 30 °C for 20 min, with agitating at 120 rpm (9). After incubation, the reaction was stopped by the addition of 10 mL of acetone and the liberated free fatty acids were titrated with 0.05 N NaOH in the presence of phenolphthalein as an indicator. The blank assays were performed by adding the extract just after the addition of the acetone solution to the flask. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol of fatty acids per minute under assay conditions. All experiments were carried out in triplicate.

Effect of incubation time

The amount of lipase produced was observed daily for a period of 8 days for SmF and 16 days for SSF.

Effect of pH

The effect of pH on lipase production was studied in a pH range of 6 to 9.5 using different buffers: sodium phosphate pH 6-7.5, Tris-HCl pH 8.0-8.5, and glycine NaOH pH 9-9.5.

Effect of temperature

To determine the effect of temperature on lipase activity, incubation was carried out at different temperatures ranging from 30 to 50 °C. Both SSF and SmF were performed at various temperatures and the enzyme assay was performed as described earlier to determine the optimal incubation temperature.

The experiments were performed in triplicate and the results were recorded as means ± SD from the 3 experiments. The results were processed using Microsoft Excel 2007 and error bars were drawn.

Effect of moisture content

The moisture content of the substrate varied from 30% to 70% to study its effect on enzyme production.

Results and discussion

The micro-organism isolated from aged rice bran oil was analysed for lipolytic activity and fungus showing lipolysis at alkaline pH of 8.5 were selected.

The microbial type culture collection (MTCC) unit of the Institute of Microbial Technology, Chandigarh, India, identified the organism as *Aspergillus fumigatus* MTCC 9657 and it was deposited. The microscopic view of the isolate is given in Figure 1. There is no information on alkaline lipase production by *A. fumigatus* isolated from aged rice bran oil using DFRB as a substrate. The fungus was selected on the basis of the utilisation of different substrates like tributyrin, olive oil, Tween 20 and Tween 80. Figure 2 shows the isolate growing on a Tween 80 plate.

Lipase production by SSF with mineral media and sterile water

Lipase production in SSF with DFRB in combination with sterile water as a moisture source had more total activity (577 U) than that supplemented with pH 8.5 Tris buffer and mineral medium (Table 1). DFRB can be used as the sole source of nutrient for lipase production without any additional supplement.

Effect of incubation time

The maximum lipase activity in SmF was observed on day 4 as shown in Figure 3. In the case of SSF, the maximum enzyme production was on day 7 (Figure 4). After day 14 it reduced due to the consumption of the nutrient materials. Lipase produced by SSF was stable for 15 days compared to SmF, and enzyme degradation started on day 5. Enzyme production began around day 2 of fermentation and the maximal activity was recorded after reaching the log phase of growth. This may be due to the fact that lipase production by *A. fumigatus* was partially associated with the growth of the fungal biomass (10).

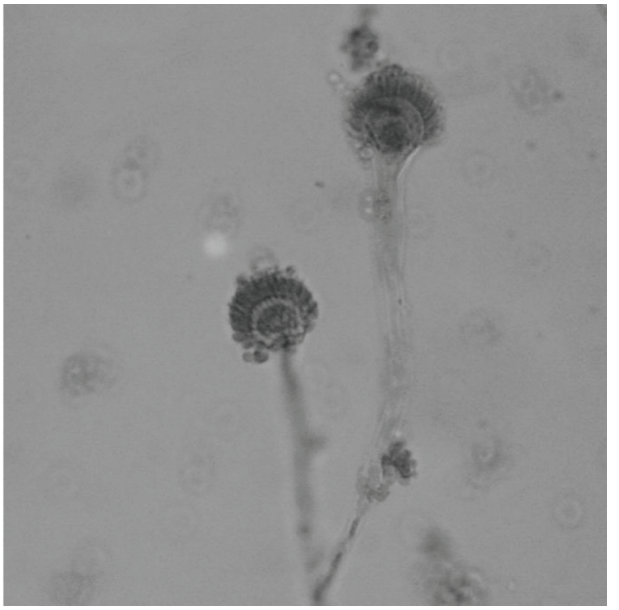

Figure 1. Microscopic view of *Aspergillus fumigatus* MTCC 9657.

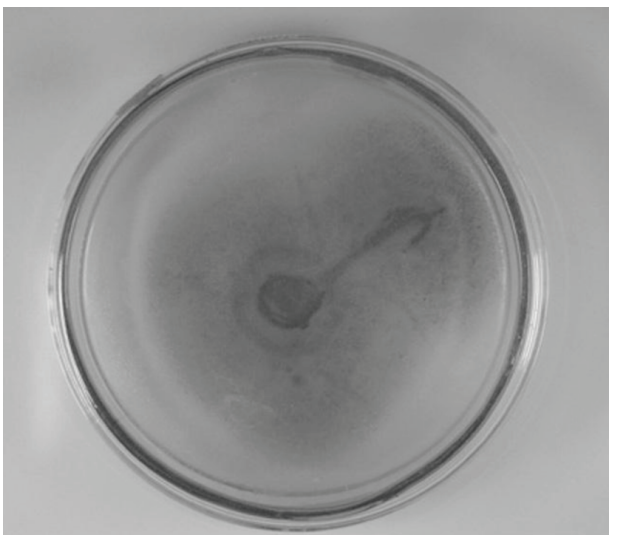

Figure 2. *Aspergillus fumigatus* MTCC 9657 on Tween 80 plate.

Table 1. Comparison of enzyme activity of DFRB.

Combination	Total lipase activity (U)
DFRB + mineral salt medium	490.0
DFRB + pH 8.5 Tris HCl buffer	516.6
DFRB + sterile water	577.5

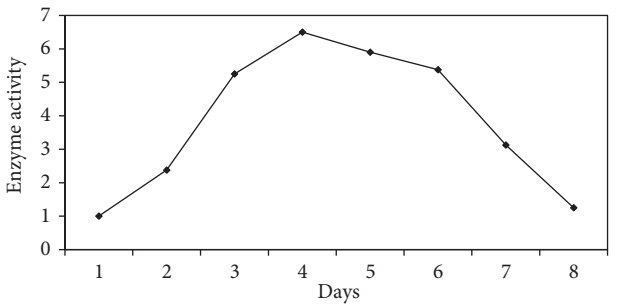

Figure 3. Optimisation of incubation time for submerged fermentation.

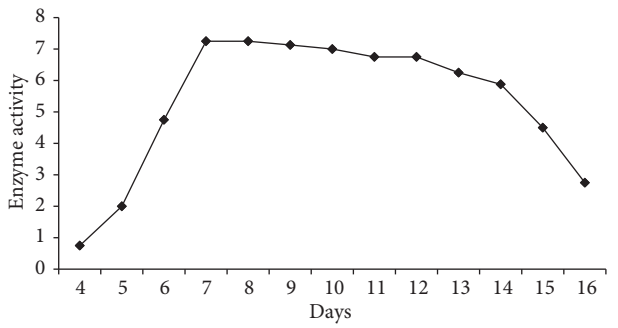

Figure 4. Optimisation of incubation time for solid-state fermentation.

Effect of pH

The maximum enzyme activity was found at pH 8.5 for both SmF and SSF (Figure 5). In the case of SmF there was a sharp decrease in enzyme activity after pH 9.0. However, SSF did not show any sharp decrease in activity. In SmF there is agitation and a change in pH is affected. SSF has a low distribution of molecules and there is no sudden shift in pH.

Effect of temperature

The maximum enzyme activity of 7.88 U was observed at 30 °C. The enzyme was stable at 35 °C, also. Therefore, room temperature was taken as ambient for lipase production (Figure 6). Pinheiro et al. (11) obtained an optimal temperature of 30

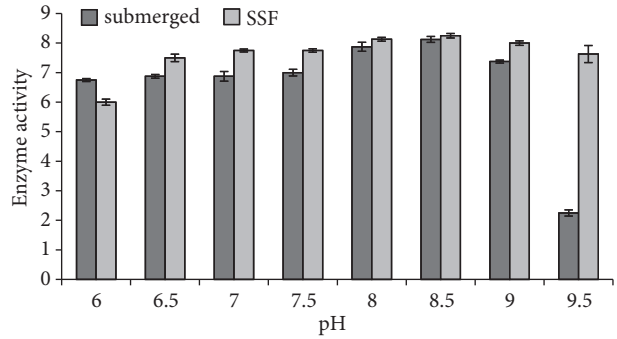

Figure 5. Effect of pH on enzyme activity.

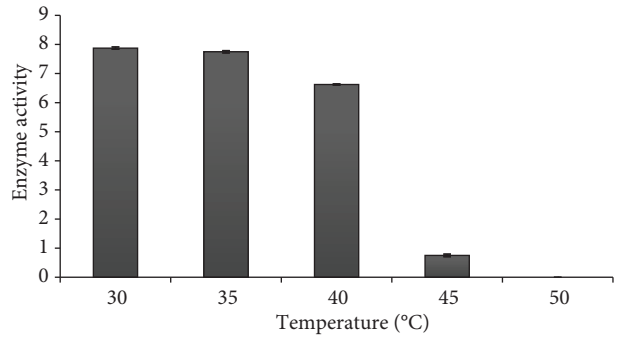

Figure 6. Effect of temperature on enzyme activity.

°C for lipase produced by the SmF of *Penicillium verrucosum*.

Effect of moisture content in SSF

The moisture content of the substrate plays a vital role for the microbial growth and biochemical activities in SSF. The maximum yield was obtained at 50% moisture content, as listed in Table 2. In SSF processes, higher moisture content leads to decreased porosity, changes in substrate particle structure, stickiness development, gas volume reduction and

Table 2. Effect of moisture content.

Moisture content (%)	Lipase activity
30	6.63
40	7.25
50	8.13
60	8.0
70	7.88

decreased diffusion. Insufficient moisture leads to a reduction in the solubility of nutrients present in the bran (12). A reduction in enzyme yield at very high moisture levels of more than 75% may be due to the steric hindrance of organism growth via a reduction in inter-particle space and decreased porosity (13).

In the present study we compared the lipase yield by SSF and SmF. As shown in Table 3, total enzyme activity for SSF and SmF was 569.10 U and 550.90 U, respectively. From this it was evident that the lipase yield is higher for SSF when compared to SmF. Enzyme titres by SSF processes were higher and stable.

Lipase activity is induced by the presence of lipid substrates in the medium. Extracellular lipase production by different micro-organisms on lipids has been extensively reported (14). Here, a solid lipid substrate—DFRB—served as the sole source of nutrient for lipase production.

SSF of DFRB by *Aspergillus fumigatus* MTCC 9657 at a moisture content of 50% yielded 8.13 U/mL of lipase in 7 days. The use of DFRB as a substrate for lipase production may have the combined benefit of utilising a low-value waste material while producing a commercially valuable product. The study showed very clearly that the SSF method is more economical than SmF mainly due to the inexpensive culture media components and stability of the enzyme over a longer period.

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Table 3. Comparison of submerged and solid-state fermentation enzyme activity.

Enzyme sample	volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Total activity (U)
Crude SSF	70	2.26	158.2	569.10
Crude SmF	70	1.98	138.6	550.90

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