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# Optimization of process parameters for production of lipase in solid-state fermentation by newly isolated *Aspergillus* species

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Of the 34 fungal species, isolated from a number of oily substrates, 9 exhibited lipase activity. AU 15, identified as *Aspergillus* sp., was found to be excellent lipase producer in submerged fermentation and was selected for solid-state fermentation (SSF). Among substrates like oil cakes of coconut, groundnut and sesame, wheat rawa, bombay rawa and soya beans (crushed), wheat rawa showed the highest lipase activity. The maximum enzyme yield (1934 U/g) was obtained with basal medium containing wheat rawa, olive oil and corn steep liquor, at 80% moisture content, *p*H 7.0 and 96 hrs incubation.

**Keywords**: lipase; *Aspergillus* species; solid state fermentation (SSF); submerged fermentation (SmF)

## Introduction

Lipases (EC 3.1.1.3) hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. They are widely distributed in animals, plants and microorganisms. Fungal lipases have received attention because of their potential use in food processing, pharmaceuticals, cosmetics, detergents and leather industry[1]. New applications, such as the resolution of racemic mixtures to produce optically active compounds, should also arise from the stereo specific acting properties of some lipases[2].

Solid-state fermentation (SSF) processes are of special economic interest for countries with an abundance of biomass and agro-industrial residues. The reports on SSF system include lipase production from *Aspergillus niger* using gingelly oil cake as substrate[3], the feasibility of obtaining lipase with *Rhizopus delemar* growing on a polymeric resin[4], production of lipase by *A. oryzae* with different solid substrates[5] and production of lipase by *Penicillium restrictum*[6]. Lipase synthesis[7] was optimized by the yeast *Candida rugosa* in SSF and the C/N ratio of medium was found to be an important parameter for lipase production. On comparing[8] the production of lipase by *Penicillium candidum* in SmF and SSF, SSF process was found superior.

The SSF offers high productivity, less capital investment and relatively easy recovery of extra cellular enzymes compared to SmF[9-12]. In the present investigation, screening was carried out to look for new potential microbial source for lipases. This paper reports the production of lipase by SSF using *Aspergillus* sp. (AU15), isolated from oily substrates and optimization of various production parameters.

## Materials and Methods

### Screening for Lipase Producing Fungi by Agar Plate Technique

Different oily substrates used for the isolation of lipase producing fungi include oil cakes of coconut, groundnut and sesame, fungal infected coconut (wet kernel), castor oil soaked cloth, soil from sesame oil mill and spoiled bread.

The substrates were inoculated into yeast extract malt extract (YEME) broth for the enrichment. To 5 ml YEME broth in test tube, small amount of each oily substrate was added separately and incubated for one week. From the broth, serial dilutions were made and 1 ml of the diluted sample was added to 50 ml of molten potato dextrose agar (PDA) medium containing 50 ug/ml of rifampicin and poured into 15.24 cm dia petriplates. The plates were incubated at 30$^{o}$C for 7 days; the discrete colonies were picked up, transferred onto PDA slants and incubated at 30$^{o}$C for 3 days. These isolates were tested for their lipolytic activity on agar plates[13] with tributyrin as substrate and victoria blue as indicator[14,15].

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**Lipase Production by SmF**

All the isolates, which exhibited lipolytic activity by agar plate technique, were subjected to SmF. A spore suspension, prepared by adding 5 ml sterile water to 72 hrs culture ($1\times10^8$ spores/ml), was added to 45 ml inoculum medium in 250 ml Erlenmeyer flasks and incubated on rotary shaker at 30°C for 48 hrs. A 10% level of inoculum was added to 45 ml production medium and incubated at 30°C for 96 hrs. The samples were withdrawn after 96 hrs and assayed for their lipolytic activity. The composition of inoculum and the production medium (*p*H 6.0) was: glucose, 10; peptone, 20; NaCl, 5 and yeast extract, 5 g/l.

**SSF Process**

The experiments were conducted in 250 ml Erlenmeyer flasks containing 10 g of solid substrate and 1 ml of salt solution. The composition of salt solution (*p*H 6.0) was: $(NH_4)_2SO_4$, 5.0; $Na_2HPO_4$, 6.0; $KH_2PO_4$, 2.0; $MgSO_4.7H_2O$, 3.0 and $CaCl_2$, 3.0 g/l. Distilled water was added in such a way that the final substrate moisture content was 70%. After sterilization by autoclaving, the flasks were cooled, inoculated with 10% inoculum (v/w) of 48 hrs culture and incubated at 30°C for 96 hrs.

**Effect of Different Physico-chemical Parameters on the Production of Lipase**

Solid substrates were procured from local market. The fermentation was carried out with 10% inoculum level at 30°C for 96 hrs. The best solid substrate was selected and used in subsequent experiments. Different incubation periods (24, 48, 72, 96, 120, 144 and 168 hrs) were employed to study their effect on lipase production. To investigate the influence of the initial moisture content (before autoclaving) of the substrate, the fermentation was carried out under various initial moisture contents (50, 60, 70, 80 and 90%) of wheat rawa, which was adjusted with distilled water. While optimizing the initial *p*H of the basal medium, the *p*H of aqueous solution was varied from 4.0 to 8.0 with 1 *N* HCl or 1 *N* NaOH. The solid substrate was supplemented with oils (1% w/w) such as groundnut, coconut, castor, sunflower and olive to study their effect on lipase production. The solid substrate was supplemented with various additives (1%) such as Tween 20, Tween 80, glycerol, glucose, peptone and cornsteep liquor to study their effect on lipase production. The fermentation was carried out as described earlier.

**Analytical Methods**

***Enzyme Extraction***

At the end of fermentation, biomass was treated with 50 ml of distilled water and agitated on a magnetic stirrer for 30 min. Whole contents were filtered through muslin cloth. The residue was again treated with another 50 ml of distilled water in the same way and filtered. The filtrates were pooled together, centrifuged and the clear supernatant was used as the enzyme source.

***Lipase Activity***

The activity of lipase enzyme was determined using olive oil substrate emulsion method[16]. The assay mixture consisted of 1.0 ml of the substrate emulsion (70.0 ml emulsifying reagent with 30.0 ml olive oil homogenized for 5 min. The emulsification reagent (NaCl 17.9 g, $KH_2PO_4$ 0.41 g, glycerol 540.0 ml, gum arabic 10.0 g and distilled water to a total volume of 1.0 L), 0.8 ml of 0.2 *M* potassium phosphate buffer (*p*H 7.0) and 0.2 ml of the enzyme were incubated at 55° C for 30 min. The reaction was terminated by adding 2.0 ml of acetone-ethanol mixture (1:1 v/v). The amount of fatty acid liberated was determined by titration with 0.01 *N* NaOH. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of fatty acid per ml per min under above assay conditions. All the fermentations and assays were carried out in triplicate and the mean value was presented.

***Estimation of Moisture Content***

Moisture content was estimated by drying 10 g of wheat rawa to a constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, wheat rawa was soaked with desired quantity of water. After soaking, the sample was again dried as described above and moisture content was calculated as follows:

Percent of moisture content (initial) of solid medium = (wt. of the wheat rawa - dry wt.) × 100/dry wt.

***p*H *Measurement***

Five milliliters of distilled water was added to 0.5 g of fermented material and the mixture was agitated vigorously. After 10 min, the *p*H of the supernatant was determined.

## Results and Discussion

Of the 34 fungi isolated from different oily substrates, 9 exhibited lipolytic activity[13-15]. Of nine iso-

lates subjected to SmF (Table 1), only one isolate showed excellent lipase activity (996 U/L). It was identified and designated as *Aspergillus* sp., AU 15[17] that was subjected to SSF studies. Maximum lipase activity (1150 U/g) was achieved with wheat rawa (Table 2).

In the present study, maximum lipase production (1152 U/g) was obtained after 96 hrs of incubation (Fig. 1), whereas earlier[18] it was 8 days of incubation using *A. niger*.

High enzyme titre (1216.0 U/g) was attained when the initial moisture level was 80% in comparison with that at low or high moisture levels (Fig. 2). The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. Increase in moisture level was believed to reduce the porosity of the wheat rawa, thus limiting oxygen transfer[19,20]. The low moisture content causes reduction in the solubility of nutrients of the substrate and low degree of swelling[21]. The maximum lipase production (1298.0 U/g) was obtained at *p*H 7.0 (Fig. 3).

Various oils have beneficial effects on the lipase production[22]. Oil type[2] was observed as a significant variable affecting the lipase yield. In the present

Table 1—Selected fungal isolates and their lipase activity

| Isolate No. | Lipase activity (U/g) |
|---|---|
| AU 3 | 700.0±12.84 |
| AU 8 | 676.0±8.00 |
| AU 15 | 996.0±12.55 |
| AU 18 | 300.0±9.21 |
| AU 19 | 600.0±13.81 |
| AU 20 | 690.0±9.55 |
| AU 24 | 298.0±4.00 |
| AU 25 | 330.0±6.55 |
| AU 31 | 529.0±9.08 |

Values are mean of three determinations with SD (±)

Table 2–Effect of different solid substrates on lipase production

| Substrate | Lipase activity (U/g) |
|---|---|
| Bombay rawa | 565.0±19.81 |
| Coconut oil cake | 620±14.21 |
| Ground nut cake | 656.0±16.55 |
| Sesame oil cake (black) | 876.0±11.00 |
| Sesame oil cake (white) | 984.0±19.84 |
| Soyabean (crushed) | 639.0±13.55 |
| Wheat bran | 901.0±12.14 |
| Wheat rawa | 1150±16.55 |

Values are mean of three determinations with SD (±)

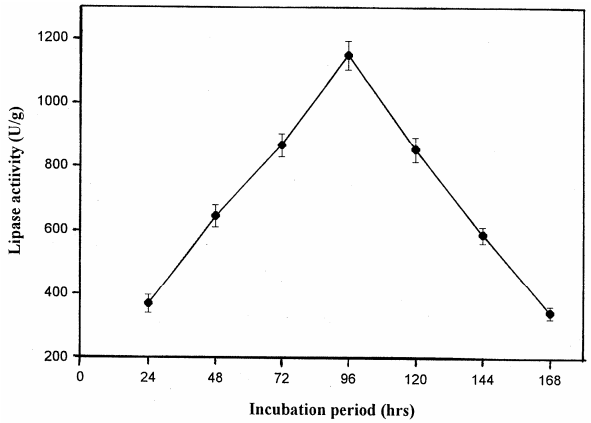


Fig. 1—Effect of incubation period on lipase production by AU 15 on SSF using wheat rawa as substrate

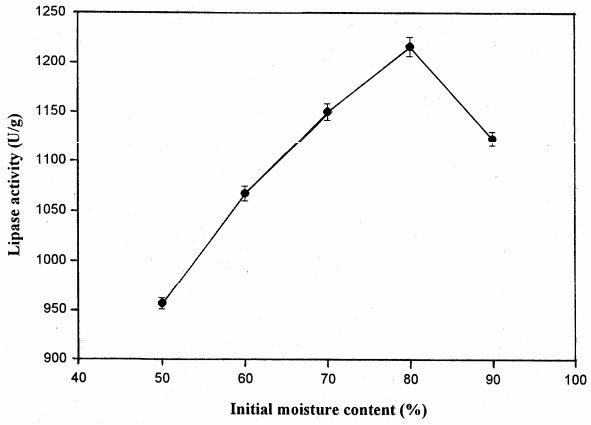


Fig. 2—Effect of initial moisture content on lipase production by AU 15 on SSF using wheat rawa as substrate

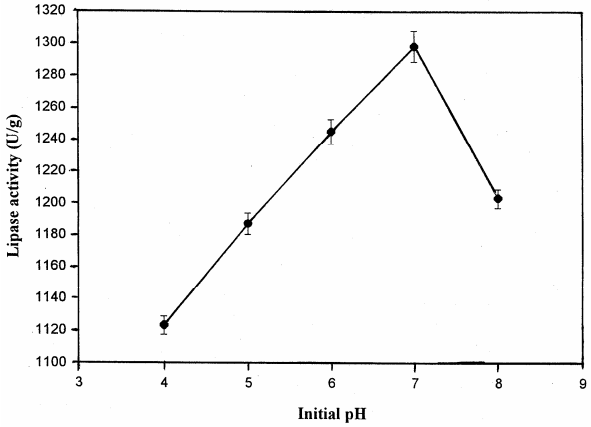


Fig. 3—Effect of initial *p*H on lipase production by AU 15 on SSF using wheat rawa as substrate

study, olive oil (1%) showed maximum enzyme production (1664 U/g) (Table 3). The incorporation of olive oil (1%) in the growth medium (C/N ratio, 9.9) gave high lipase production[23]. A thermostable lipase was produced when olive oil was used as a carbon source[24]. Addition of small amount of Tween 80 and olive oil increased lipase production from 1.6 to 100 U/ml[25]. An increase of 1.8 times is reported[6] with 2% olive oil. In the present study, results are in agreement with reported data.

Among the various additives investigated (Table 4), corn steep liquor exhibited the maximum lipase production (1789 U/g). A high lipase production is reported[26] when corn steep liquor and olive oil are incorporated in the fermentation medium. High nitrogen concentration in culture media is effective in enhancing the production of lipase by microorganisms[5,27,28]. Results of the present study are in agreement with the previous reports.

With all the above-optimized factors, the productivity of lipase with SSF was 1934 U/g. The optimum factors in the basal medium include wheat rawa with olive oil (1% w/w), corn steep liquor (1% w/w), 80% moisture content, *p*H 7.0 and 96 hrs incubation.

Table 3—Effect of supplementation with different oils on lipase production by AU15 in SSF using wheat rawa as the solid substrate

| Oil | Lipase activity (U/g) |
|---|---|
| Ground nut | 1128±15.84 |
| Coconut | 1025±14.00 |
| Castor | 710.0±16.55 |
| Sunflower | 904.0±17.21 |
| Olive | 1664.0±10.81 |

Values are mean of three determinations with SD (±)

Table 4—Effect of supplementation with different additives on lipase production by AU15 in SSF using wheat rawa as the solid substrate

| Additives (1%) | Lipase activity (U/g) |
|---|---|
| Corn steep liquor | 1789.0 ± 18.00 |
| Peptone | 1719.0 ± 15.55 |
| Tween 80 | 1676.5 ± 16.00 |
| Tween 20 | 1600.0 ± 17.84 |
| Glycerol | 1508.0 ± 10.55 |
| Glucose | 1361.0 ± 17.21 |

Values are mean of three determinations with SD (±)

Present study with SmF showed 996 U/L lipase productivity. The production of lipase by SSF was better than by SmF technique. The accumulation of lipase by SSF was 1.94 times higher than the SmF. This study has shown that SSF is most suitable technique for lipase production employing *Aspergillus* sp. (AU 15). Further, short fermentation time (96 hrs) makes SSF as promising system, which has commercial importance.

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