# [BIO26] Selection and optimization of lipase production from *Aspergillus flavus* USM A10 via Solid State Fermentation (SSF) on rice husks and wood dusts as substrates

**How Shiao Pau and Ibrahim Che Omar**

Fermentation and Enzyme Technology, School of Biological Sciences, Universiti Sains Malaysia, Minden, 11800, Penang, Malaysia

## Introduction

Solid state fermentation (SSF) involves the growth of microorganisms on moist solid substrates in the absence of free flowing water. The necessary moisture in SSF exists in a complex form which is absorbed within the solid matrix, and this is considered advantageous for growth because of the possible efficient oxygen transfer process. In SSF, the water content is quite low and the microorganism is almost in contact with gaseous oxygen in the air, unlike in the case of submerged fermentation [1]. Many microorganisms are capable of growing on solid substrates, however, only filamentous fungi which are able to grow effectively in the absence of the free water. Bacteria and yeasts grow on solid substrates at 40-70% moisture level while filamentous fungi was reported to grow under a much lesser water content [1]. SSF has many advantages over submerged fermentation including economy of space needed for fermentation, simplicity of fermentation media, no requirement of complex machinery, equipment and control systems, compactness of fermentation vessel owing to lower water volume, superior yields, less energy demand, low capital and recurring expenditure [2, 3]. Thus, such a system finds greater applications in solid waste management, biomass energy conservation and in the production of secondary metabolites. Based on the low production cost coupled with other advantages, SSF is expected to have enormous potential for commercialization particularly for developing countries like Malaysia. A number of success stories have been documented in the commercialization of the SSF systems for the production of microbial metabolites mainly in countries like India, France and Eastern Europe.

With the ultimate goal to commercialize the SSF system for enzyme production, the selection of a potential lipase producer via SSF was studied. The optimization of the SSF system based on the medium composition and cultural conditions was carried out. This paper described the findings of the selection of *Aspergillus flavus* USM A10 as the potential lipase producer and the enhancement of the enzyme production through the modification of the medium composition and cultural conditions.

## Materials and Methods

### *Isolation of microorganisms and screening of lipolytic activities*

All the microorganisms used in this study were isolated from environmental samples from Penang and South Kedah, Malaysia. The isolation procedure was performed based on the method described by Nakayama (1981) [4]. Preliminary screening of lipolytic filamentous fungi was carried out on Potato Dextrose Agar, (PDA) plates supplemented with 3% (v/v) tributyrin. Culture plates were incubated at 37°C and periodically examined for 7 days. Colonies showing clear zones around them were selected and examined for lipase production in the SSF system.

### *Solid state fermentation (SSF) cultivation systems*

Three different substrates that were paddy husks, rubber wood saw dusts and palm kernel cake were examined for the cultivation of filamentous fungi. All the cultures were grown in 250-ml conical flasks containing 10g of substrate, 90% (v/w) distilled water and 10% (v/w) of olive oil as inducer with the inoculum size of 5 x $10^5$ spores $g^{-1}$ substrate used. The inoculum was prepared using a 3 to 5 day old slant cultures. A known volume of sterile distilled water was added to the cultures and gently vortexed to release the spores. The number of spores in the suspension was determined using a haemocytometer.

After the inoculation with the spore suspension, the substrate was homogeneously mixed and was incubated at room temperature (28 ± 3°C) for 4 days. After 4 days, cultures were collected were mixed with 70 ml of sodium phosphate buffer (pH 7.0, 0.2 mM) and homogenised to extract the enzyme. The biomass slurry was centrifuged at 5000 rpm for 15 min at 4°C. The biomass was used to determine the growth of the fungus, while the supernatant was used as the source of crude lipase preparation.

***Identification of the potential lipase producer***

Fungal identification was carried out based on the colony morphologies and structural characteristics as observed under the light and electron microscopy. The fungal characteristics were described and identification was performed based on manual described by Gilman (1967) [5].

***Optimization of medium composition and cultural conditions***

Optimization of the medium composition and cultural conditions was carried out through the modification of several growth parameters. The optimum temperature was examined in the range of 28 – 45$^{o}$C, while for the moisture content in the range of 60 – 100%, inoculum size in the range of 5 x $10^3$ – 5 x $10^7$ and the sizes of the paddy husks were examined in the range 0.5 – 2.0 mm. The effect of lipid materials as inducer and the effect of supplementation of additional carbon and nitrogen sources in the SSF system were also examined in enhancing the lipase production.

***Analyses***

Lipase activity was determined based on the olive oil-polyvinyl alcohol emulsion method described by Mustranta *et al.* (1992) [6]. The substrate was prepared by emulsifying 30 ml of olive oil with 70 ml of emulsifier by homogenizing for 3 min. The emulsifier consisting of NaCl (17.9 g), $KH_2PO_4$ (0.41 g), glycerol (540 ml), polyvinyl alcohol (10 g), and distilled water was prepared by vigorous homogenisation (Ika-Werk, Janke Kunke, RW20, Germany) for 5 min at the ambient temperature (28 ± 3°C). The reaction mixture consisting of 5 ml of the emulsion, 4 ml of 0.2 M sodium phosphate buffer (pH 7.0) was incubated for 10 min at 37°C and 200 rpm. About 1.0 ml of the crude enzyme solution was added to the reaction mixture and incubated for 30 min at 37°C and 200 rpm. The reaction was stopped by the addition of 10 ml of an acetone-ethanol mixture (1:1). Fatty acid liberated from the hydrolysis of the substrate was determined using the pH stat (Titramate 20, Mettler Toledo) at pH 9.0. One unit of lipase activity was defined as the amount of enzyme which liberated 1 μmol equivalent of fatty acid at 37°C and pH 7.0.

The growth of the fungi was determined based on the glucosamine method described by Swift (1972) [7].

**Results and Discussion**

***Isolation and identification of the potential producer of lipase***

A total of 35 fungal isolates were capable of showing lipase activities with different degree on tributyrin agar. These isolates were then transferred to the SSF system consisting of 10 g of paddy husks with either wood dust or palm kernel cake at the ratio of 1:1 and incubated at ambient temperature (28 ± 3°C) for 96 hours. Based on the results obtained (data not shown), an isolate which was designated as USM A10 was found to give the highest lipase activity of 4.58 ± 0.18 $Ug^{-1}$ substrate consisting of paddy husk: rubber wood saw dust. Other isolates showed varying degrees of enzyme production depending on the types of substrate used in the SSF. Palm kernel cake (PKC) does not result in high enzyme activity although PKC contained relatively higher lipid content than other substrates [8]. The results indicated that the addition of olive is adequate for the induction of lipase production.

***Identification of isolate USM A10***

Isolate USM A10 grew significantly on potato dextrose agar (PDA and malt extract agar (MEA) exhibiting colonies of greenish grey coloration. Under the light microscope, the conidia were found to be globose with the size ranging from 2.7 x 3.3 – 4.1 x 4.6 μm (Fig.1). The conidiophores appeared to be rough or spiny with diameter of 8 – 10 μm. Phialides were available with the diameter ranging from 3 – 4 μm. Screlotia with white colouration initially changed to brownish after maturity. Cleisthothecia were not found.

Based on the morphological and structural characteristics described by Gilman (1967) [5], isolate USM A10 was identified as Aspergillus *flavus*. *A. flavus* has also been reported as a producer of cell bound lipase in submerged cultivation system [9].

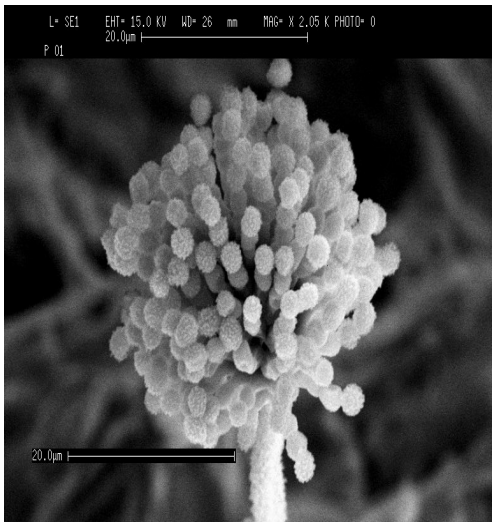


FIGURE 1 Scanning electron micrographs of *Aspergillus flavus* USM A10 showing the sporangium and mycelia.

***Optimization of the SSF system for lipase production by Aspergillus flavus USM A10***

*Effect of substrate size*

The effect of the size of paddy husks were examined at 0.5 mm, 0.75 mm, 1.0 mm and 2.0 mm mixed with wood dusts at the ratio of 1:1 for lipase production. The paddy husks which were not ground were used as the control. The size of unground paddy husks range from 0.5 – 2.0 mm. The results obtained were shown in Fig. 2. The maximum lipase activity of 4.667 ± 0.057 $Ug^{-1}$ substrate with the growth of 0.17 ± 0.03 mg glucosamine $g^{-1}$ substrate obtained using the paddy husks of size 2.0 mm indicated that oxygen is a prerequisite for the growth of the fungus. Smaller size substrate particles resulted in compact substrate bed which hindered mycelial penetration and gaseous exchange.

*Effect of moisture content*

Water is present in very limited amount in the SSF system and thus an optimum content is important as it determines the productivity of a SSF process [2]. Moisture content in SSF system can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and liberation through fungal metabolism and also due to environmental factors. The moisture content in the substrate also depends on the types of microorganisms and the substrate used in the SSF. At the same time, the amount of moisture content also varied depending on the water binding characteristics of the substrates. The effect of moisture content present in paddy husks and wood dusts on lipase production was examined and the result obtained is shown in Fig. 3. The water content added to the substrate was adjusted to vary from 60 – 100% (v/w). As shown in the figure, the optimum lipase production was observed at 90% indicating that the water binding capacity of paddy husks and wood dusts was low. Although the results do not indicate the actual moisture content in the system, however it gives an indication of the initial water content which is required to give the maximum lipase production and fungal growth. Considering the water loss, consumed and produced during the fermentation, the actual water required by the system is normally not known since water exists in a complex form within the solid matrix as a thin layer either absorbed to the surface of the substrate particles or less tightly bound within the capillary region of the solids. The water that exists or absorbed within the solid matrix is important for fungal growth as it will allow efficient oxygen transfer process [10].

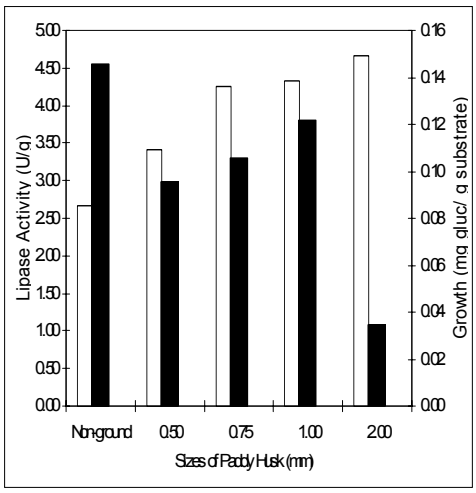


FIGURE 2 Effect of sizes of paddy husks on the growth and lipase production by *Aspergillus flavus* USM A10.
(□ ) Lipase activity, (●) growth

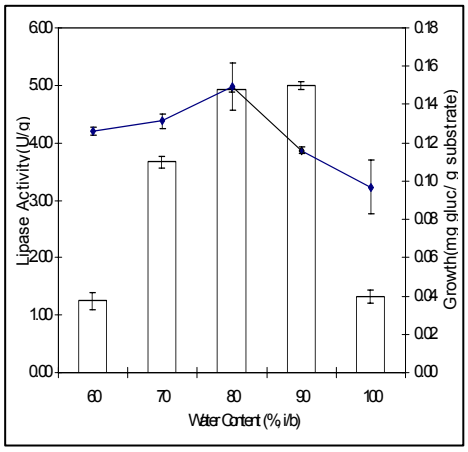


FIGURE 3 Effect of moisture content on the growth and lipase production by *Aspergillus flavus* USM A10.
(□ ) Lipase activity, (●) growth

*Effect of inoculum size*

Fig.4 (a-e) show the lipase production and growth of *A. flavus* USM A10 using different sizes of inoculum. Higher inoculum size is expected to reduce the lag phase of the fungal growth and therefore the maximum lipase production can be achieved in a much shorter time. The results shown in the figures indicated that increasing inoculum size resulted in a rapid initial growth, although the enzyme production did not increase significantly. Upon comparison, it is observed that the maximum lipase productivity were 0.11, 0.26, 0.31, 0.11, 0.13 U $h^{-1}$mg glucosamine$^{-1}g^{-1}$ substrate using the inoculum sizes of 5 x $10^3$, 5 x$10^4$, 5 x $10^5$, 5 x $10^6$ and 5 x $10^7$ spores $g^{-1}$ substrate, respectively. The results indicated that the optimum inoculum size was 5 x $10^5$ spores $g^{-1}$ substrate giving the lipase activity of 5.1 $Ug^{-1}$ after 4 days cultivation. Leong and Ibrahim (2002) [11] have shown that inoculum size did not show any significant effect on benzyldehyde production but will only affect the fungal growth in the SSF system. Raimbault and Alazard (1980) [12] deduced that the decline in enzyme production with high inoculum size was related to the high biomass which is responsible for the reduction in enzyme biosynthesis under nutrient limited conditions in the fermentation mashes.

*Effect of cultivation temperature*

The effect of fermentation temperature in the range of 28 – 45°C was examined on the production of lipase. Temperature plays important role in SSF processes as it significantly affects the germination of spores. However, as the spore germinates, the optimum temperature for mycelial propagation may change (Raimbault and Alazard,. 1980). The temperature shift is made more complex considering SSF generated substantial amount of heat throughout the process which will affect the water content in the SSF system. As in the case of *A. flavus* USM A10, the optimum temperature was found to be in the range of 28 – 30°C with the maximum lipase activity of 5.9 ± 0.03 $Ug^{-1}$ substrate with the growth of 0.17 ± 0.01 mg glucosamine $g^{-1}$ substrate (Fig 5). Higher temperature resulted lower lipase activity which may be related to the low enzyme stability at higher temperature since the growth was not affected at the temperature in the range of 30 – 45°C.

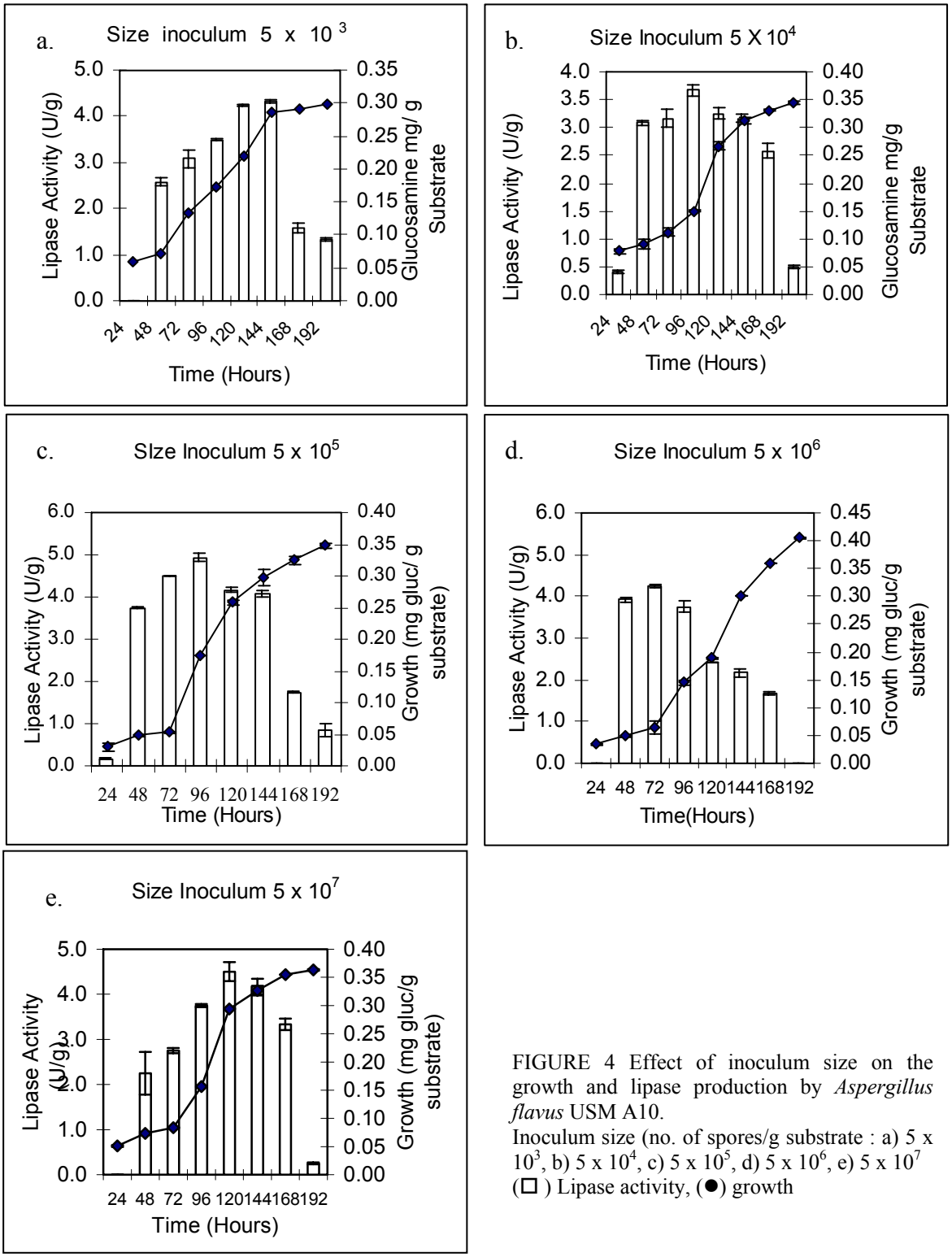


FIGURE 4 Effect of inoculum size on the growth and lipase production by *Aspergillus flavus* USM A10.
Inoculum size (no. of spores/g substrate : a) 5 x $10^3$, b) 5 x $10^4$, c) 5 x $10^5$, d) 5 x $10^6$, e) 5 x $10^7$
(□ ) Lipase activity, (●) growth

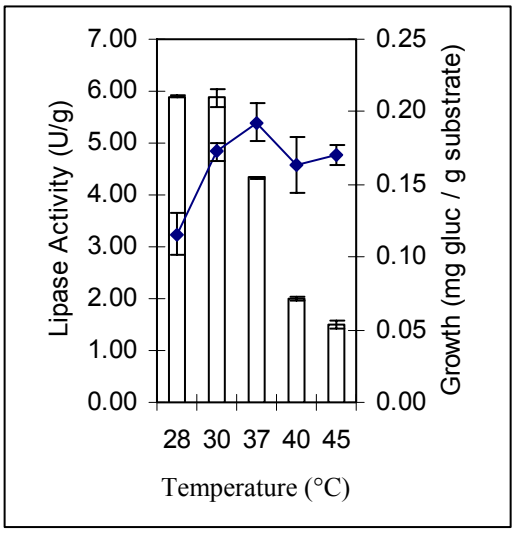


FIGURE 5. Effect of temperature on the growth and lipase production by *Aspergillus flavus* USM A10.
(☐ ) Lipase activity, (●) growth

*Effect of lipid materials as inducer for lipase production*

Numerous research have shown that lipase are highly inducible in the presence of lipid materials such as fats and oils, triacylglycerol, fatty acids, glycerol, surface active compounds and other lipid compounds in the production medium [13,14,15] . The lipid materials can be the substrates of lipolytic enzymes, hydrolytic products of lipids, lipid derivatives and the substrate analogs. The effect of lipid materials at 10% (v/w) addition in the SSF medium for lipase production was examined (Fig.6a). As shown in the figure, sesame oil was found to give the highest lipase production of about 5.0 U/g substrate with the growth of 0.25 ± 0.01 mg glucosamine $g^{-1}$ substrate. The higher growth compared to the system in the absence of lipid material indicated that the lipid materials can also act as the substrate for fungal growth. The results obtained also showed that the lipase was specific towards lipids fatty acids with longer chain length which are present in oil such as sesame oil, olive oil and corn oil. Triglyceride with shorter chain length such as tributyrin showed much lower lipase production.

The effect of sesame oil concentration was examined on the lipase production in the range of 4 – 14% (v/w) and the results are shown in Fig.6b. The maximum lipase production was observed at the sesame oil concentration of 6% with the lipase production of 5.7 $Ug^{-1}$ substrate. Higher concentration of the oil does not improve the lipase production or the fungal growth. Higher oil content resulted in the formation of a biphasic system which prevented not only the accessibility of water to the fungus but also prevented oxygen transfer and nutrient assimilation by the fungus from the substrate [2, 16].

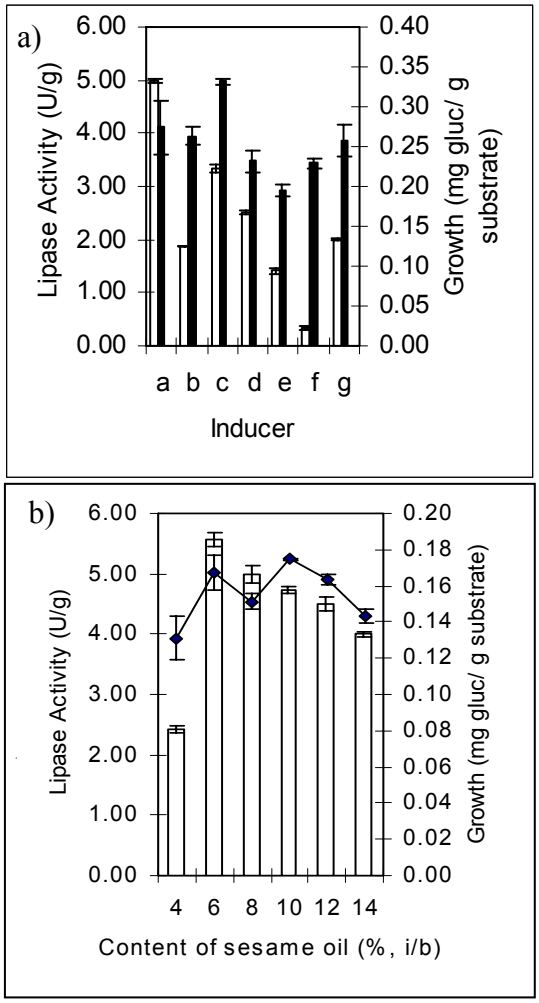


FIGURE 6 Effect of lipid materials as inducer on the growth and lipase production by *Aspergillus flavus* USM A10.
[(☐ ) Lipase activity, (●) growth]
a) Types of lipid materials : A; sesame oil, B; castor oil, C; corn oil, D; oleic acid, E; olive oil F; tributyrin, G; Tween 20
b) Effect of sesame oil amount

*Effect of additional supplementation of carbon and nitrogen sources*

The nutrients available in the SSF system are derived completely from the degradation of organic compounds present in the solid substrates. Low production of the degrading enzymes may result in poor growth due to limited availability of the nutrients. In many cases, the production of primary metabolites is affected by good microbial growth. Therefore, it is important to enhance the growth mainly during the initial stage of the fermentation which will initiate the production of degrading enzymes. Therefore it is important to ensure rapid initial growth which can be done by supplementing the solid substrates with additional carbon or nitrogen sources.

A total of 13 types of carbohydrates were supplemented to the solid substrate as carbon sources. The results of the supplementation of the carbon sources at 0.1% (w/w) on the production of lipase and growth of *A. flavus* USM A10 is shown in Fig. 7a. As shown in the figure, the growth and lipase production show great variation with different carbon sources supplemented. The results suggested that the fungus exhibited a preference on the utilization of the carbon sources. As shown by the large variation in the growth with the range of 0.06 – 0.14 mg $g^{-1}$ substrate, the results indicated that the growth although was rapid but ceased immediately after achieving the maximum growth. The low growth of the fungus, thus indicated that the poor production of degrading enzymes will subsequently resulted in a low nutrient content in the SSF system. No lipase production was observed with the addition of lactose, xylose or soluble starch. However, in the case of maltose, the good growth of the fungus enable the fungus to simultaneously utilized the nutrients from the solid substrates and lactose resulting in a higher lipase production of about 6.0 U $g^{-1}$ substrate. A maximum lipase production was obtained with the maltose concentration of 0.15% (w/w) added to the substrate giving a production of 6.750 ± 0.029 U $g^{-1}$ substrate and growth of 0.16 ± 0.03 mg glucosamine $g^{-1}$ substrate.

The effect of supplementing the solid substrates with organic and inorganic nitrogen sources at 0.1% (w/w) were examined on the lipase production and growth of the fungus. The supplementation of nitrogen sources resulted in an increase in growth in the range of 0.20 – 0.25 mg glucosamine $g^{-1}$ substrate. However, not all nitrogen sources resulted an increase in lipase production (Fig. 8a). Among them, only meat extract, yeast extract and $NH_4H_2PO_4$ showed significant effect on lipase production with $NH_4H_2PO_4$ gave the highest production of 6.5 U/g substrate and growth of 0.32 ± 0.03 mg glucosamine $g^{-1}$ substrate. When cultivation was carried out with the addition of $NH_4H_2PO_4$ at 0.05% (w/w) and 0.15% (w/w) of maltose, a maximum production of 7.00 ± 0.252 U $g^{-1}$ substrate was obtained (Fig. 8b). Based on these results, it is clear that the increase in enzyme production is generally related to the growth of the fungus

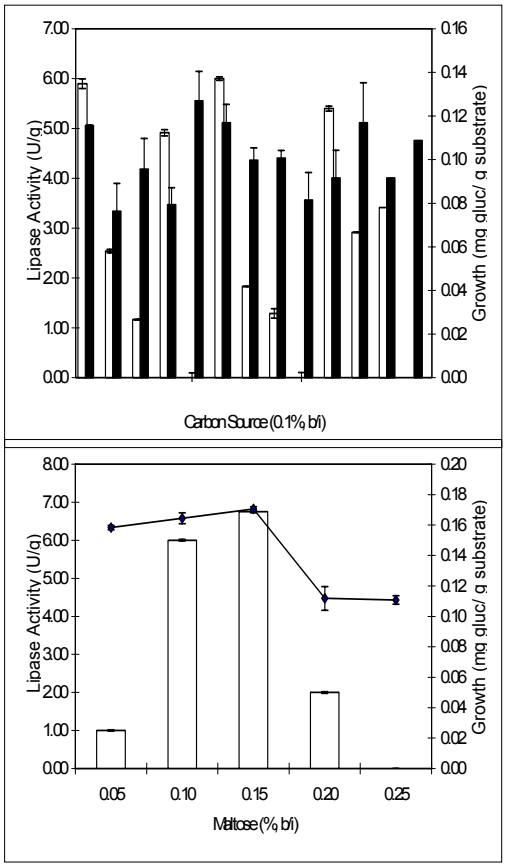


FIGURE 7 Effect of supplementation of carbon sources on the growth and lipase *Aspergillus flavus* USM A10. [(□ ) Lipase activity, (●) growth]

a) Types of carbon sources: a; D-glucose, b; D-fructose, c; L-arabinose, d; lactose, e; maltose, f; sucrose, g; dextrose, h; soluble starch, i; cellulose, j; xylose, k; myo-inositol, l; D-sorbitol

b) Effect of amount of maltose supplementation

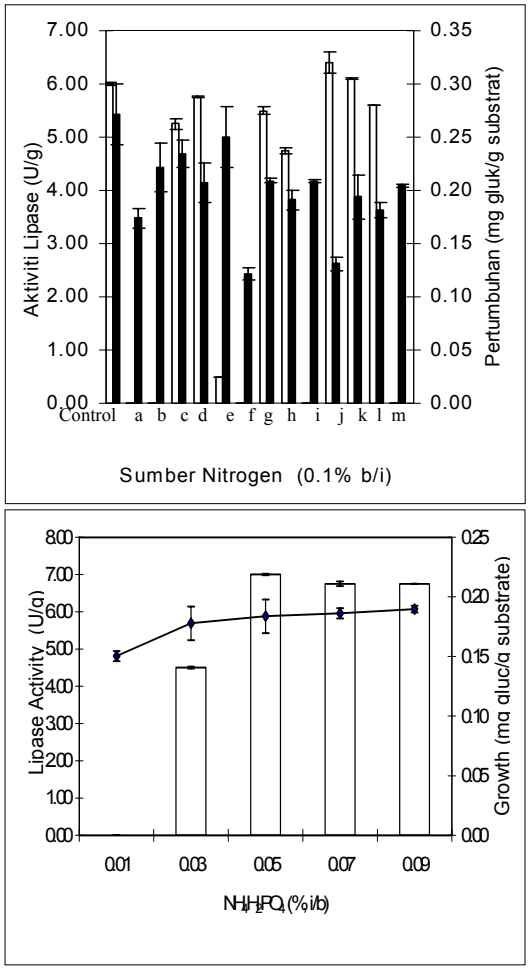


FIGURE 8 Effect of supplementation of nitrogen sources on the growth and lipase production by *Aspergillus flavus* USM A10 (□) Lipase activity, (●) growth

a) Types of nitrogen sources: control; control no addition of any nitrogen sources, a; casein, b; meat extract, c; malt extract, d; peptone, e; tryptone, f; yeast extract, g; $NaNO_3$, h; $NH_4H_2PO_4$, i; $KNO_3$, j; $NH_4NO_3$, k; $(NH_4)_2PO_4$, l; $(NH_4)_2SO_4$, m; $NH_4Cl$

b) Effect of amount of $NH_4H_2PO_4$ supplementation

*Profiles of lipase production by Aspergillus flavus USM A10 via SSF.*

The time course profiles of lipase production by *A. flavus* USM A10 shows maximum production of 7.00 ± 0.252 U $g^{-1}$ substrate with the growth of 0.205 mg glucosamine $g^{-1}$ substrate after 24 h (Fig. 9b). The lipase production dropped gradually thereafter which is related to the low stability of the lipase at higher temperature resulted from the increase in the metabolic activity of fungus over time. When compared to the profiles before optimization, the maximum lipase production was 4.583 ± 0.184 U $g^{-1}$ after 96 hr. The productivity of lipase before and after optimization were 1.42 and 0.2 U/$h^{-1}$ $mg^{-1}$ glucosamine $g^{-1}$ substrate, respectively which is an increment of about 700%.

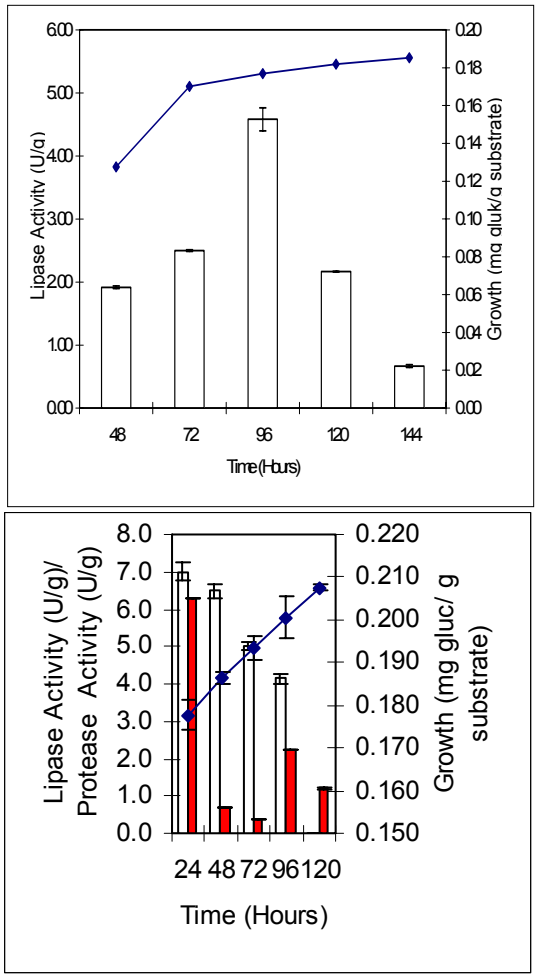


FIGURE 9 Profiles of growth and lipase production before (a) and after (b) optimization of cultural condition and medium composition by *Aspergillus flavus* USM A10
(□) Lipase activity, (●) growth,
(□) Protease activity

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