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Investigation on extracellular lipase production by *Aspergillus japonicus* isolated from the paper nest of *Ropalidia marginata*

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Abstract

Aspergillus japonicus isolated from the paper nest of *Ropalidia marginata* was screened for its extracellular lipase production. Maximum lipase activity (120 U/ml) was achieved by manipulating the culture environment. The optimum pH was 7.5 and the temperature was 40°C. Sucrose served as the best carbon source whereas peptone was the best nitrogen source for lipase production. An animal fat, pig fat in the culture medium enhanced lipase production. These investigations will be helpful in further scaling-up processes of this industrial enzyme. Owing to many applications in high-value syntheses and as bulk enzymes, lipases have high impact on bioprocessing.

Keywords*:* *Aspergillus japonicus*; paper nest; pig fat; *Ropalidia marginata*; lipase; Tween-20.

Introduction

Lipases (Triacylglycerol acyl hydrolase E.C.3.1.1.3) are lipolytic serine esterases that are secreted by many fungi, yeasts and bacteria. Lipases are active at their interface between their hydrophobic lipid substrate and the hydrophilic space medium (oil-water interface) cleaving water-insoluble glycerides into molecules that can be readily imbibed by cells (Jaegar & Eggert, 2002). Microbial lipases have assumed a great deal of importance as industrial enzymes in view of their potential for use in various biotechnological processes. Fungi are important enzyme producers since they produce enzymes extracellularly (Ferreira & Peralta, 1999). Lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. Lipases are the most widely used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases (Gitlesen *et al*., 1997). Besides their role in synthetic organic chemistry, these enzymes also find extensive applications in chemical, pharmaceutical, food and leather industries (Gulati *et al*., 2005). Promising areas for the application of lipases include the biodegradation of plastics (Gombert *et al*., 1999) and the resolution of racemic mixtures to produce optically active compounds (Muralidhar *et al*., 2001). These attributes of lipases owe to their broad specificity for a wide spectrum of substrates, stability in organic solvents and enantioselectivity (Snellman & Colwell, 2004).

The production of lipases is influenced by many factors such as pH, temperature, carbon and nitrogen (George *et al*., 1999). *Aspergillus* spp. are natural 'factories' for the production of enzymes such as cellulases, xylanases, amylases, proteases, and lipases. Owing to their ability for elaborating an array of extracellular enzymes, aspergilli play an important role in the production of industrial enzymes (Lockington *et al*., 2002). The industrial demand for lipolytic enzymes continues to stimulate the search for new enzyme sources. In view of the diversity in applications, renewed interest is evinced in the development of new sources of lipases. Sources of bacteria, yeasts and moulds produce lipases with different enzymological properties and specificities but moulds are known to be more potent lipase producers (Choo *et al*., 1998). *Aspergillus* spp. are among the most well known lipase producers and their enzymes are suitable for use in many industrial applications (Fu *et al*., 1995). Although many lipases have already been described, the search for new lipases with greater thermostability and substrate selectivity is on (Mateos *et al*., 2006).

Fig. 1. Paper nest of Ropalidia marginata

In this study, *A. japonicus* isolated from the paper nest of *Ropalidia marginata* was investigated for its ability to elaborate lipase enzyme vis-a-vis the culture environment. The enzyme production was optimized against the environmental variables such as culture medium, carbon and nitrogen sources and the influence

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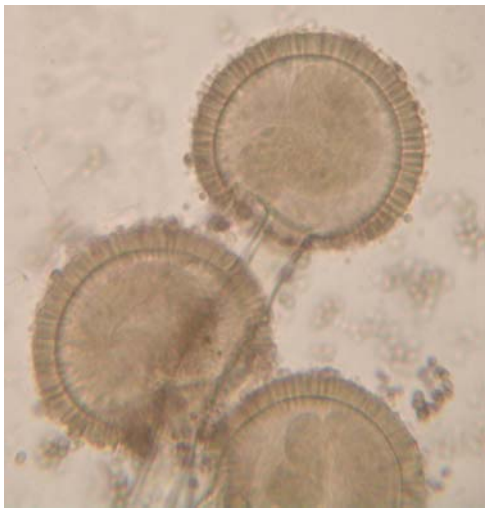
of pH and temperature as also certain lipid inducers. The enzyme was grown in a minimal production medium containing pig fat for enhanced lipase production for further scaling up process.

Materials and methods

Isolation of A. japonicus from the paper nest of Ropalidia marginata

The paper nest of *Ropalidia marginata* was collected from a glass house at CAS in Botany, University of Madras (Fig. 1). The paper nest was surface sterilized with 0.1% mercuric chloride for 2 min and washed thoroughly in sterile distilled water for 2 min and the bits plated on PDA and incubated at 25°C for a week. A fungus, identified as *A. japonicus* (Deuteromycete: Hyphomycetes), was isolated from the paper nest of *R. marginata* (Fig. 2). After isolation, the fungus was subcultured and the pure culture was stored in PDA at 4°C.

Fig. 2. Spore morphology of A. japonicus



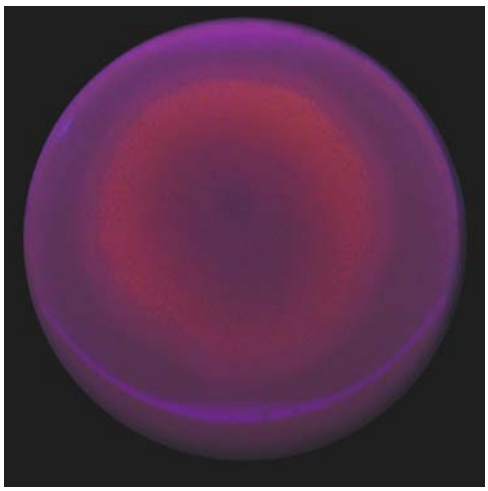
Lipase activity on Rhodamine-B agar plates

The fungal strain was tested for lipase production on agar plates that contained 15 g, olive oil 10 ml, Rhodamine-B 0.01g and Tween- 80 0.001% in 1000 ml of distilled water. After 7 days of incubation at 28°C, the plate was irradiated with UV light (350 nm), with a bright pink fluorescent halo confirming lipolytic activity (Valeria *et al*., 2003).

Lipase production medium

Modified lipase medium (YEP) was prepared by adding 10 g peptone and 1 g yeast extract to 1000 ml of 0.1M phosphate buffer, pH 7.0 (Roberts *et al*., 1987). Then, 1% filter sterilized glucose was added to the sterile peptone–yeast extract solution. Sterile erlenmeyer flasks (250 ml) containing 100 ml sterile yeast-extract-peptone medium with glucose were inoculated with 2 ml spore suspension from 7 day old culture of the test fungi and incubated with incidental illumination at 30°C in a rotary shaker at 120 rpm. After 5 days of incubation, the cultures were filtered through a filter paper to remove the mycelium. The sterile culture filtrates were stored at -20°C for further use. The culture filtrate was used as the enzyme source.

Fig. 3. Extracellular lipase activity by A. japonicus on Rhodamine-B plate irradiated under UV



Spectrophotometric assay for lipase activity

Lipase activity was assayed quantitatively by using ρ-nitro phenyl palmitate as the substrate (Winkler & Stuckmann, 1979). 10 ml isopropanol containing 30 mg ρ-nitro phenyl palmitate (Sigma) was mixed with 90 ml 0.05 M sodium phosphate buffer (pH 8) containing 207 mg sodiumdeoxycholate and 100 mg gum arabic. A total volume of 2.4 ml freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1 ml enzyme solution. After 15 min incubation at 37°C, absorbance at 410 nm was measured against a blank. One enzyme unit was defined as 1µmol of ρ-nitrophenol enzymatically released from the substrate in milliliters per minute.

Effect of pH

The effect of pH was studied by adjusting the YEP medium to different pH values–5.5 to 9.5. Erlenmeyer flasks (250 ml) containing 100 ml of YEP medium were inoculated with a 7 mm mycelial disc of 7 day old culture of *A. japonicus* and incubated over a period of 7 days at room temperature. The lipase activity was measured in the culture filtrate after the incubation period.

Effect of temperature

The effect of temperature was studied by incubating the test fungi at different temperatures. Erlenmeyer flasks (250 ml) containing 100 ml of YEP medium with pH 7.5 were inoculated with a 7mm mycelial disc of 7 day old culture of *A. japonicus* and incubated over a period of 7 days at different temperatures (20°C-80°C). Lipase activity was estimated in the culture filtrate after the incubation period.

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Effect of carbon source

Glucose, sucrose, galactose, lactose, fructose, maltose, ribose, mannitol, starch or glycogen were added to the lipase production medium at 1.0%, inoculated with 7 mm mycelial discs from 7 day old culture of *A. japonicus* and incubated for a period of 7 days at optimum pH and temperature. Lipase activity was estimated in the culture filtrate after the incubation period.

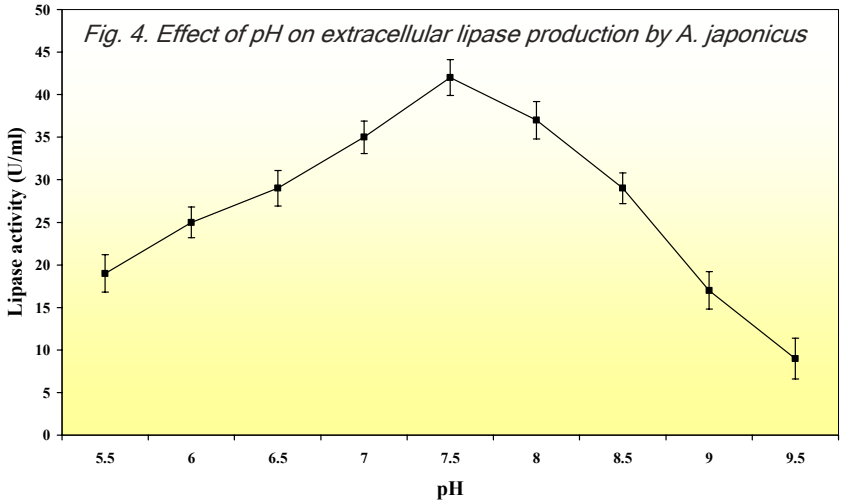

Fig. 4. Effect of pH on extracellular lipase production by A. japonicus

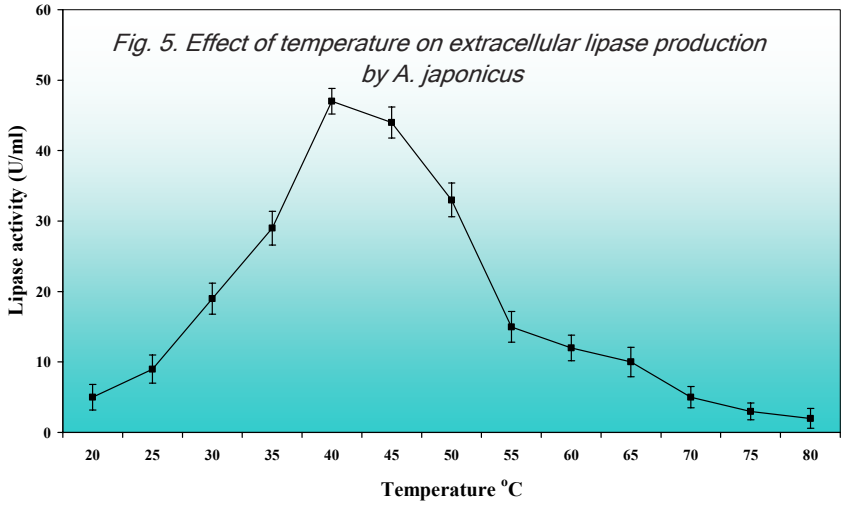

Fig. 5. Effect of temperature on extracellular lipase production by A. japonicus

Effect of nitrogen source

Organic nitrogen sources like peptone, soybean meal extract or yeast extract and inorganic nitrogen sources such as ammonium chloride, ammonium sulphate, ammonium nitrate or urea was added to YEP medium at 1.0%, inoculated with 7 mm mycelial discs from seven day old culture of *A. japonicus* and incubated for a period of 7 days at optimum pH and temperature. The lipase activity was measured in the culture filtrate after the incubation period.

Effect of inducers

Different lipid sources at 1.0% were added to the lipase production medium as inducers of lipase. Tween-20, Tween-40, Tween-60, Tween-80, Tween-100, tributyrin or likewise, different vegetable oils or an animal fat (pig fat) was amended to the lipase production medium at 1.0% each. Olive oil, sunflower oil, castor oil, mustard oil or groundnut oil were used as vegetable oil source. *Aspergillus japonicus* was inoculated into the production medium and incubated as above. The culture filtrate after 7 days incubation served as the enzyme source.

Effect of incubation time and aeration

The lipase production medium inoculated with the test fungus was incubated for a period of 7 days in both shaken and stationary states. The enzyme activity was assayed for every 24 h, in both shaken and stationary culture filtrates and the results were plotted against the biomass of the culture filtrate.

Biomass

The biomass of the test fungus grown in the lipase production medium was estimated by measuring the dry weight. The mycelium from the liquid culture was harvested and dried in an oven and the dry weight was estimated. The harvested mycelium was dried in a preweighed filter paper by incubating it in an oven at 60°C to a constant weight (Prescott *et al.,* 1993).

Statistical analysis

All the experiments were carried out in triplicate; the data were analyzed by a two-way ANOVA with an Agres statistical software package (Agres, 1994). The least significant difference (LSD) analysis was performed to group the treatment mean values. The values presented in the graphs are those of the mean of three independent experiments and the error bars indicate standard deviation.

Results and discussion

The influences of various environmental factors such as pH, temperature, nitrogen, carbon and lipid sources on lipase production were well documented. After having screened the fungus isolated from the paper nest which has a waxy coating, for the production of extracellular lipase in the laboratory, the necessary confirmation test for the enzyme was carried out. Subsequently, the enzyme production was quantified by a suitable spectrophotometric method by manipulating the culture environment. Extracellular lipase activity on Rhodamine-B agar was witnessed as a bright pinkish red fluorescent zone around the colonies indicating that the enzymes produced are but lipases (Valeria *et al*., 2003). The enzyme is believed to interact with the dye, Rhodamine-B leading to a complex- formation which fluoresces when exposed to ultraviolet radiation (Fig. 3).

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The culture environment has a dramatic influence on enzyme production (Elibol & Ozer, 2001). Enzyme yields can be enhanced several fold by providing the suitable cultural conditions both physical and chemical. The initial pH of the culture medium is crucial for enzyme production. A near neutral pH (7.5) was found to have favoured lipase secretion in *A. japonicus* (Fig. 4). In contrast, Jonsson and Snygg (1974) observed elevated growth and lipase production in *Mucor caseolyticus* at an acidic pH (4.0). Temperature as one of the important factors of the physical environment also profoundly influences enzyme secretion (Oso, 1978). *Aspergillus japonicus* lipase was most active at 40°C, pointing to its thermotolerance (Fig. 5). This attribute is advantageous for application in industries as most industrial processes take place at elevated temperatures. *Fusarium solani* FS1, *F. oxysporum* f. sp. *vasinfectum* are known to elaborate thermotolerant lipases (Hoshino *et al*., 1991; Maia *et al*., 2001). Carbon and nitrogen sources play a crucial role in enzyme induction in microorganisms. Sucrose favoured maximum lipase activity in *A. japonicus* while, an organic nitrogen peptone exerted the same effect (Fig. 6 & 7). As for lipid substrates as inducers, although triglycerides are well known for their positive effects on lipase induction in microorganism, in this study pig fat favoured maximum activity of the enzyme (Fig. 8). Similar trend was observed by Yadav *et al*. (1998). The production medium with pH 7.5 was inoculated with *A. japonicus* with sucrose, peptone and pig fat at 1.0% each at 40°C over a period of 7 days in both shaken and stationary states to study the effect of aeration. The enzyme activity was estimated at 24 h intervals in both shaken and stationary culture filtrates. The biomass and the concomitant enzyme activity were maximum on the 7th day. The biomass was 1.08 g ml^{-100} in stationary cultures while the same was 1.23 g ml^{-100} in shaken cultures. The lipase activity was also maximum on the 7th day. Lipase activity was 105 U/ml in stationary cultures whereas it was 120 U/ml in shaken cultures. There was a gradual decrease in lipase activity from 8th day onwards of incubation and no change in biomass was observed either (Table 1).

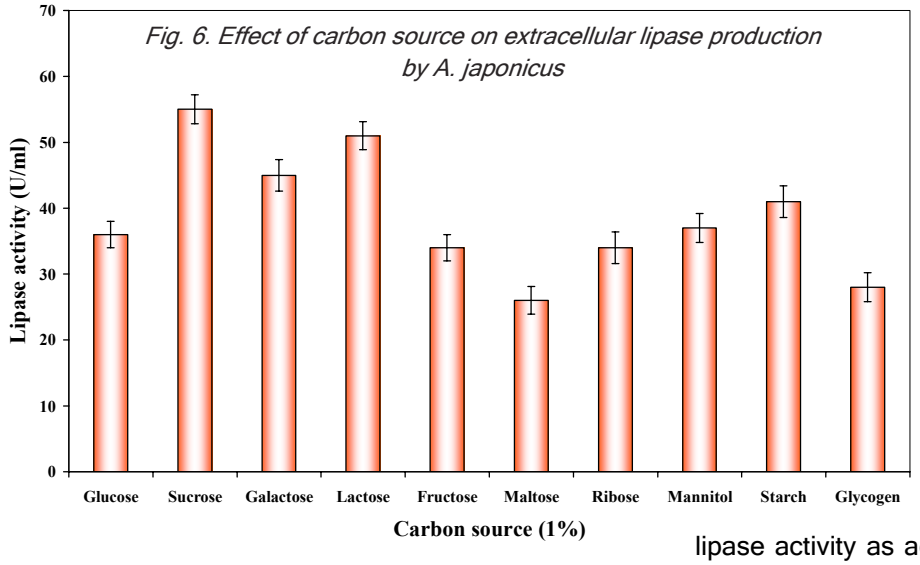

Fig. 6. Effect of carbon source on extracellular lipase production by A. japonicus

Aeration also exerts its own influence on enzyme synthetic processes in microbes. In this study, shaken cultures yielded maximum lipase activity as against static culture. Incubation period also is crucial for enzyme elaboration. A 7 day incubation offered good yield of the enzyme in the test organism.

Therefore, these environmental factors need a careful and judicious management so as to reap a good harvest of this industrial enzyme. This investigation reveals that the environment greatly influences enzyme production and that its optimization needs a careful manipulation of the cultural environment. The indication that the enzyme showed a tendency towards thermotolerance merits further consideration for developing the same towards this goal for better industrial application.

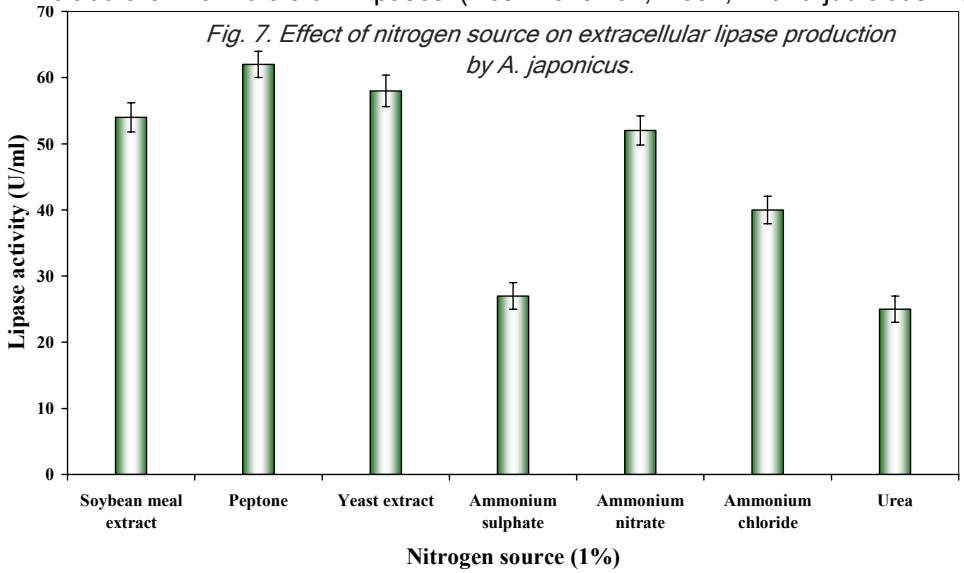

Fig. 7. Effect of nitrogen source on extracellular lipase production by A. japonicus.

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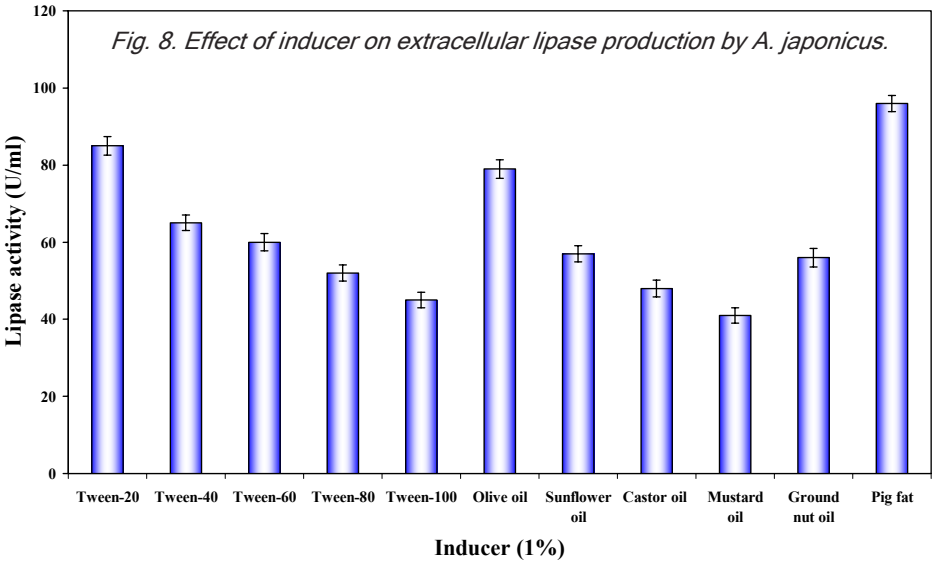
Table 1. Relationship between biomass and lipase secretion in A. japonicus

Incubation period (hrs)	Biomass (g/ml^{-100})		Lipase activity (U/ml)	
	Stationary	Shaken	Stationary	Shaken
24	0.98 ± 0.02^{k}	1.10 ± 0.02^{fg}	87 ± 1.7^{n}	92 ± 1.8^{i}
48	1.01 ± 0.02^{ij}	1.13 ± 0.02^{e}	91 ± 1.8^{m}	101 ± 2.0^{h}
72	1.00 ± 0.02^{j}	1.15 ± 0.02^{d}	93 ± 1.8^{k}	107 ± 2.1^{e}
96	1.02 ± 0.02^{i}	1.16 ± 0.02^{d}	97 ± 1.9^{j}	107 ± 2.2^{b}
120	1.04 ± 0.02^{h}	1.18 ± 0.02^{c}	100 ± 2.0^{i}	110 ± 2.1^{e}
144	1.06 ± 0.02^{g}	1.21 ± 0.02^{b}	103 ± 2.0^{g}	115 ± 2.2^{d}
168	1.08 ± 0.02^{f}	1.23 ± 0.02^{a}	105 ± 2.1^{f}	120 ± 2.2^{a}
192	1.08 ± 0.02^{g}	1.23 ± 0.02^{b}	103 ± 2.0^{g}	118 ± 2.2^{c}

Values are means of three replicates and the values in the columns with same letter are not significantly different at p= 0.05 level



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