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Solid state bioprocessing for scale up of *Aspergillus tamarii* MTCC5152 lipase and its degreasing effect on cow hide

A. Dayanandan*, S. Hilda Vimala Rani, M. Shanmugavel, A.Gnanamani, and G. Suseela Rajakumar
Department of Microbiology, Central Leather Research Institute (CSIR- New Delhi) Adyar, Chennai-600020, India
asdaya17@gmail.com

Abstract

To scale up lipase production process, the effects of different oil cakes and oils as substrates in solid state fermentation (SSF) were investigated. Combination of wheat bran (WB) with gingelly oil cake (GOC) and gingelly oil was observed to be suitable medium for the scale up of lipase. Wheat bran and gingelly oil cake in the ratio of 8:2 along with 0.5% gingelly oil showed 998 u/g of lipase activity in perforated steel trays in koji room cultivation. When compared to flask both enamel tray and perforated steel trays in koji room level scale up studies showed an enhanced production by 12.1% and 26.1%, respectively. A combination of gingelly oil cake and wheat bran exhibited bulk production of lipase in the koji room with shorter period of incubation. Application of 3% of *Aspergillus tamari* lipases offered maximum degreasing in the hide and the results are comparable to the commercial enzymatic process.

Keywords: Wheat bran, oil cakes, lipase, scale up production, Aspergillus tamarii MTCC5152.

Introduction

Among the allknown microbial enzymes, lipase constitutes the versatile biotechnological tool and most important group of biocatalysts for biotechnological applications (Reetz 2002; Kishore *et al*., 2011; Sangeetha *et al*., 2011). Lipases are versatile catalyst and have drawn the attention of much research for the production of biodiesel (Zhang *et al*., 2011; Lee *et al*., 2011). Blends of lipase with other enzymes are used in detergent industries and as dietary supplement (Harwood 1989). Fungal lipases are one of the commercially important industrial enzyme and has many applications viz food, detergent and leather industries and are an important tool in studying the structure of protein and peptides (Jaeger & Reetz 1998; Dierick& Decuupere, 2002).

Well known microbial lipases are derived from a wide variety of yeasts, molds and bacteria (Balaji & Ebenezer, 2008; Jayaprakash & Ebenezer, 2010). Fungal lipases offer a distinct advantage over bacterial enzymes in terms of ease of downstream processing. A large proportion of commercially available lipases are currently derived from *Aspergillus* strains, although potential use of fungal lipases is being increasingly realized (Contesini, 2010). In recent years, solid state cultures gained much consideration in production of microbial lipases through solid state fermentation (SSF) by filamentous fungi which utilizes low-cost agricultural residues as substrates, economic space, and gives high yield (Gombert *et al*., 1999; Pandey *et al*., 2000; Gutarra *et al*., 2005). Agro based substrate not only supports and stimulates the secondary metabolite production under optimized conditions but also gives nutrients and anchorage to the cells (Emtiazi *et al*., 2003; Wang *et al*., 2008).

About 40-45 % of the production cost of industrial enzymes is estimated to be the cost of the growth medium. Therefore, there is a strong need to optimize the conditions for cost-effective scale up production of lipase. Environmental pollution caused by chemical-based industries necessitates the development of environmentally friendly processes as an alternative to currently employed chemical method. In this regard, lipase based enzyme technology has potential to replace the conventional method (Dayanandan *et al*., 2003).The present study was investigated to optimize various process parameters using low cost oil rich solid substrate for the production of lipase and its application potential for degreasing operation in leather processing. The aim of the present work is to analyze the efficiency of microbial source and efficacy of mixed oil rich substrates containing various oil cakes for scale up production of lipase by *A.tamarii*MTCC5152 and evaluate the degreasing potential of lipase in ecofriendly leather processing.

Materials and methods

Analytical grade chemicals purchased from Hi-media were used in this study. Wheat bran, different oil cakes (gingelly, groundnut, cotton seed and coconut) and oils (olive, gingelly, coconut and groundnut) have been purchased from local supplier. *Aspergillus tamarii* MTCC 5152 used in the present work was isolated from tannery effluent soil and maintained on CzapekDox's agar slants. Spore suspension from 7 day old slant culture raised on Czapek Dox's agar incubated at 28 ± 2°C containing 0.1% Tween-80 was used as an inoculum after adjusting the desired spore count using a haemocytometer. Spore suspension was filtered aseptically and appropriate volume was used.

SSF and fermentation conditions

Erlenmeyer flasks (250 ml) containing 8 g of wheat bran and 2 g of powdered oil cakes (gingelly, ground nut, cotton seed and coconut oil cakes) were moistened with 7.0 ml water (70% v/w) and sterilized at 121 °C for 15 min. After cooling, the flasks were inoculated with spore suspension (1.0 ml) containing 10^6 spores/ ml. The contents of each flask were mixed thoroughly with a sterile glass rod for uniform distribution of fungal spores in the medium and incubated at 28 °C in temperature controlled incubator at static condition for the fungal growth. After the incubation period, the ferment from each

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flask were extracted with phosphate buffer and the enzyme activities were estimated every 24 h intervals for a period of 6 days. In addition the effect of various oils on enzyme production was studied by adding 0.5% w/w of olive, gingelly, coconut and groundnut oil to the best substrate selected in this study. The flasks were then inoculated, incubated, extracted and enzyme activities were assayed as described above.

Extraction and enzyme assay method

The mouldy agricultural residue was thoroughly mixed with 1:10 ratio of 0.1M phosphate buffer, pH 7.0 containing 0.1% Tween-80, in an orbital shaker filtered and centrifuged at 10,000 rpm for 15 min in a refrigerated centrifuge and used as a crude enzyme extract. Two methods were adopted to assay the lipase activity. Enzyme activity was assayed through alkali titration with olive oil emulsion as the substrate, using a modified version of the procedure described by Saxena *et al*. (2003). In brief, the assay mixture consisting 5 ml of olive oil emulsion, 4 ml 0.1 M phosphate buffer (pH 7.0) and 1 ml crude enzyme extract was mixed well and incubated for 20 min at 37°C. The reaction was stopped by the addition 20 ml acetone. The mixture was then titrated with 0.05M NaOH in the presence of phenolphthalein (0.1ml) as indicator. The titre values were used to calculate for lipase activity. One unit of lipase activity is defined as the amount of enzyme required to release 1µmol of fatty acid per minute under the standard assay conditions. To confirm the lipase activity spectrophotometric method was also performed (Kwon and Rhee, 1986) and a good agreement has been noted between the results obtained using the two methods. Protein content of the crude enzyme extract was analyzed by the method of Lowry *et al*. (1951) and total free fatty acid content released was estimated according to the method of Saxena *et al*. (2003).

Process optimization studies for lipase production

Solid state cultivation in flasks: For the flask production wheat bran 8 g and oil cakes 2 g were used for the growth conditions were described in section 2.2. The parameters studied were effect of various oil cakes, addition of different oil sources, initial moisture content, and inoculum size and incubation period. The parameters optimized each time were incorporated in subsequent experiments conducted during the present study. Lipase activities were expressed in units per gram of dry weight of the fermented substrate (u/g).

Scale up process: The fungal strain was cultivated in enamel trays (20 x 8 x 5 cm^3) containing 300 g substrates (240 g of of wheat bran and 60 g of gingelly oil cake) moistened to 70% v/w with distilled water. All other conditions were maintained as optimized for 250 ml Erlenmeyer flasks as mentioned earlier. The trays were covered with perforated aluminium foil and sterilized at 121° C for 20 min, cooled, and then inoculated with 5% of 7 day old inoculum. The trays were incubated in a temperature controlled incubator at 27 ° C, at 90-95% relative humidity for 144 h. Samples were withdrawn at desired intervals, and lipase was assayed as described in section 2.3. Scales up studies were done in a koji room with 3 kg of substrate in each perforated steel trays (3 ft by 1ft by 2 inch). The substrate chosen for flask level studies were used in scale up studies also. The substrates were inoculated with 5% of 7 day old inoculum and incubated for 144 h. All the parameters and other conditions studied were as optimized in 250 ml Erlenmeyer flasks as mentioned earlier. The koji room temperature was maintained at 27° C and the humidity was kept at 90 to 95%. The scale up studies conducted in Koji room was effect of moisture content, inoculum size and incubation period. Samples were withdrawn at desired intervals, and lipase activity was assayed.

Degreasing of cow hide using lipase: Hides were processed as per the standard procedure (Puvanakrishnan & Dhar, 1998). The dehaired hide up to 15 x 20 cm were subjected to degreasing using *A. tamarii* MTCC5152 lipase and commercial enzymatic degreasing agent and the samples were kept under shaking in a drum for 4 hours. The liquor was collected and estimated for the amount of free fatty acid liberated from the hide according to Saxena *et al*. (2003). All

Fig. 1. Effect of oil cakes at flask level (10g) lipase production

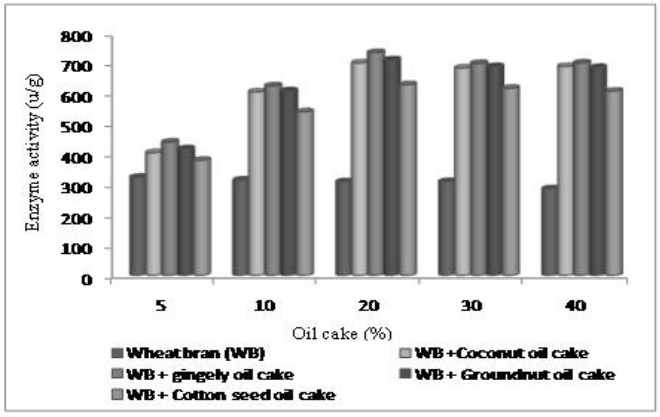
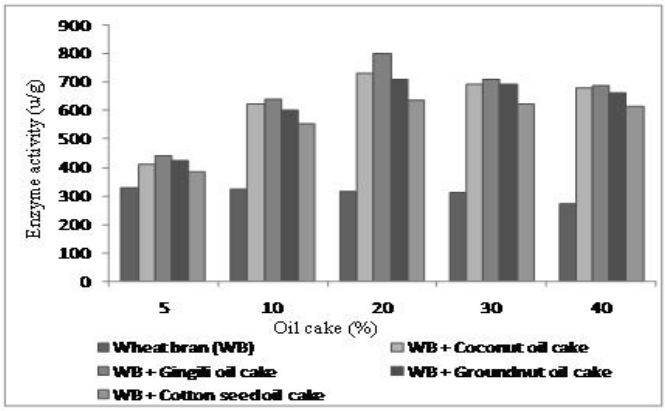


Fig. 2. Effect of oil cakes at enamel tray level (300 g) lipase production



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experiments were done in triplicate and the values presented are the means of three independent determinations.

Results

Effect of combination of wheat bran with various oil cakes on enzyme production

Ideal agro-based oil cakes for lipase production in a solid-state fermentation process based on cost and availability of such substrates were identified and screened. The results of the present study (Fig. 1 & 2) indicate that lipase production pattern varied with the type of agro-oil cakes. Maximum enzyme activity was obtained when combination of wheat bran (80%) and gingelly oil cake (20%) was used, compared to the other substrate combination.

Table 1. Effect of addition of oils on lipase production in flasks

Substrate	96h	120h
	(Lipase u/g)	
WB	552	574
WB +GOC	611	735
WB + GOC + Gingellyoil	753	792
WB + GOC + Coconut oil	652	757
WB + GOC + Groudnut oil	665	761
WB + GOC + Olive oil	748	782

Fig. 3. Effect of concentration of gingili oil on lipase production

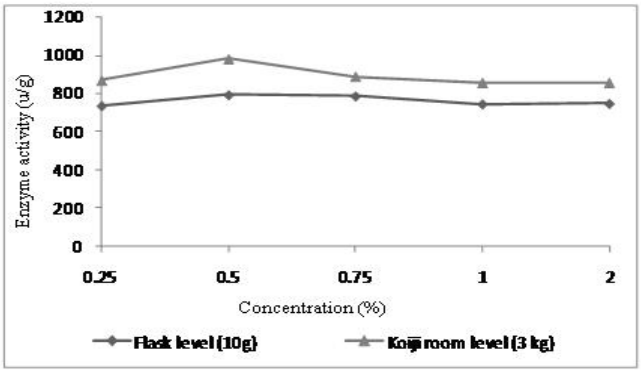
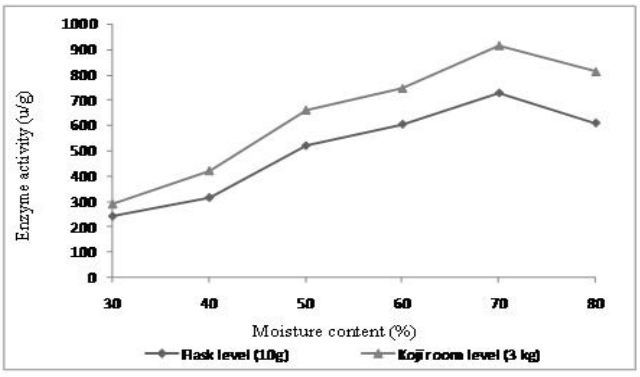


Fig. 4. Effect of initial moisture content on lipase production



Effect on addition of various oils on the production of lipase

Addition of various oils in different combination with wheat bran and gingelly oil cake on enzyme production was studied. It is revealed that the maximum lipase activity (Table. 1) was obtained when 0.5% gingelly oil was added to wheat bran containing gingelly oil cake (977 u/g).

Effect of various concentration of gingelly oil on lipase production

Fig. 3 shows the results on the effect of addition of different concentration of gingelly oil on lipase production. Maximum lipase activity was found when 0.5% gingelly oil was added to wheat bran containing gingelly oil cake (989 u/g).

Effect of initial moisture content

The initial moisture content is a crucial factor that affects the product formation through solid state fermentation. To check the influence of moisture on lipase activity during SSF, the flask (10 g) and Koji room level (3 Kg) fermentation substrates were moistened with different percentage (30-80%) of moisture and the results are shown in Fig. 4. Wheat bran and gingelly oil cake at 70% moisture content produced maximum lipase activity (914 u/g). In flask level experiment the enzyme

Fig. 5. Effect of inoculum size on lipase production

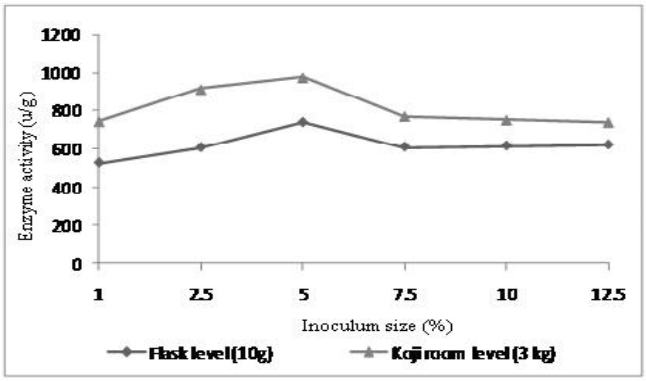
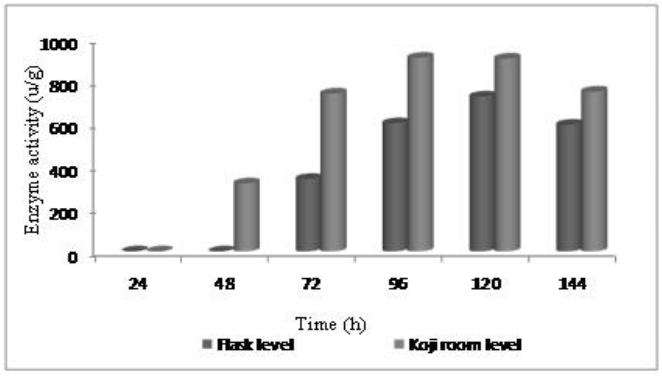


Fig. 6. Effect of incubation time on lipase production



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production was found to be declined when the moisture content was above or below 70%.

Effect of inoculum size

Fig. 5 shows the results on the effect of different inoculum level (1-12.5%) for the maximum production of lipase in flask (10g) perforated steel trays in Koji room level (3 kg) studies. 5% inoculum shows the maximum lipase activity in both types of cultivations. Lower or higher inoculum size did not support the increase in production.

Effect of incubation period

During the course of study, enzyme activity was detected in the culture extracts of all the experimental samples with a different level of production. Maximum activity was found after 120 h in flask studies with an activity of 731 u/g. In Koji room cultivation, maximum activity (914 u/g) was obtained after 96 h of incubation (Fig. 6). A steady decline in enzyme yield was observed after 120 h of incubation.

Table 2. Comparison of lipase production by A.tamarii in various levels of SSF

Substrate	Flask	Enamel tray	Koji room	Comparison between flask and tray culture	Comparison between flask and Koji room culture
	(Lipase u/g)			(% increase)	
WB + GOC	736	801	908	8.8	23.3
WB+OC+ Gingelly oil	791	887	998	12.1	26.1

Comparative study on lipase yield in flask, enamel trays and Koji room production

Several researches have claimed that the SSF in tray culture gives greater enzyme yield than flask (Solis-Pereira *et al*., 1993; Pandey *et al*.,1999) and the data shown in Table. 2 shows a comparative evaluation on the lipase yield by *A. tamarii* MTCC5152 in flasks, enamel tray and perforated steel trays in Koji room level production. When compared to other two levels, perforated steel trays in Koji room levels culture accomplished 23.3 % increase in enzyme yield using gingelly oil cake. It is further enhanced to 26.1% of enzyme production by the addition of gingelly oil.

Degreasing studies using A. tamarii MTCC5152 lipase

Table. 3 shows that the application of 4% of *A. tamari* MTCC 5152 lipase shows maximum degreasing effect. The results were comparable with control samples processed using commercial degreasing agents.

Discussion

Maximum production of lipase was obtained with combination of wheat bran and gingelly oil cake as a fermentation media. It could be attributed to the solid material's dual role: supply of nutrients for microbial growth and anchorage for the growing mycelia. SSF by *Aspergillus* species using certain oil cakes were showed to induce lipase production (Kamini *et al*., 1998; Iluyemi *et al*., 2006). In this investigation gingellyoil cake was used for further studies. Supplementation of oil source showed a mild improvement in the production. Addition of olive oil induce the lipase production of *Staphylococcus* sp. Lp12 (Pogaku *et al*. 2010) and *A. niger* (Colin *et al*., 2010). Immanuel *et al*. 2008 observed that lipases are inducible enzymes and can be induced in appreciable amount with gingelly oil by *Serratia rubidaea*. The lipase activity was improved by the addition of castor oil in *A. niger* grown in solid-state fermentation (Dutra *et al*., 2008).

Table 3. Degreasing effect of commercial enzymatic agents and A.tamarii lipase in of hide

Hide treated with Lipase	Free acid released (%)
Control [Commercial enzymatic degreasing agent (4%)]	100
A.tamarii lipase 2 %	50
A.tamarii lipase 3 %	69
A.tamarii lipase 4 %	92
A.tamarii lipase 5%	90

From the growth parameters, it is evident and also established, that lower moisture levels leads to particle agglomeration, limitation in gas transfer, and competition from microbes. Szendefy *et al*. (2006) reported that the highest yield of xylanase by *A. oryzae* was attained at 80% initial moisture content on eucalyptus and bagasse pulp as substrate. 5% inoculum is sufficient for optimal level of mycelium production and enzyme as well. Toshiko *et al*. (1989) reported that 5% of inoculum gave maximum production of lipase by *Rhizopus oligosporous*. Increase in mycelial mass at higher inoculum level, reduces the production of enzyme due to exhaustion of nutrients in the fermentation medium. Maximal lipase production by *Streptomyces* sp. was reported after 120 h of incubation (De Azeredo *et al*., 2004). *Penicillium* sp. in SSF showed maximum activities after 72 h (Yang *et al*., 2000; Agarwal *et al*., 2003). The decline in lipase production after the optimum period in both the experimental set up (flask, koji room) could be due to depletion of nutrients available to the fungus cells. During the microbial cultivation, the depletion of nutrients decreases the production of enzymes (Chu *et al*., 1992; Gupta *et al*., 2002).

It is significant to note that in Koji room, lesser period of incubation was sufficient to achieve optimum enzyme production. This might be due to conducivegrowth condition and anchorage to the mycelia as well as availability of nutrients in the SSF medium. There was an increased production of lipase in koji room compare to flasks and enamel tray, may be due to proper aeration followed by better biomass, and heat transfer from the fermentation media. Scale up studies of xylanase shows an increment in production (Archana & Sathyanarayan, 1997). It has been reported that combination of gingelly oil cake and wheat bran and rawa induces better lipase production (Edwinoliver *et al*., 2010). Based on scale up studies results from koji room investigation, pilot scale production (25 kg) of lipase by *A. tamarii* MTCC5152 was

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conducted. It may be possible to achieve higher level of lipase production with increase in quantities of the potential solid substrate wheat bran and gingelly oil cake using *A. tamarii* MTCC5152 in the koji room. Application of *A. tamarii* lipase on hide shows a best degreasing effect. Afsar and Cetinkaya (2008) reported that an application of lipase to goat skin shows the best degreasing effect and a combination of alkaline lipase and alkaline protease gave a better degreasing.

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