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PRODUCTION, DETOXIFICATION AND PURIFICATION OF LIPASES USING DE-OILED SEED CAKE AS A SUBSTRATE.

Asmita R. Shringeri* and G. R. Naik

Department of Post Graduate & Research in Biotechnology, Gulbarga University, Kalaburagi-585106, Karnataka, India.

***Corresponding Author: Asmita R. Shringeri**
Department of Post Graduate & Research in Biotechnology, Gulbarga University, Kalaburagi-585106, Karnataka, India.

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ABSTRACT
Lipases (triacylglycerol acylhdrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. Lipases are enzymes that can be secreted by several microorganisms able to produce these enzymes. Many agro-industrial residues can be used as potential substrate for production of enzymes. Lipolytic bacteria was isolated from contaminated garage soil sample and grown on tributyrin media containing 1% of Tributyrin. This article discusses the enzyme assay, production of lipase using solid state fermentation, detoxification of seed cake, purification of lipase using ammonium sulphate precipitation, column chromatography**,** gel filtration chromatography and use of microbial lipases. Our results suggested that solid state fermentation process is a tool to utilize the low-cost substrate like simarouba seed cake, jatropha seed cake, pongamia seed cake for the production of industrially important enzyme such as lipase. In view of the increasing understanding of lipases and their many applications in high-value syntheses and as bulk enzymes, these enzymes are having an increasing impact on bioprocessing.

KEYWORDS: Lipases, Detoxification, Solid state fermentation, Column Chromatography, Gel Filtration Chromatography SDS-PAGE.

INTRODUCTION
Lipases are one of the most important classes of industrial enzymes. They hydrolyse triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. In recent years, there has been an increasing interest in the study of lipases mainly due to their potential applications as medicines (digestive enzymes), food additives (flavour modifying enzymes), clinical reagents (glyceride-hydrolysing enzymes) and cleaners (detergent additives) (Sharma et al., 2001). Lipases would be economically manufactured in solid state fermentation.

Solid state fermentation (SSF) is defined as the fermentation of solids in the absence of free water; however, the substrate must possess enough moisture to support the growth and metabolism of microorganisms. Recently, several reports have been published indicating the application of this culture in upgrading the food and industrial wastes and in the production of fine chemicals and enzymes. The utilization of by-products and wastes from food and industrial sources has several advantages over submerged fermentation such as superior productivity, simple techniques, reduced energy requirements, low wastewater output, improved product recovery and the reduction in production costs (Ashok, 2003). In SSF, any type of substrate, including industrial wastes, could be used to enhance the production of enzymes because of their richness in fatty acids, triacylglycerols and /or sugars. The use of cheap raw materials would diminish the operating costs of the process. Moreover, total capital investment for lipase production has been reported to be significantly lower in solid state fermentation than in submerged fermentation (Castilho et al., 2000). Most studies on lipolytic enzymes production with bacteria, fungi and yeasts have been performed in submerged fermentation; however, there are only few reports on lipase synthesis in solid state fermentation. In recent years, considerable research has been carried out using agricultural wastes, which are renewable and abundantly available to produce value-added products. For example babassu oil cake (Gombert et al., 1999), olive cake and sugar cane bagasse (Cordova et al., 1998), gingelly oil cake (Kamini et al., 1998), wheat bran (Mahadik et al., 2002), rice bran (Rao et al., 1993) and *Jatropha curcas* seed cake (Mahanta et al., 2008) have been used as the substrates for lipase production.

Since they are rich in fatty acids, triacylglycerols and / or sugars and other nutrients, they can serve as the potential

substrates for lipase production using SSF. Hence an attempt is made in this paper to utilize the simarouba seed oil cake as a substrate for the production of lipase by solid state fermentation. It was under taken to optimize the key process variables, including incubation time, inoculum level, initial moisture content, carbon level and nitrogen level of the medium for the production of lipase using this oil cake under SSF.

MATERIALS AND METHODS

Enzyme assay

The reaction mixture consisted of 0.1 ml of 10% tween in 50 mM Tris hydrochloride pH 8.0 and $CaCl_2$ solution of 1 M. Reaction was started by addition of suitable quantity of Lipase solution in 1.8 ml of Tris buffer. The crude sample obtained was used for the assay. The crude enzyme was used in different concentration. Reagent blank was prepared with buffer instead of enzyme and incubated at 40^0C for 1 h.

As a result of Tween hydrolysis the fatty acids released during the reaction from insoluble fatty acid salt giving a precipitate that can be measured turbidometrically at 400 nm. 1 unit of lipase activity was defined as that of the amount of enzyme which after 1 h under the condition of the assay resulted in an increase at 400 nm of 0.01. Results were tabulated and activity was calculated. The specific activity was calculated according to the following formula,

$$\text{Specific Activity} = \frac{\text{Activity of the enzyme in units}}{\text{Concentration of protein in mg/ml}} = \_\_\_\_\_ \text{ Units/ mg of Protein}$$

In vitro assay of lipase

The plate screening enzyme assay was carried out for three isolates. The reaction mixture consisted of 0.1 ml of 10% tween in 50mM Tris hydrochloride pH 8.0 and $CaCl_2$ solution of 1 M. Reaction was started by addition of suitable quantity of Lipase solution in 1.8 ml of Tris buffer. The crude enzyme sample was used in different concentration. Reagent blank was prepared with buffer instead of enzyme and incubated at 40^0C for 1 h. As a result of Tween hydrolysis the fatty acids released during the reaction from insoluble fatty acid salt giving a precipitate that can be measured turbidometrically at 400 nm. 1 unit of lipase activity was defined as that of the amount of enzyme which after 1 h under the condition of the assay resulted in an increase at 400 nm of 0.01. Results were tabulated and activity was calculated. The specific activity was calculated according to the following formula,

$$\text{Specific Activity} = \frac{\text{Activity of the enzyme in units}}{\text{Concentration of protein in mg/ml}} = \_\_\_\_\_ \text{ Units/ mg of Protein}$$

Estimation of total protein

The protein estimations for all the enzyme extracts were carried out according to Bradford (1976). The Bradford reagent was prepared by dissolving the CBB G-250 in 50ml of 95% ethanol. 100ml of o-phosphoric acid was added later and the whole reagent was diluted to 200 ml to make 5X concentrated dye. In clean and dry test tubes, 5 ml of 1X Bradford reagent was pipette out. 0.1 ml of the enzyme extract was added to each test tube and mixed well. The optical density of protein-dye complex was read within 30 minutes at 595 nm. The concentration of protein was calculated using standard graph.

Production of Lipase

Parameters Involved In Solid State Fermentation

With reference to the plate screening for lipase production, two isolates (ARS_21) were selected for further study, based on the larger zone of hydrolysis.

Agrowaste Screening

About 5g of sugarcane baggasse, Pigeon Pea waste, Rice bran, sawdust, Dal husk and the seed cakes (after detoxification) were dispensed into conical flasks. To that about 5ml of nutrient broth media was added. Then conical flasks were autoclaved, after cooling bacterial culture was added (5%) and incubated for 24 h in an incubator. After 24 h enzyme was extracted by adding 10 ml of Tris buffer (50 mM, pH 8.0) and vigorously shaking for 10 min. The mixture was filtered through muslin cloth and centrifuged at 10,000 rpm for 10 min in cold. The supernatant was considered as crude enzyme and assay was done as said before. According to the results Simarouba cake gave maximum activity. So all the coming parameters were done using Simariouba cake.

Detoxification of seed cake

The seed cakes were detoxified by ethanol extraction (Saetae and Suntornsuk, 2010) in order to remove phorbol esters. Five grams of the seed cake (Honge, Susa honge, Simaruba, Neem and Hippe cake) was extracted by 15 mL of 90% (v/v) ethanol with a shaking speed of 150 rpm for 5 min at room temperature. This extraction was repeated four times. The seed cake passed through this process was referred to as the detoxified seed cake.

Large-scale production

500 ml of nutrient broth was prepared 1% tributyrin was added to the broth. The culture was inoculated to the broth and kept for incubation at 37^0C for 24 h. After a day incubation the broth was centrifuged at 10,000 rpm for 3 min. the enzyme content supernatant was collected.

Partial purification of Lipase

Ammonium sulphate precipitation

The 200ml broth was centrifuged and the supernatant was saturated to 80% (w/v) with ammonium sulphate. The saturation was carried by adding ammonium sulphate slowly into the crude extract with constant stirring. The stirring was continued for 2 more hours after ammonium sulphate was completely dissolved to aid the precipitation. Whole process was carried out for 8 hrs in cold. The precipitated protein was separated by centrifuging at 10,000 g for 10mins. The protein pellet

was dissolved in minimal volume of 25mM Tris buffer (pH 8.0).

Dialysis

The precipitated proteins were further purified by dialysis. The protein samples were dialyzed using the low cut off membrane against 500ml of 5mM dialysis buffer containing 5mM Tris buffer (pH 8.0) for 8 hrs with 2 changes in buffer.

Column Purification

For the purification of Lipase enzyme two column matrices were used. The first approach was carried with gel filtration column followed by ion-exchange column.

Gel filtration chromatography

The Sephadex G-75 column material was activated by heating the matrix at 50^{o}C for 30 minutes with 50mM Tris buffer. After cooling the floating fines were removed and loaded into a column without forming any air bubble the column was allowed to settle and was washed with 100 ml of 50mM extraction buffer (pH 8.0). The dialyzed enzyme (3ml) was loaded on to a Sephadex column pre-equilibrated with extraction buffer with simultaneous fraction collection. 25 fractions of 2ml each were collected and analyzed for the eluted protein by measuring the absorbance at 595nm followed by screening for the active fractions by finding the activities of the Lipase. The specific activity of the enzyme eluted at every step of purification and the total fold purification was calculated.

Ion exchange chromatography

Ion exchange Matrix preparation

Pre swollen anion exchanger matrix, DEAE Cellulose, was used for the matrix preparation. The pH of the ion exchange slurry was brought to 8.0 with acidic/basic component of the buffer and allowed to settle.

Packing of column

The prepared ion exchange slurry was packed in 30ml column without forming any air bubble. The column was allowed to settle and washed with 50mM of Tris buffer (pH 8.0). The pooled active fraction of the gel filtration column (2ml) was loaded onto a DEAE Cellulose column pre equilibrated with extraction buffer. The bound enzyme was eluted with linear gradient of 25-500mM extraction buffer. The 25 fractions of 2ml were collected. The fractions were analyzed for the eluted protein by reading the absorbance at 595nm. The fractions were screened for Lipase activity and the active fractions were pooled together.

SDS-PAGE analysis of purified product

10% separating gel mix containing 2ml of deionized water, 1.65ml of 30% Acrylamide, 1.25ml of 1.5M Tris-Cl buffer (pH 8.8), 50µl of SDS, 50µl APS and 10µl TEMED was prepared and poured in between the clean, grease free glass plate, pre sealed at the bottom with agar. After 5-10min, the stacking gel containing 1.7ml of deionized water, 415 µl 30% Acrylamide, 315µl of 1.5M Tris-Cl buffer (pH 6.8), 25µl SDS, 25µl APS, 10µl TEMED was prepared and poured onto the polymerized separating gel. Immediately the comb was inserted and the gel was incubated to polymerize completely.

The protein sample (80µl) was mixed with 30µl of loading dye Bromophenolblue. The protein sample and dye was boiled for 3min and loaded into the washed wells of polyacrylamide gel. For electrophoresis, the buffer of pH 8.3 containing 3g of Tris buffer, 14.4 g of glycine, 1g of SDS dissolved in 1L of distilled water was used. The electrophoresis was carried out at 10min at 100V and then later carried out at 50-100V at room temperature until the dye front reaches the bottom of the gel.

Visualization of Protein in gel using $AgNO_3$ staining

$AgNO_3$ staining was carried out after electrophoresis of the samples. The gel was fixed in the fixing solution for 2hrs with continuous shaking and washed with 50% EtOH for 3 times with continuous shaking. Treat with pretreatment solution for exactly 1min followed by 3 washes in distilled water (20sec each). Soak gel in $AgNO_3$ solution for 20min followed by 2 washes with distilled water (20sec each). Develop in developer till suitably colored bands without background staining are visible. Finally wash gel briefly in distilled water and stop reaction by adding the fixing solution.

RESULTS

Bulk Production

The enzyme was produced by inoculating the culture in 500 ml of culture media. The supernatant obtained was used for purification.

Enzyme assay

Bulk product of lipase extracted by centrifuge and assayed. The activity was found to be 1.17 U/ml.

Estimation of total protein

The total protein was estimated by Bradford's method with the help of BSA standard curve. The protein was 4.51 mg/ml and specific activity was 14.86 U/mg proteins.

Purification of lipase enzyme

Lipases have been extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature and effects of metal ions and chelating agents. In many cases, lipases have been purified to homogeneity and crystallized. Purification methods used have generally depended on nonspecific techniques such as precipitation, hydrophobic interaction chromatography, gel filtration and ion exchange chromatography.

The enzyme was purified to homogeneity by ammonium sulfate precipitation and dialysis, sephadex column, ion-exchange chromatography and SDS-PAGE.

Lipase was purified to by a combination of DEAE-Sephadex G-75 ion exchange chromatography.

- The lipase activity assayed in the dialyzed extract was found to be 1.18 U/ml.

Column purification

Gel filtration column

The dialyzed protein was located on to sephadex G-75 column for separation; the fractions were collected in 25 fractions of 2ml each (Fig.1).

The sephadex fractions were screened for lipase activity by performing the lipase assay method the enzyme was eluted in to all the 25 fractions. The active fraction of highest O.D (i.e. 5-15) were selected for further study and stored in the deep freezer.

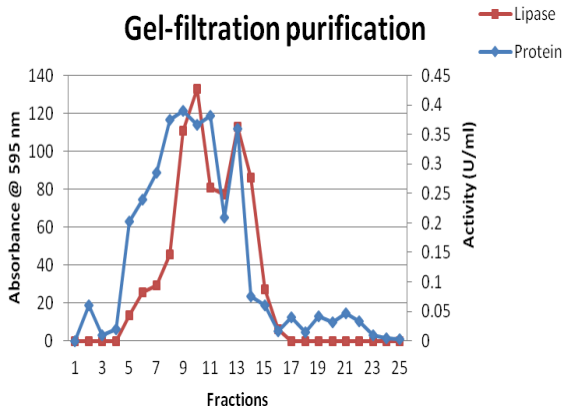

Fig.1- Elution profile of lipase from gel-filtration column.

Ion-exchange column purification

Totally 4ml of the active fractions collected from get filtration column purification, was loaded on to DEAE cellulose columns. 25 fractions were eluted of 2ml each (Fig.2).

The eluted DEAE fractions were screened for lipase activity by performing the lipase assay method the enzyme was eluted in to all the 25 fractions. The active fraction of highest O.D (i.e. 14-19) were selected for further study and stored in the deep freezer.

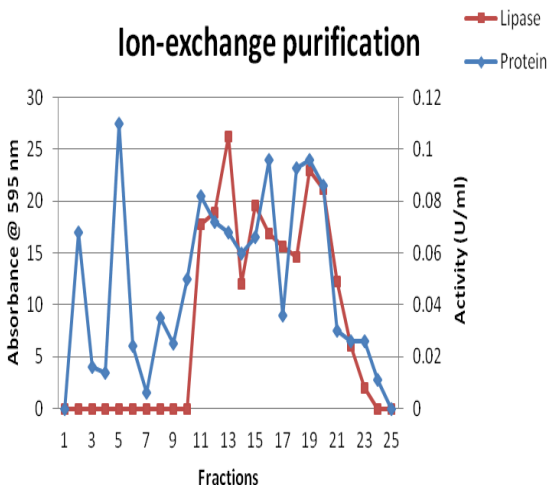

Fig.2- Elution profile of lipase from ion-exchange column.

Table-1: Purification of lipase.

Purification step	Activity (Units/mll)	Protein Concentration (mg/ml)	Specific activity (U/mg)	Yield (%)	Fold purity (%)
Crude	1.17	4.51	14.86	100	1
Dialysis	1.8	7.31	9.16	162.14	61.66
Ion-exchange	69.8	3.45	19.40	76.57	130.56
Gelfiltration	666.38	1.83	36.54	40.66	245.87

SDS-PAGE analysis

The PAGE analysis indicated three protein bands in the ion-exchange active fractions. The proteins were of molecular weight ~25kd (Fig.3).

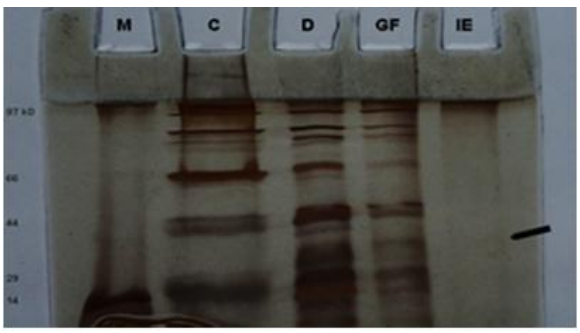

Fig.3- PAGE analysis of Lipase.

Where,

M=Marker, C=control, D=dialysis, GF=gel filtration and IE=ion exchange.

SUMMARY AND CONCLUSION

Lipases have found a wide range of applications in various industries such as chemical industry, food industry, textile, leather and pulp industries, synthetic chemistry and detergents etc. SSF has revealed the possibilities of effective utilization of agro-industrial residues for value addition through biotechnological means, lipase production by SSF using seed cake brings down the cost of production and it also provide an alternative path for the effective utilization of such nutrients rich seed cake residues.

The present study indicates that simabrouba seed cake is potential substrate for lipase production under SSF. Results obtained with study are comparable hence process can be exploited for commercial application after a comprehensive study.

REFERENCES

1. Ashok P (2003) Solid state fermentation. Biochem Eng J, 13: 81-84.
2. Castilho LR, Polato CMS, Baruque EA, Sant Anna GL Jr, Freire DMG (2000) Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. Biochem Eng J, 4: 239-247.
3. Cordova J, Nemmaoui M, Ismaili-Alaoui M, Morin A, Roussos S, et al. (1998) Lipase production by solid state fermentation of olive cake and sugar cane bagasse. J Molecular Catalysis B: Enzymatic, 5: 75-78.
4. Gombert AK, Pinto AL, Castilho LR, Freire DMG (1999) Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. Proc Biochem, 35: 85-90.
5. Joshi C, Mathur P, Khare SK (2011) Degradation of phorbol esters by *Pseudomonas aeruginosa PseA* during solid-state fermentation of deoiled *Jatropha curcas* seed cake. Bioresour Technol, 102(7): 4815–4819.
6. Kamini NR, Mala JGS, Puvanakrishnan R (1998) Lipase production from *Aspergillus niger* by solid-state fermentation using gingelly oil cake. Proc Biochem, 33: 505-511.
7. Mahadik ND, Puntambekar US, Bastawde KB, Khire JM, Gokhale DV (2002) Production of acidic lipase by *Aspergillus niger* in solid state fermentation. Proc Biochem, 38: 715-721.
8. Mahanta N, Gupta A, Khare SK (2008) Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa PseA* in solid-state fermentation using *Jatropha curcas* seed cake as substrate. Bioresour Technol, 99: 1729-1735.
9. Mochizuki M, Hirano M, Kanmuri Y, Kudo K, Tokiwa Y (1995) Hydrolysis of polycaprolactone by lipase: effects of draw ratio on enzymatic degradation. J Appl Polymer Sci, 55: 289-296.
10. Openshaw K (2000) A review of *Jatropha curcas*: an oil plant of unfulfilled promise. Biomass Bioenergy, 19: 1–15.
11. Rao P V, Jayaraman K, Lakshmanan CM (1993) Production of lipase by *Candida rugosa* in solid state fermentation. 1: determination of significant process variables. Proc Biochem, 28: 385-389.
12. Sharma R, Chisti Y, Banerjee UC (2001) Production, purification, characterization and applications of lipases. Biotechnol Adv, 19: 627-662.
13. Yana L, Thara S, Jemil Y, Nicolas S (2010) Fermentable sugar release from Jatropha seed cake following lime pretreatment and enzymatic hydrolysis. Bioresour Technol, 101: 6417–6424.