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## RESEARCH ARTICLE

### IMPORTANCE OF LIPASES OF OIL SEEDS IN THE PROCESS OF ECO-FRIENDLY BIODIESEL

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#### ABSTRACT

The current rapid growth in demand for fuel and global climatic changes due to the oil processing has resulted in the quest for alternatives for renewable fuels. Recent developments in the oil industry has indicated a growing interest in the biodiesel production and has proved to be an environmentally acceptable fuel source. Many plant oils have been suggested and used as fuels across the globe. However, the processing methods for converting the plant oils into feasible biofuels need to be simplified and generalized while keeping the environmental challenges minimal. Accordingly, in comparison to the chemical processing of oils, enzymatic catalysis in the transesterification of oils is found to require simple chemicals as well as lower temperatures. In this work it has been estimated the efficiency and concentrations of lipases of selected oil seeds viz., *Arachis hypogaea* (Groundnut), *Glycine max* (Soya), *Helianthus annuus* (Sunflower), *Sesamum indicum* (Gingelly) for their candidacy in the bioprocessing of biodiesel. Our data indicated the lipases from Gingelly to be the most efficient with the highest specific activity suggesting high turnover per unit protein and lowest Km suggesting highly active nature of the enzyme. Though our data is indicative of the fact that the Gingelly lipases could be used for the bio processing of the biodiesel, this enable to trust further necessary investigations with different oil samples.

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## INTRODUCTION

There is an exorbitant global demand for clean renewable fuel for replacing the fossil oil owing to the environmentally hazardous trails. Recent developments in the global diplomacy as well as participation of most countries in ensuring zero harmful emission have resulted in a majestic quest for alternate fuel options. This has rapidly grown interest in the biodiesel production among the global leaders (Sippula et al., 2019). The main reasons which strongly support biodiesel production include increasing energy demand, limited fossil fuel resources and climate change which can be altered using renewable bio fuels for the transport sector and lowering emissions of ecologically harmful particles such as sulphur, carbon monoxide and hydrocarbons (Kumar et al., 2018). Biodiesel is mainly obtained by the transesterification of triacylglycerols (TAG) with an alcohol like methanol, ethanol via acid/ base catalysis. TAGs are major constituents in vegetable oils and animal fats. The current industrial-scale protocols of transesterification of oils which is the primary step of the biodiesel production needs pure acid/ alkali and other chemicals and pose a dangerous environmental threat.

The mixtures obtained after trans-esterification are composed of fatty acid mono alkyl esters and are classified as biodiesel (Shivayogimath et al., 2009; Montoya et al., 2014; Tamilalagan et al., 2019). In the biodiesel production, alkaline trans-esterification is the most used industrial method, however enzymatic catalysis is more efficient than acid and alkali catalysis as it works under moderate reaction conditions, small amount of methanol required in the reaction and impacts no environmental challenges (Wang et al., 2014). Enzymatic catalysis using lipases is an alternative to chemical catalysis processes, is simple to perform, at low investment cost and therefore potentially easier to disseminate, especially in developing countries. Contrary to alkaline catalysts, enzymes do not form soaps and can esterify both free fatty acid and triacylglycerides in one step without the need of subsequent washing step (Yellapu et al., 2017). Although microbial lipases have been extensively studied, little research has been focused on the use of plant lipases in biodiesel production (Bharathiraja et al., 2017). These lipases can, however, be readily extracted from the plant seeds and they are less expensive to use than microbial lipases. The basic objective is to obtain alkyl esters from oils using enzymatic method. Enzymatic pathways are clearly being studied to an higher extent for industrial-scale biodiesel production (Khot and Ghosh, 2017). Although microbial lipases have been extensively studied and used to

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catalyze transesterification for biodiesel production, plant lipases are a promising alternative to microbial lipases due to easy availability, low toxicity and higher biodiesel yield (>96%) (Mendoza-Lopez *et al.*, 2017). Accordingly, lipases can also be readily extracted from the plant seeds, leaf, stem, bran or latex which are promising alternative to catalyze trans-esterification for biodiesel production (Azocar *et al.*, 2010). Most of the oils are extracted from seeds and after the oil is obtained the remaining debris is sold as fodder base or potting mix. In this investigation it has been proposed to use the proteins in this debris to isolate value added products like lipases which can be used for biodiesel production enzymatically.

## METHODS

**Seed collection and germination:** Seeds of Groundnut (*Arachis hypogaea*), Soya (*Glycine max*), Sunflower (*Helianthus annuus*) and Gingelly (*Sesamum indicum*) were procured from the local market. The seeds of each species separately soaked in water for six hours and then allowed to germinate for 24h at room temperature (28±2°C).

**Isolation of Lipases from Oil Seeds:** Germinated seeds of Sunflower, Soya beans, Groundnuts and Gingelly were de-husked manually according to previous methods of Gadge *et al.* (2011) with minor modifications. The endosperm of the seeds were homogenized in Tris HCl buffer (0.1M, pH 7.5) containing EDTA (1mM) and beta-mercaptoethanol (0.1mM). The homogenates were filtered through muslin cloth and filtrates were centrifuged subsequently at 5000rpm for 10min at 4°C. The supernatant was added with 30% ammonium sulphate, the precipitated proteins were separated by centrifugation at 5000rpm for 10min at 4°C. Further, the pellet were stored at 4°C for next processing. The filtrate was further added with ammonium sulphate solution to reach a final concentration of 60%. The pellets were collected by filtration and centrifugation. The pellets obtained were dialyzed at 4°C using a semipermeable membrane bags (10kd) in 10mM Phosphate buffer (pH 8.0) for 48 hours with the spent buffers exchanged with fresh batches every 6 hours. The dialyzed protein suspension was freeze dried and the powder was weighed and stored at -20°C.

**Estimation of protein in the enzyme preparation:** Amount of protein in the enzyme preparations were estimated by using method prescribed by Lowry *et al.* (1951) with minor modifications. 10mg of the dry powder was dissolved in water and an aliquot of the sample was incubated with copper sulphate alkaline medium (10min). Subsequently, Folin – Ciocalteu's phenol was added and the blue color developed over 20 minutes was measured at 750nm using a UV-Visible spectrophotometer. The amount of protein was quantified using bovine serum albumin as the standard.

**Estimation of Specific Activity of Lipases from the seed samples:** The lipase activity in the seed preparations was estimated using the titrimetric method of Madhikar *et al.* (2011). Sunflower oil emulsion was prepared in 180mL distilled water containing 20mL sunflower oil, 0.4g of sodium benzoate. Assay mixture contained 5mL sunflower oil emulsion, 5mL 0.1M Trisbuffer (pH 8) and 1mL crude enzyme preparation and incubated at 35°C for 10min. The reaction was stopped by 10mL of acetone and methanol mixture (1:1).

Each sample was titrated against 0.01 N KOH using phenolphthalein as an indicator. The volume of KOH used in the titration was noted and used for enzyme activity calculations. One unit of lipase is defined as the amount of enzyme required to liberate 1µmol of free fatty acid from olive oil per min under the standard assay conditions.

**Effect of pH and temperature:** Optimum pH for lipase activity was determined covering the range (3-9) using 0.1M buffers of different pH. The buffers were: pH 3-6 (acetate); pH 7 (phosphate); pH 8-9 (Tris-Cl). For optimum temperature, the enzyme assay was performed as discussed above except that incubation was done at temperatures from 20-70°C.

**Estimation of Enzyme kinetics:** Lipase was assayed in reaction buffer (pH 8) at 24°C with different concentrations (10-120mg mL<sup>-1</sup>) of olive oil emulsion as a substrate. The value of Km (Michaelis constant) was calculated from Lineweaver-Burk (LB) plot by inverting the activity and the substrate concentrations.

**Statistical analysis:** All experiments were conducted in triplicates, analyses and data presented as Mean ± Standard Error. The data were processed by Student's 'T' test by using Graph pad Prism software.

## RESULTS AND DISCUSSION

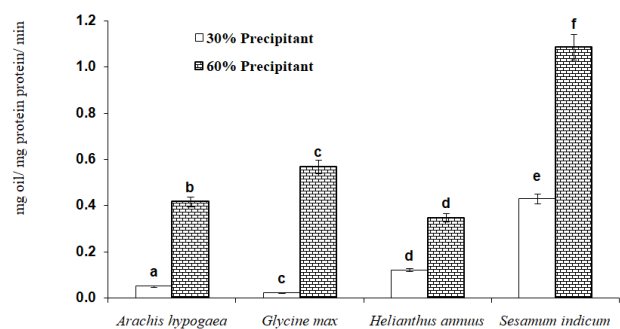


Fig 1. Specific activity of the lipase fractions from the selected oil seeds

Different letters indicate the significant difference between the groups. Results are expressed as Mean ± SE. The data was analyzed by one way ANOVA ( $p \leq 0.05$ ) followed by Post hoc Tukey's test to compare among the groups. Protein fractions obtained from different oil seeds demonstrated luxurious presence of lipases. The two sequential precipitate fractions (at 30% and subsequently 60% ammonium sulphate precipitation) from each oil seed had marked activity profile, however, their specific activity was varied significantly. Evidently, the 60% precipitate showed higher specific activity indicating the higher enzyme efficiency per unit protein. In addition, the gingelly (*S. indicum*) showed the highest specific activity (60% precipitate) in comparison to other fractions. Specific activity in comparison to activity per volume provides a greater information about the activity efficiency of the enzyme, as it indicates the enzyme units in a definite protein concentration which is in parallel to the observations of Amid *et al.* (2016). Specific activity always is an excellent tool to identify the efficiency of the enzyme catalysis since it was not influenced by the dilution or concentration of the enzyme sample. In the present data, accordingly the second fraction of gingelly seeds turned out to be the most acting enzyme in terms of trans-esterification of the oils.

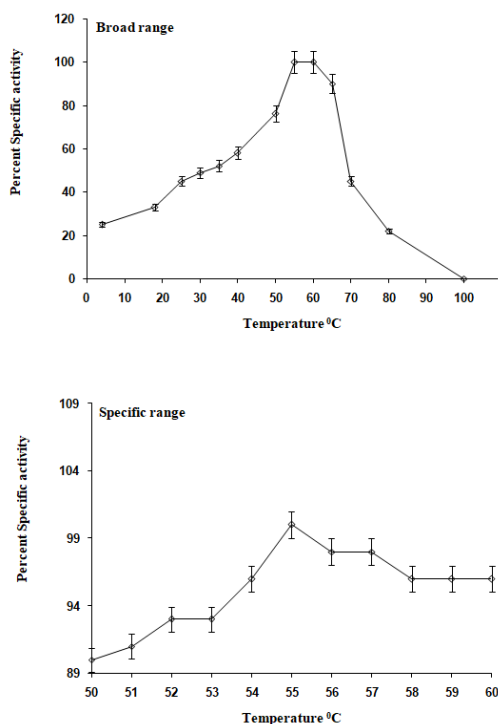


Fig 2. Optimum activity temperature of the lipases isolated from the selected oil seeds

Table 1. Optimum pH of 30% and 60% fractions of lipases isolated from the selected oil seeds

Oil seeds	pH (30% and 60% enzyme fractions)
<i>Arachis hypogaea</i>	7.8 ± 0.05
<i>Glycine max</i>	7.2 ± 0.02
<i>Helianthus annuus</i>	7.6 ± 0.05
<i>Sesamum indicum</i>	7.8 ± 0.08

Different letters indicate significant difference between the groups. Results are expressed as Mean ± SE. The data are analyzed by one way ANOVA ( $p \leq 0.05$ ) followed by Post hoc Tukey's test to compare among the groups. All the enzymes are proteins except for ribozymes. The factors influencing the optimum activity are the same as those affecting the tertiary structure of the proteins. The factors affecting the enzyme activity are pH, temperature, salt concentration etc. The temperature at which the enzymes demonstrate their highest rate of the catalysis is the point when they are in their best functioning structure including the orientation and readiness of the active site. Such point is called optimum temperature. At a lower temperature the enzyme protein would not be in the required orientation and tertiary structure; while at a higher temperature the protein is denatured and shows any evident activity. Accordingly all the fractions from the four oil seed samples tested, the optimum temperature was tested to be 55°C.

One of the major factors which affect the protein structure is the hydrogen ion concentration around the vicinity. Similarly, the enzymes are also affected by changes in pH. The pH at which the enzyme functions the greatest with the highest of its activity is called optimum pH. Lower pH and higher pH than the optimum point would change the ionic atmosphere around the protein structure. Also, the change in the pH would also affect the ionic surface of the active site structure inducing the entry or escape of water molecules into the active site crevices. This would contrastingly affect the substrate binding, hence, the enzyme catalytic efficiency or velocity.

In the present study it was found that there was an optimum pH range of 7.2 to 7.8 for all the lipase fractions from the four oil seeds.

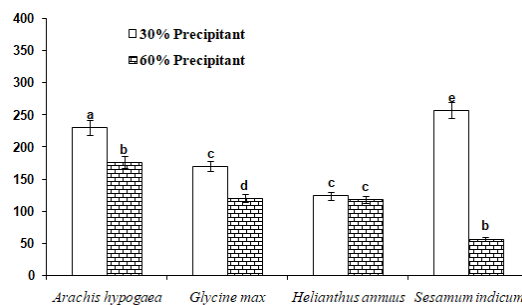


Fig 3. Km values of the lipase fractions from the selected oil seeds

Different letters indicate significant difference between the groups. Results are expressed as Mean ± SE. The data are analyzed by one way ANOVA ( $p \leq 0.05$ ) followed by Post hoc Tukey's test to compare among the groups. Rate of an enzyme catalyzed reaction varies according to factors including the concentrations of both enzyme molecules and substrates, pH, temperature as well as presence of activators and inhibitors (Sihia *et al.*, 2019). Unfortunately the response between the enzyme velocity and the substrate concentration is not linear (Khyade *et al.*, 2019).

Hence, the double reciprocal plots come to the rescue which convert the curve into a straight line. Since, this method provides a most accepted way of calculating the enzyme kinetics, it has been used the same. Accordingly, it has been calculated Km which was the concentration of the substrate required for achieving half of the maximum rate. In lines with the specific activity of the data, the second fractions in all the samples showed lowest Km indicating that they reach highest activity and start releasing the products at a lower substrate concentration. It is sure that the specific fractions were assessed at the optimum temperature and respective optimum pH. Interestingly, the second fraction of the gingely seeds showed the least Km value in comparison to the fractions of the other oil seed samples.

## Conclusion

From these exclusive data about the lipases isolated from the four oil seeds, it is evidenced that the 60% precipitation fraction from the *Sesamum indicum* (Gingely) seeds have highest specific activity and the lowest Km. Present findings indicated the efficiency of the lipase fraction to be most efficient and allowed to work with lower enzyme concentrations reaching highest activity faster. Such efficient enzymes are promising contributors in improving the industrial biodiesel production meanwhile enhancing the profits. The debris after the gingely oil extraction is usually a low economy organic fertilizer for plants which can be diverted to isolate value added product like lipases for the biodiesel production. Hence, it has been proposed to study the structure of lipases from different fractions which pave way for further understanding into the molecular details of the enzyme efficiency.

**Conflict of interest:** The authors declare that there is no conflict of interest.

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