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An Intermediate Temperature Stable, Extracellular and Alkaline Lipase from *Pseudomonas aeruginosa* and Its Application in Biodiesel Production

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ABSTRACT

An intermediate temperature stable, alkaline lipase from a newly isolated *Pseudomonas aeruginosa* strain was produced on a defined minimal medium and was found to have 800 IU/L of lipase under un-optimized conditions. Crude lipase produced by *Pseudomonas aeruginosa* was characterized for its biochemical properties and it was found to have temperature optima of 60°C with broad range of temperature activity from 40°C to70°C and pH optima was found to be 9.0 with sudden decline in lipolytic activity at pH other than 9.0. Crude enzyme was concentrated by ammonium sulfate precipitation and it was immobilized onto Celite® for is application for biodiesel production.

Keywords: Biodiesel, Enzyme characterization, Lipase and Pseudomonas aeruginosa.

1. INTRODUCTION

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) catalyze the hydrolysis of triacylglycerols into glycerol and fatty acids under aqueous conditions but under low water conditions and non- aqueous conditions they can catalyze the reverse reaction [1]. Apart from their activity over a broad range of pH, temperature and variety of substrate, their ability to catalyze enantioselective, chemoselective and regioselective [2] reactions make them the biotechnological tool of choice for biocatalysis and synthetic chemistry. Owning to their unique properties, they occupy a distinct position among all the enzymes and they have diverse applications in a wide variety of industries [3]. Although numerous applications of lipases have been documented in literature but it is still a challenge to identify the most suitable lipase for specific need such as enantioselectivity and regioselectivity, and best reaction conditions for an efficient application, because property of each lipase varies from other and its application is largely governed by its characteristic properties. Frequently used methods such as immobilization and optimization of the reaction medium cannot be transferred from one reaction system or substrate to another, this makes the task more challenging for finding a suitable lipase for a particular application. Industrial demand for highly active preparations of lipolytic enzymes with appropriate specificity and stability to pH, temperature, ionic strength and organic solvents continues to stimulate the search for new enzyme sources of their unique industrial applications [4]. One such application which has remarkably gained the momentum in recent decade is, its application in nano-biotechnology

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and biodiesel production, biodiesel being a renewable, biodegradable, and nontoxic fuel, has come up as a potential alternative fuel to meet the future fuel demands.

Biodiesel is defined as the fatty acid methyl esters (FAME) which are derived from triglycerides by transesterification with methanol [5]. Several successful attempts for biodiesel production have been made in past using soybean oil [6,7], sunflower oil [8,9], cotton seed oil [10], rapeseed oil [11], palm oil [12,13] restaurant kitchen waste [14] and Jatropha oil [15].

Here, we report an extracellular, alkaline and intermediate temperature stable lipase from *Pseudomonas aeruginosa* with its applicability and catalytic potential as biotechnological tool for biodiesel production.

2. MATERIALS AND METHODS

All analytical reagents and media components were locally purchased from established brands such as SRL, India; Hi-Media, India; Merck, India and Celite® 209 and standards (99.99% pure by GC) of methyl oleate and methyl linoleate were purchased from Sigma Aldrich, USA.

2.1 Bacterial strain and inoculum preparation

Strain of *Pseudomonas aeruginosa* was isolated from the soil of waste disposal site of Mic- Mac canteen of Delhi Technological University, Delhi and it was identified using Gaby-Hadley Reagents A and B [16-17] and was also confirmed as described by [18]. Initially, it was grown at 37°C in nutrient broth (pH 7.0) with shaking at 250 rpm for 24hrs. For lipase production, 1ml of 4hrs old culture was used to inoculate the production flasks containing 100ml production media (Table 1). The extracellular lipolytic potential of the isolated stain was confirmed by tributyrin agar plate assay and gel diffusion assay [19-20].

Table 1.: Defined minimal media for the production of lipase from *Pseudomonas aeruginosa*

S. No.	Media Component	Composition (%)
1.	Glucose	2.00
2.	Olive oil	1.50
3.	K_2HPO_4	0.30
4.	KH_2PO_4	0.10
5.	$MgSO_4.7H_2O$	0.01
6.	NH_4Cl	0.50

2.2 Enzyme production and lipase assay

The enzyme was produced at shake flask level in 250ml flasks on a defined minimal media (Table 1). pH of the production media was adjusted to 7.0 by 0.1N NaOH before autoclaving. Olive oil was used from the stock of 10% olive oil emulsion

with water in presence of 2% gum acacia. After the inoculation, production media was incubated at 37°C, 250rpm for 24hrs. Cell free culture broth was collected as supernatant by centrifugation at 15,000g for 20min. at 4°C using Eppendorf® 5810R centrifuge (Eppendorf, Germany) and its extra cellular lipolytic activity was again confirmed as described in [21-24] and total protein was estimated by Lowry's method [25]. The supernatant was concentrated by 60% ammonium sulfate precipitation followed by dialysis against pH 9.0 Tris-HCl buffer before its immobilization onto Celite® 209 (Sigma Aldrich, USA) as described in [15, 26-27].

2.3 Lipase unit

One International Unit (IU) of lipase is defined as the amount of enzyme required to liberate 1 μmol of fatty acid per ml per minute under standard assay conditions.

2.4 Characterization of lipase

Temperature and pH optima

Using pNPP as a substrate [23], initially, lipase assay was performed at neutral pH and at different temperature ranging from 20°C-90°C for identifying the temperature optima of lipase activity and thereafter, lipase assay was performed at known optimal temperature and at varied pH (3.0- 11.0) range for ascertaining the pH optima of the lipase [28].

Substrate specificity

To study the substrate specificity, lipase activity on different emulsified oils was calculated against olive oil as the standard using the method as described in [29, 30] at defined optimal temperature and pH.

2.5 Application of lipase in biodiesel production

One of our previous report [31] suggested that the maximum conversion of olive oil into its respective methyl esters (methyl oleate and methyl linoleate) can take place just in six hours using 2000 IU of *Pseudomonas aeruginosa* lipase and hence six hours was fixed as reaction time. Synthesis of biodiesel under solvent conditions by transesterification was done by using 5ml of olive oil and 5ml of methanol and they were allowed to react in presence of varied lipase units (200- 3000 IU) of Celite immobilized *Pseudomonas aeruginosa* lipase at 60°C and 200 rpm for 6hrs and the final volume of methanol was raised to 15ml by adding first dose of 5 ml methanol after 2hrs of reaction and second dose of 5ml methanol after 4hrs of reaction so as to reach the final equimolar concentration of free fatty acid and methanol and to minimize the deactivation of lipase in presence of methanol.

The reaction was terminated by collection of solvent layer after separation by centrifugation at 15000g at 4°C for 10 minutes using Eppendorf® 5810R centrifuge (Eppendorf, Germany). Excess of solvent was evaporated by vacuum evaporation and the residue was dissolved in 1.0ml of n-hexane for GC analysis. The percentage yield was also estimated and percent yield was expressed as the percentage molar conversion of olive oil to ester after titrating the residual fatty acid against 0.01 N KOH using phenolphthalein as an indicator.

2.6 Gas Chromatography analysis

GC analysis was done as described in [31]. For GC analysis, 1.0 µl aliquot of the sample was injected onto capillary column using AOC- 20i auto injector in split mode on a Shimadzu GC-2010 Gas Chromatography (Shimadzu, Japan). The GC was equipped with Restek® Rxi®-5Sil MS capillary column with 30-meter column length and 0.25 mm ID which was having a coating of 5% diphenyl and 95% dimethyl polysiloxane as stationary phase with uniform phase/ film thickness of 0.25µm. The column pressure cut off was kept at 130.0 kPa and a constant flow rate of 1.2 mL/min was maintained. The initial column oven temperature was kept at 140°C and the final temperature was raised to 280°C for a total run time of 40 min. The FID detector was used which was set at 270°C and hydrogen was used as carrier gas and the helium was used as makeup gas while air flow was maintained at 400ml/min.

3. RESULTS AND DISCUSSION

3.1 Temperature and pH optima

The extracellular lipase produced by *Pseudomonas aeruginosa* was purified 1.5-fold (Table 2) using ammonium sulfate precipitation followed by dialysis against pH 9.0 buffer and immobilization. The lipase was active over a broad range of temperature (40°C- 70°C) with temperature optima of 60°C (Fig. 1a). Similar trend has been observed for *Pseudomonas aeruginosa* MTCC 2488 lipase [32]. It has been observed that the lipases from various *Pseudomonas spp*. are active over a broad temperature range between 30°C- 80°C and some of them have been found to have temperature optima as high as 70°C [33, 34]. Some of the bacterial lipases have been found to be stable upto 70°C [35-36]. Although, a few lipases may operate at 100°C but they have short half-life as for example lipase from *P. fluorescens* [37].

Table 2: Summary of partial purification of *Pseudomonas aeruginosa* lipase

Purification steps	Total activity (IU/L)	Total protein (mg)	Specific enzyme activity (IU/mg protein)	Yield (%)	Fold purification	
					With respect to previous step	Over all
Cell free fermentation broth						
Crude	800.00	65.00	12.30	100.00	1.00	1.00
Ammonium Sulfate precipitation and dialysis						
Partially purified	720.00	48.00	15.00	90.00	1.21	1.21
Immobilization on Celite®209						
Immobilized	480.00	26.00	18.46	66.66	1.23	1.50

The pH optima and activity studies were performed at 60°C and the enzyme was found to be active within the narrow pH range of 7.0- 9.0 and there was a sharp decline in lipase activity on the either side of this pH range (Fig. 1b). This makes this enzyme alkaline in nature which can be used for various industrial processes and biocatalytic reactions operating at alkaline

pH. The enzyme was optimally active at pH 9.0 and the activity at pH 9.0 was considered as 100% activity. Lipase from *Bacillus thermoleovorans* have been reported to be active at pH 9.0 [38] and lipases from *Bacillus pumilus* LV01 and *Pseudomonas fragi* have been reported to be optimally active at pH 9.0 [33, 39].

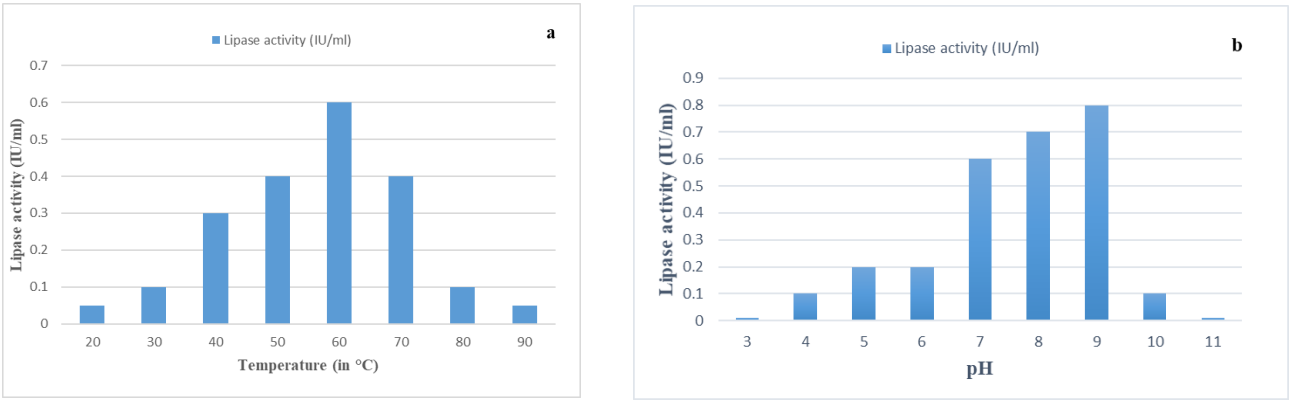


Fig. 1: *Pseudomonas aeruginosa* lipase activity at various (a) temperature and (b) pH

3.2 Substrate specificity

Activity using olive oil as substrate was considered as 100% and the relative activity was calculated for rest of the oils as substrate with respect to the activity on olive oil. Relative activity for castor oil was found to be 123.40% which was the highest among all the oils whereas lowest relative activity (4.4%) was observed in case of sunflower oil.

Thus, this lipase from *Pseudomonas aeruginosa* selectively catalyzes the hydrolysis of castor oil, mustard oil and olive oil whereas a decline in activity of 40% or more was observed in case of other oils (Table 3 and Fig. 2a). Since major fatty acid composition of castor oil is ricinoleic acid (12-hydroxy cis-Δ^9-octadecenoic acid) which is 18 carbon fatty acid and major fatty acid composition of olive oil is oleic acid (cis-Δ^9-octadecenoic acid) which is also 18 carbon fatty acid while for mustard oil, olive oil is second major constituent fatty acid after erucic acid.

This indicates that the *Pseudomonas aeruginosa* lipase is selective for the glycerides of 18 carbon fatty acids (ricinoleic acid and oleic acid). A similar affinity of *Pseudomonas* sp. lipase has been reported by Rathi *et al.* [40]. The higher affinity of the lipase for castor oil is of potential use in the castor oil based industry since the major component of castor oil is ricinoleic acid, which has numerous industrial application [41].

On olive oil as emulsified substrate, K_m and V_{max} for the *Pseudomonas lipase* were found to be 20µM and 12 µmol per ml per second respectively (Fig. 2b) which confirms its greater affinity towards the glycerides of cis-Δ^9-octadecenoic acid (olive oil) and its derivatives, such as 12-hydroxy cis-Δ^9-octadecenoic acid (castor oil).

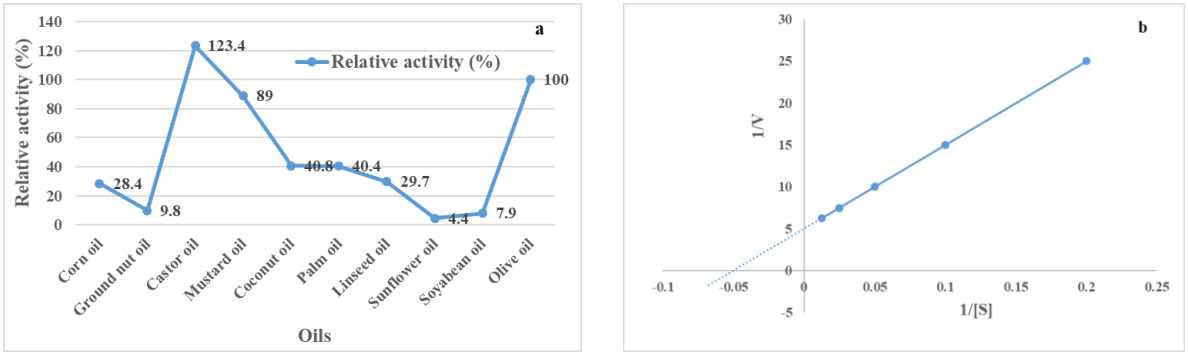


Fig. 2: *Pseudomonas aeruginosa* lipase (a) relative activity on various oils as emulsified substrate and (b) Lineweaver-Burk plot using emulsified olive oil as substrate

Table 3: Substrate specificity of *Pseudomonas aeruginosa* lipase at 60°C and pH 9.0 in aqueous conditions using various emulsified oils as substrate.

S.No.	Oil as substrate	Relative activity (%)		S.No.	Oil as substrate	Relative activity (%)
1.	Corn oil	28.40		6.	Palm oil	40.40
2.	Ground nut oil	9.80		7.	Linseed oil	29.70
3.	Castor oil	123.40		8.	Sunflower oil	4.40
4.	Mustard oil	89.00		9.	Soyabean oil	7.90
5.	Coconut oil	40.80		10	Olive oil	100.00

Table 4: Synthesis of biodiesel (major olive oil methyl esters) using immobilized *Pseudomonas aeruginosa* lipase and percent yield of biodiesel.

S. No.	Enzyme units (IU)	Percent yield	
		Methyl oleate	Methyl linoleate
1.	200	1.00	nd$^{\neq}$
2.	500	32.27	27.00
3.	1000	57.78	50.53
4.	1500	83.14	76.38
5.	2000	93.26	86.46
6.	2500	94.00	86.92
7.	3000	93.86	86.53

nd$^{\neq}$ not detected

3.3 Biodiesel production and GC analysis

Synthesis of biodiesel (FAME) by transesterification of major constituent fatty acids (oleic acid and linoleic acid) of olive oil with the help of Celite® 209 immobilized *Pseudomonas lipase* into their respective methyl esters revealed that the majority of biodiesel (methyl oleate and methyl linoleate) was synthesized while using 2000 IU of lipase and further

increase in enzyme concentration could not enhance the conversion rate and over all yield whereas 200 IU lipase could not produce any significant results (Table 4 and Fig. 3). It is evident from the results that the *Pseudomonas aeruginosa* lipase can successfully mediate the esterification reactions for the synthesis of biodiesel.

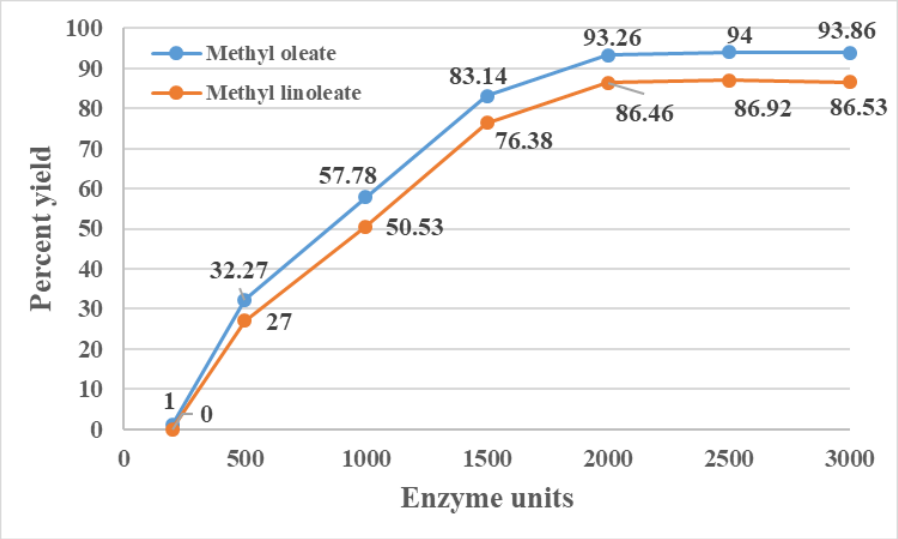
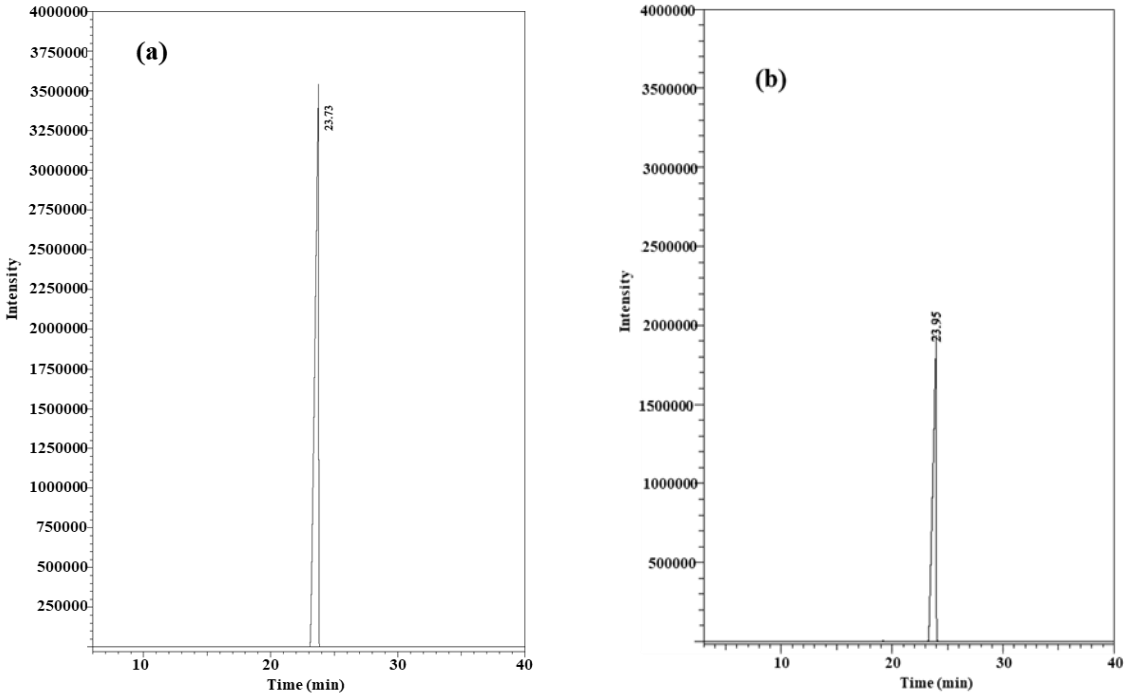


Fig. 3: Percent yield of biodiesel (methyl oleate and methyl linoleate) using olive oil and methanol as substrate in presence of *Pseudomonas aeruginosa* lipase



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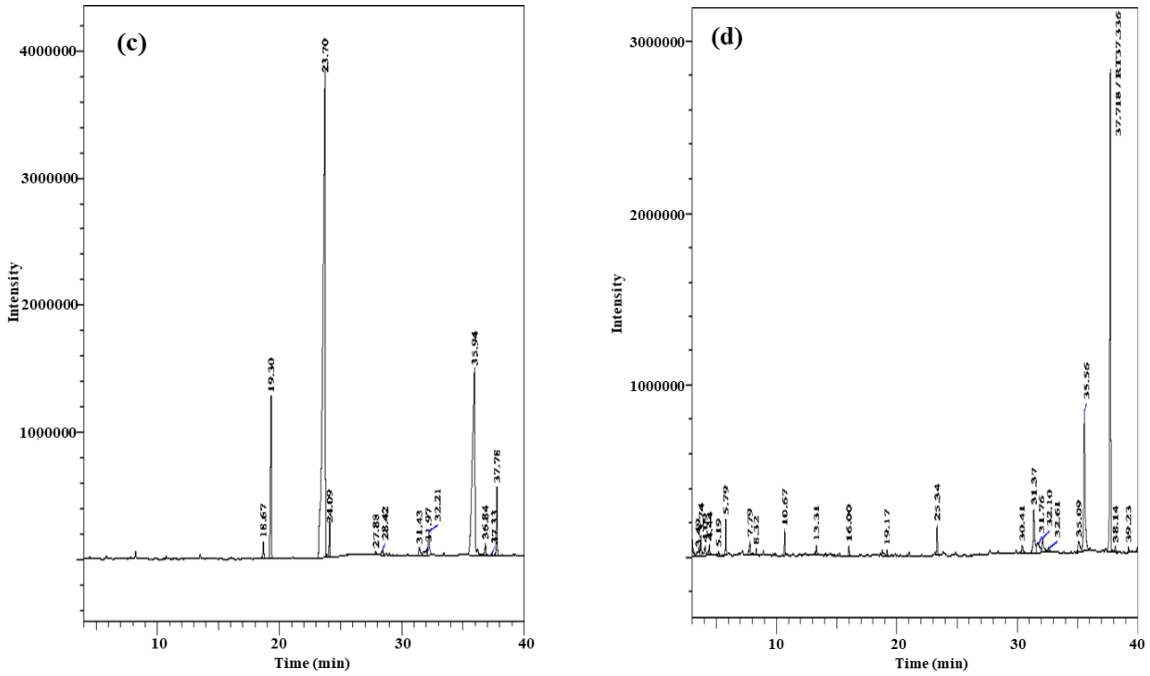

Fig. 4: GC chromatogram of standard (a) methyl oleate, (b) methyl linoleate and (c) test sample with 2000 IU lipase units (d) control.

GC analysis revealed that retention time (RT) of methyl oleate and methyl linoleate are very close (23.73 min and 23.95 min respectively) and this can be attributed to the fact that they do not differ much in chemical formula, structure and properties (Fig. 5) and hence their chemical behavior remains almost same towards the GC column stationary phase.

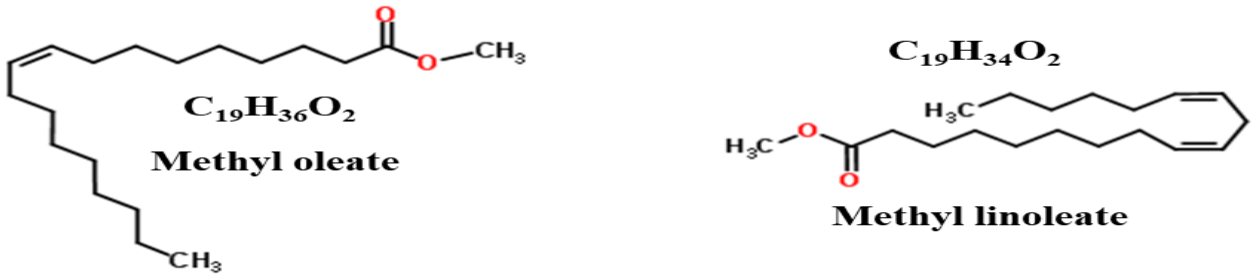

Fig. 5: Structure of methyl oleate and methyl linoleate

Synthesis of olive oil esters using various alcohols over a range of temperature (10- 90°C) have been reported by Kenz *et al.* [42] wherein they could achieve a maximum of 17.0% methyl oleate synthesis at 50°C in 3hrs while Marzuki *et al.* [43] reported 90.90% yield of methyl oleate using nano-bioconjugates *Candida rugusa* lipase.

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4. CONCLUSION

We have presented here lipase production under unoptimized conditions which indicates quite low levels of lipase and to make the process economical viable we need to further optimize the production conditions.

However, these are preliminary investigations with respect to the lipase production from the laboratory isolate of *Pseudomonas aeruginosa* but downstream processing and characterization of partially purified lipase indicates that it's an intermediate temperature stable alkaline lipase which can be used for the biocatalysis and other industrial process operating at alkaline pH. There are several similar reports for the production, downstream processing and characterization of lipase but all of them says either enzyme is active at pH 9.0 or 60°C but none of them suggests a lipase operating at alkaline pH and 60°C temperature simultaneously hence, it's a unique report with respect to the properties such as pH and temperature.

The downstream processing which we have tried could just enhance the purification of lipase by 1.5-fold. It has been observed that sometimes properties of enzymes vary with degree of their purity and hence we further need to enhance the purification of this lipase to escalate its catalytic efficacy to make it more suitable for industrial needs.

Affinity of this lipase for the glycerides of cis-Δ^9-octadecenoic acid (olive oil) and its derivatives, such as 12-hydroxy cis-Δ^9-octadecenoic acid (castor oil) makes it a suitable biocatalytic tool for the industries based on olive oil and castor oil.

Since this lipase can effectively and efficiently catalyze the synthesis of fatty acid methyl esters this indicates that it can be a potential enzyme for green chemistry for esterification and transesterification. Non-edible oils should to be tried for biodiesel production using this lipase so that its scope in biodiesel industry can be proposed.

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