Asian Journal of Applied Science and Technology (AJAST)
Volume 1, Issue 7, Pages 38-46, August 2017

Time Kinetics Studies of Enzyme Catalyzed Hydrolysis of Triolein and Transesterifications of Olive Oil for the Synthesis Major Olive Oil Methyl Esters Using Pseudomonas Aeruginosa Lipase

Vineet Kumar Goswami[1] and Jai Gopal Sharma[2]

[1,2]*Laboratory of Environmental and Industrial Biotechnology, Department of Biotechnology, Delhi Technological University, Delhi, India.*

Article Received: 20 July 2017 *Article Accepted: 05 August 2017* *Article Published: 08 August 2017*

ABSTRACT

The enzyme lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is a special class of esterase enzyme that acts on fats and oils and hydrolyze them into the respective glycerides and fatty acids, and finally on complete hydrolysis into glycerol and fatty acid. The hydrolysis of triolein and olive oil using Pseudomonas aeruginosa lipase has been carried out under controlled conditions at 60°C and 200 rpm for varied time intervals (3- 72hrs). The analysis of the triolein hydrolytic products on TLC plates showed the presence of glycerol, triolein, diolein, monoolein and oleic acid in 15hrs as compared to 24hrs for the production of oleic acid and glycerol. However, complete hydrolysis of triolein took place in 24hrs but the synthesis of methyl oleate and methyl linoleate by transesterification of olive oil in presence of methanol as acyl donor was initiated as early as 3hrs and it reached to its maximum level of respective methyl ester synthesis just in 6hrs.

Keywords: Catalysis, Gas chromatography, Lipase, Methyl linoleate, Methyl oleate, Olive oil and Transesterification.

1. INTRODUCTION

Lipases (triacylglycerol ester hydrolases E.C.3.1.1.3) are hydrolytic enzymes [1&2]. They are special class of esterase enzymes that act on fats and oil and hydrolyze them into the respective glycerides and fatty acids, and finally on complete hydrolysis into glycerol and fatty acid. Lipases are used as versatile biocatalysts in modern organic chemistry especially for modifications of fats and oils via hydrolysis, esterification, transesterification, interesterification reactions [3,4 & 5] and as a tool for nano-biosensor [6]. On the other hand, this enzyme can efficiently carry out the reverse reaction of synthesis under water limiting conditions [7].

Olive oil is the primary edible oil of Spain, Italy and several other countries. The approved limits for its various constituent fatty acids have been published by European Union (European Community Regulation, 1991; 2001) and International Olive Oil Council. The respect of these limits does not characterize olive oil (especially extra virgin and virgin as largely defined). Fatty acid composition differs from sample to sample and is influenced by the olive variety, production zone, climate and stage of maturity of the drupes when they are collected. The composition of major fatty acids, oleic acid and linoleic acid in olive oil varies from 49-79% and 3.1-24% respectively depending upon the session of harvest and region of

© 2017 AJAST All rights reserved.

cultivation of crop [8&9]. In the present study, the rate of hydrolysis and the rate of transesterification reactions catalyzed by the immobilized lipase enzyme produced by *Pseudomonas aeruginosa* were studied for the analysis of time kinetics of hydrolysis of triolein and for the transesterification kinetics of olive oil into respective methyl esters.

2. MATERIAL AND METHODS

A natural isolate of *Pseudomonas aeruginosa* produced 22,000 IU/L lipase at share flask level on a defined media with composition (%): Casein Acid Hydrolysate (2.000), Tween 80 (1.000), KH_2PO_4 (0.300), KH_2PO_4 (0.100), $MgSO_4.7H_2O$ (0.085), NaH_2PO_4 (0.680) and pH 6.3±0.2. The crude enzyme from fermentation broth was concentrated and partially purified by 60% ammonium sulfate precipitation followed by dialysis against pH 9.0 Tris-HCl buffer and was further concentrated using 60kDa membrane and was immobilized onto the alumina before its use in biocatalysis. Product analysis was carried out using Thin Layer Chromatography (TLC) and Gas Chromatography (GC) and percent yield was determined by titration of initial and residual fatty acids contents according to the procedure [10]. Olive oil (Figaro brand) was purchased from the local market at Delhi and the standards of methyl oleate and methyl linoleate were procured from Sigma Aldrich, USA.

One International Unit (IU) of lipase activity is defined as the amount of enzyme required to release one micro-mole of fatty acid per ml per minute under the standard assay conditions.

Triolein hydrolysis was carried out in the aqueous system at 60ºC and 200 rpm for varied time (3-72hrs). 1 ml of triolein and 2000 IU of immobilized *Pseudomonas aeruginosa* lipase and 4 ml of Tris- HCl buffer pH 9.0 were incubated at 60°C for 72hrs with shaking at 200 rpm and aliquots of 200µl were withdrawn at a regular interval of time upto 72hrs and the reaction were terminated using 5ml of diethyl ether each time. The products were analyzed by thin layer chromatography (Silica Gel 60 F_{254}, Merck, Germany) using the solvent system consisted of petroleum ether: diethyl ether: acetic acid in ratio of 80:30:1. Spots were visualized in a saturated iodine chamber.

For the synthesis of major olive oil methyl esters (methyl oleate and methyl linoleate) under non- aqueous conditions by transesterification, 5ml of olive oil and 5ml of methanol was allowed to react in presence of 2000 IU of immobilized *Pseudomonas aeruginosa* lipase at

© 2017 AJAST All rights reserved.

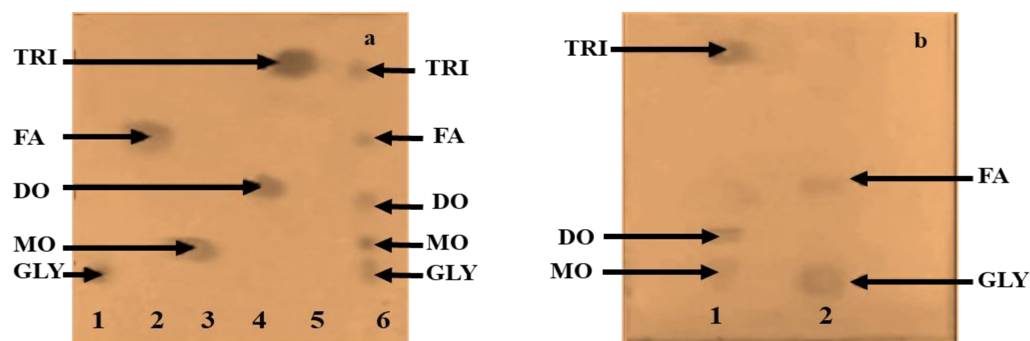
60°C and 200 rpm for varied time intervals (3- 72hrs) and the final volume of methanol was raised to 15ml by adding additional first dose 5 ml of methanol at one-third time of termination time and second dose of 5 ml methanol at two-third time of termination time so as to reach the final equimolar concentration 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 and 4°C for 5 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.

2.1 GC Analysis

A1.0 μl aliquot of the sample was injected onto capillary column using AOC- 20i auto injector in split mode on a Shimadzu GC-2010 Gas Chromatograph (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 phase/ film thickness of 0.25μm. The column pressure cut off was held at 130.0 kPa and a constant flow rate of 1.2 mL/min was maintained. The initial column oven temperature was held 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 DUISCUSSION

The hydrolysis of triolein using *Pseudomonas aeruginosa* lipase was studied in relation to time. The analysis of the hydrolytic products on TLC plates showed the presence of free fatty acid, glycerol, triolein, diolein, monoolein and oleic acid at the reaction time of 15hrs (Fig. 1a) but no free fatty acid was observed on TLC plate for initial 3hrs sample (Fig. 1b) indicating very poor or no hydrolysis during initial 3hrs while for 24hrs sample, only glycerol and free fatty acid could be spotted on TLC plate indicating complete hydrolysis of triolein in 24hrs (Fig 1b) after which the concentration of oleic acid did increased in the reaction system (Fig. 2 & table 1).



Lane 1- 5: Standards **Lane 1: 3hrs.**

Lane 6: Triolein hydrolysis at 15hrs. **Lane 2: 24hrs.**

TRI=Triolein, FA= Fatty acid, DO= Diolein, MO=Monoolein, GLY= Glycerol

Fig. 1: TLC analysis of hydrolysis products of triolein using *Pseudomonas aeruginosa* lipase

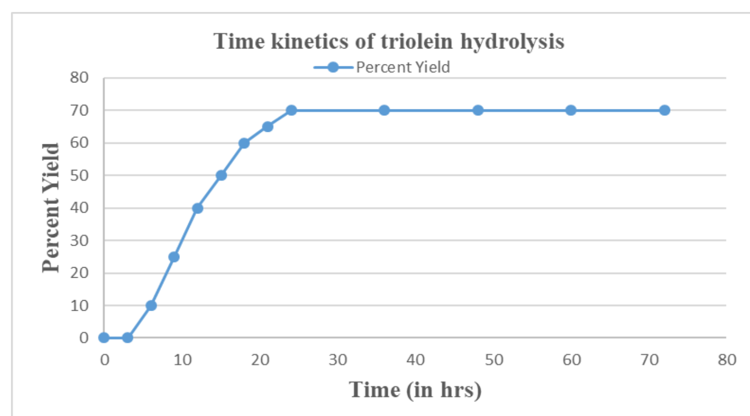


Fig. 2: Time kinetics of triolein hydrolysis by *Pseudomonas aeruginosa* lipase

Table 1: Triolein hydrolysis profile and time kinetics of percent yield

S.No.	Time (hrs)	Triolein Hydrolysis	
		Compounds (Seen on TLC)	Percent yield
1.	3	TO, DO, MO	--
2.	6	DO, MO, FA	10
3.	9	DO, MO, FA	25
4.	12	DO, MO, FA	40
5.	15	MO, FA, GLY	50
6.	18	MO, FA, GLY	60
7.	21	MO, FA, GLY	65
8.	24	FA, GLY	70

© 2017 AJAST All rights reserved.

9.	36	FA, GLY	70
10.	48	FA, GLY	70
11.	60	FA, GLY	70
12	72	FA, GLY	70

TO=Triolein, DO=Diolein, MO=Monoolein, FA=Fatty acid, GLY=Glycerol

This showed that *Pseudomonas aeruginosa* lipase could very efficiently catalyze the hydrolysis of triolein and it converts all the TO, DO, MO into free oleic acid and glycerol within 24hrs and no further increase in free oleic acid concentration was observed after 24hrs, indicating complete hydrolysis within 24hrs.

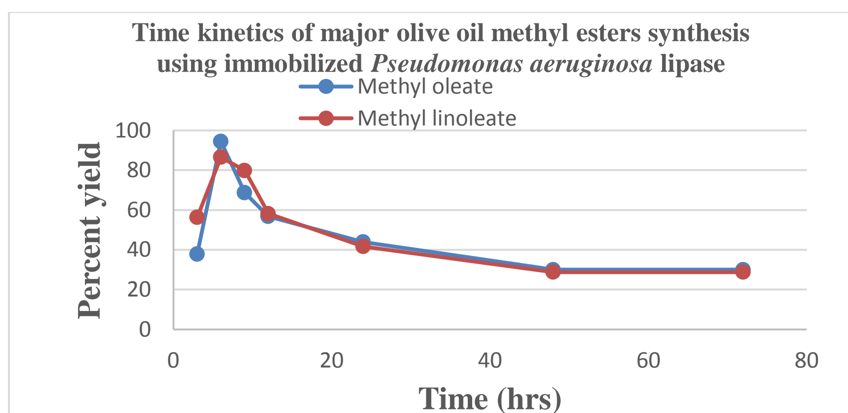


Fig. 2: Time kinetics of major olive oil methyl esters synthesis using immobilized Pseudomonas aeruginosa lipase

For the transesterification of major constituent fatty acids (oleic acid and linoleic acid) of olive oil with the help of immobilized *Pseudomonas lipase* into their respective methyl esters, it was observed that the reaction proceeded in forward direction very rapidly and 37.76% of methyl oleate could be synthesized within initial 3hrs and the transesterification reaction reaches to its maximum level (94%) within 6hrs (Fig. 2), after which reaction proceeded in backward direction leading to the decrease in the overall production of methyl oleate (table 2). For the synthesis of methyl linoleate, a similar pattern to that of methyl oleate synthesis was observed and the maximum transesterification (86%) was observed in 6hrs (Fig. 2) after which reaction went into reverse direction and the lowest catalytic turnover (37%) was observed in 72hrs (table 2). However, it is evident from the results that the *Pseudomonas*

aeruginosa lipase could successfully mediate the esterification reactions for the synthesis of methyl oleate and methyl linoleate but if the product is not withdrawn from the reaction mixture on regular interval then reaction can proceed in backward direction leading to the hydrolysis of methyl oleate and methyl linoleate into their respective fatty acids.

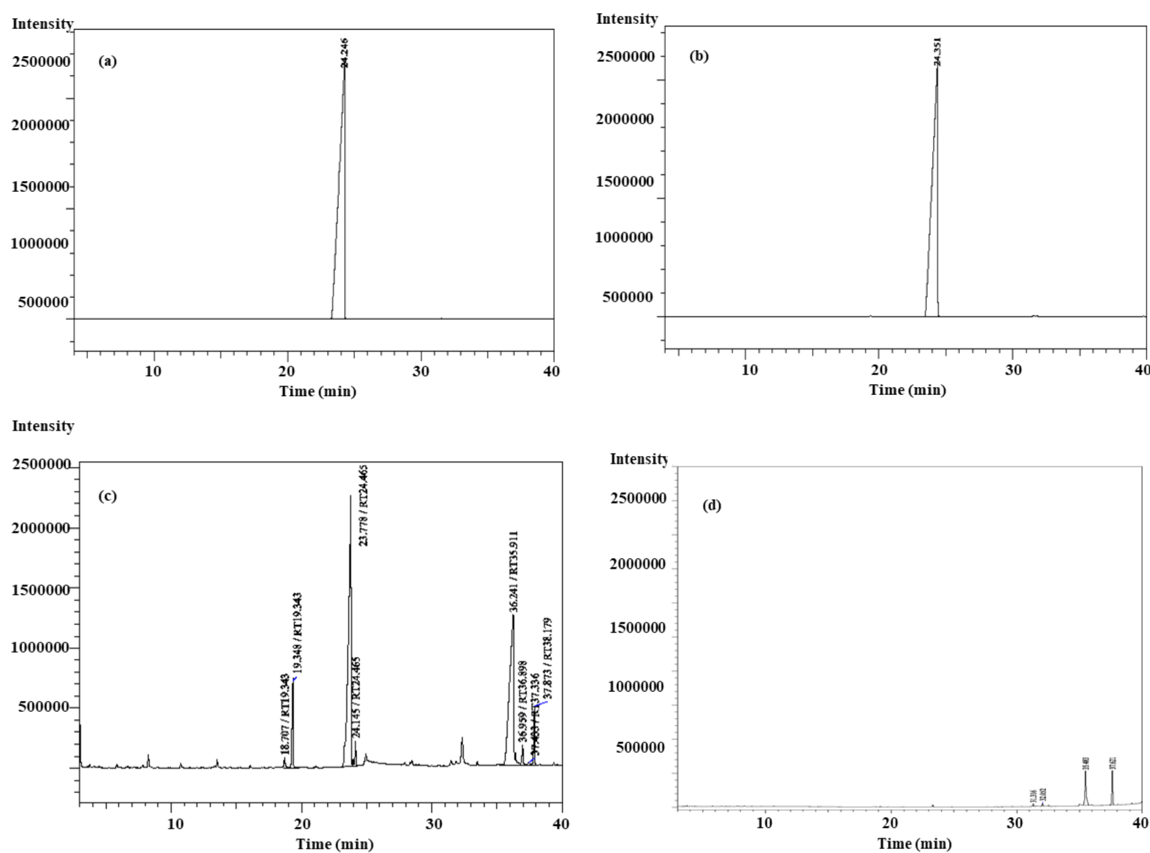


Fig 3: GC chromatogram of (a) standard methyl oleate (b) standard methyl linoleate (c) test 6hrs (d) control 6hrs

Table 2: Synthesis of major olive oil methyl esters using immobilized *Pseudomonas aeruginosa* lipase

S.No.	Time (hrs)	Products	Percent yield
1.	3	Methyl oleate	37.76
		Methyl linoleate	56.28
2.	6	Methyl oleate	94.460
		Methyl linoleate	86.53
3.	9	Methyl oleate	68.66
		Methyl linoleate	79.83
4.	12	Methyl oleate	56.80
		Methyl linoleate	58.15
5.	24	Methyl oleate	43.89
		Methyl linoleate	41.67

6.	48	Methyl oleate	30.00
		Methyl linoleate	28.82
7.	72	Methyl oleate	30.00
		Methyl linoleate	28.82

Slight variations in retention time (RT) was observed for pure/ standard and synthesized methyl oleate and methyl linoleate which was correlated and confirmed by co-elution of standards with test sample. GC chromatograms for standards, test and control sample are placed in figure 3.

Catalytic potential of lipase lies in the fact that they act on fats and oils on oil- water interface and through the series of intermediates it finally breaks the oil into respective fatty acids and glycerol [11]. There are several reports wherein successful efforts have been made to hydrolyze triolein using lipase in macroemulsion [12 & 13], mono-phase reaction system containing cyclodextrin [14], immobilized lipase [15] and all these reports advocates the effect of the initial enzyme concentration, the interfacial area and the initial concentrations of triolein and a fatty acid on the entire process of the triolein hydrolysis.

Synthesis of olive oil esters using various alcohols over a range of temperature (10- 90°C) have been reported by [16] wherein they could achieve a maximum of 17.0% methyl oleate synthesis at 50°C in 3hrs while [17] reported 90.90% yield of methyl oleate using nano-bioconjugates *Candida rugusa* lipase. However, in this study we have not used lipase in emulsion or in aqueous phase which is otherwise inhibitory for ester synthesis due to high water activity but our results are in concurrence of the previous reports [11-17].

4. CONCLUSION

Esters, such as methyl oleate, are functionally important compounds in many industrial sectors, mainly as components for manufacturing of emulsifiers, detergents, intermediate stabilizers and wetting agents. This study has established that the lipase produced by the laboratory isolate of *Pseudomonas aeruginosa* shows both the lipolytic (hydrolytic) and esterolytic activity as evident from its efficacy to hydrolyze triolein and to synthesize the oleic acid methyl esters by transesterification. Owning to this property it can be inferred that the lipase from this new isolate of *Pseudomonas aeruginosa* may have vast industrial application. Other industrially important aspects such as enantio-selectivity and regio-

specificity of this lipase need to further investigated for its commercialization so that its applicability for other industrial uses can be ascertain.

5. ACKNOWLEDGMENTS

Authors acknowledge the financial support from Delhi Technological University to carry out this investigation.

REFERENCES

1. Raku, T., Kitagawa, M., Shimakawa, H., and Tokiwa, Y. J., Enzymatic synthesis of trehalose esters having lipophilicity, *Biotechnol*., **100,** (2003), 203-208.
2. Shintre, M. S., Ghadge, R. S. S., and Sawant, S. B., Kinetics of esterification of lauric acid with fatty alcohols by lipase: Effect of fatty alcohol, *J. Chem. Technol. Biotechnol*., **77**, 10, (2002) 1114-1121.
3. Nagayama, K., Yamasaki, N., and Imai, M., Fatty acid esterification catalyzed by *Candida rugosa* lipase in lecithin microemulsion-based organogels, *Biochem. Eng*. J., **12, (**2002), 231-236.
4. Piao, J., Kobayashi, T., Adachi, S., Nakanishi, K., and Matsuno, R., Synthesis of mono- and dioleoyl erythritols through immobilized-lipase-catalyzed condensation of erythritol and oleic acid in acetone, *Biochem. Eng. J*., **14**, (2003), 79-84.
5. Lu, X., Luo, Z., Yu, S. and Fu, X., Lipase catalyzed synthesis of starch palmitate in mixed ionic liquids, *J. Agric. Food. Chem*., **60**, (2012), 9273-9279.
6. Ansary A. E. and Faddah L.M., Nanoparticles as biochemical sensors, *Nanotechnology Science and Applications*, **3**, (2010);65-76.
7. Kidwai, M., Mishra, P., Bhushan, K. R., Saxena, R. K., Gupta, R. and Singh, M., Synthesis of novel antibacterial cephalosporin derivatives using microwaves, *Indian J. Chem*., **38**, (1999), 993-997.
8. Mailer, R., Testing olive oil quality: chemical and sensory methods, *Primefacts*, **231**, (2008), 1-5.
9. Poiana, M., and Mincione, A., Fatty acids evolution and composition of olive oils extracted from different olive cultivars grown in Calabrian area *Mincione, Grasas y Aceites*, *International J. of Fats and Oils*, **55,** 3, (2004), 282-290.

10. Naka, Y. and Nakamura, T., The effect serum albumin and related amino acids on pancreatic lipase and bile salts inhibited microbial lipases, *Biosci. Biotechnol. Biochem.* **56**, (1992), 1066-70.
11. Saxena, R. K., Ghosh, P. K., Gupta, R., Davidson, W. S., Bradoo, S. and Gulati, R, Microbial lipases potential biocatalysts for the future industry, *Curr. Sci.,* **77**, (1999), 101-115.
12. Burrier, R. E. and Brecher, P., Hydrolysis of triolein in phospholipid vesicles and microemulsions by a purified rat liver acid lipase, *The J. of Biol. Chem*., **258,** 19, (1983), 12043-12050.
13. Jing, F., Ana, X. and Shen, W., The characteristics of hydrolysis of triolein catalyzed by wheat germ lipase in water-in-oil microemulsions, *Journal of Molecular Catalysis B: Enzymatic,* **24-25**, (2003), 53-60.
14. Lee, H. Y., Kim, T. K., Shin, H. D. and Park, D. C., Enzymatic hydrolysis of hydrophobic triolein by lipase in a mono-phase reaction system containing cyclodextrin; Reaction characteristics, *Biotechnol. Bioprocess Eng*. **3**, (1998), 103-108.
15. Plou, F. J., Barandiaran, M., Calvo, M. V., Ballesteros, A. and Pastor, E., High yield production of mono- and di-oleylglycerol by lipase-catalyzed hydrolysis of triolein, *Enzyme and Microbial Technology,* 1996, **18,** 66-71.
16. Kenz, Z., Leitgeb, M., Zavrsnik, D. and Lavric, B., Synthesis of oleic acid esters with immobilized lipase, *Fat Sci Technol.*, **92,** 4, (1990), 169-172.
17. Marzuki, N. H. C., Huyop, F., Enein, H. Y. A., Mahat N. A. and Wahab, R.A., Modelling and optimization of *Candida rugosa* nanobioconjugates catalysed synthesis of methyl oleate by response surface methodology, *Biotechnology & Biotechnological Equipment*, **29,** 6, (2015), 1113-1127.