Exploration And Biochemical Characterization Of A Novel Lipase From Wrightia Tinctoria Latex

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Abstract

Plant derived Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3.), has attracted a lot of attention in recently due to its ease of preparation, cost-efficiency and diverse biotechnological applications. In the present investigation, the novel lipase source from latex of Wrightia tinctoria (WTL) was studied. Their activities on p-nitrophenyl palmitate were optimal at pH 8.0 and 40 °C. Moreover, the activities of the lipases were inhibited by ethylenediaminetetraacetic acid, phenyl methyl-sulfonyl fluoride, and 4-(2-aminoethyl) benzene sulfonyl fluoride, and were reactivated by Ca2+ and Mg2+, indicating that both lipases are metalloenzymes and serine-type enzymes. Similarly, the influence of organic solvents like acetonitrile, methanol, isopropanol, ethanol acetone, and DMSO) and emulsifier agents (PEG 6000, Triton X100, SDS, CTAB Tween-20) were analysed. WTL maintained above 80% of its initial activity at a wide range of temperatures (20-70 °C) and pH values (6-11), with an optimal temperature of 40°C and optimal pH at 9.0 with p-nitrophenyl palmitate as a substrate. In addition, the kinetic models, Michaelis—Menten and Hill kinetic model were suggested. The obtained results were compared, and found Hill kinetic model describes better experimental compared to Michaelis— Menten model system. Our finding further insights, WTL would be suggested an excellent novel lipase candidate for industrial and biotechnological applications.

INTRODUCTION

Lipases have emerged as leading biocatalysts in recent years and account for almost ten percent of the enzyme market (Brady et al., 2009). Lipases have shown their ability to contribute to the tens of billion-dollar market of the bioindustry and are used in the manufacturing of paper (pitch control), biodynamics (lipid exclusion), medicinal products, detergents (cleaning agents), additives of food (enzyme-modifying flavour), cosmetics (lipid exclusion), leather (fat removal from animal skin), and wastewater treatment (decomposition and oil removal). Lipases also find their applications in polymer biodegradation and fatty waste degradation (Hubbe et al., 2021). Lipases have attracted interest from various industries owing to their desirable features, such as substrate specificity, regiospecificity, stereospecificity, chiral selectivity, activity on various substrates (monoglycerides, diglycerides, triglycerides, low- and high molecular- weight esters, amides, thiol esters, polyol/polyacid esters, free fatty acids in trans-esterification, etc.), tolerance to pH and temperature fluctuation, ability to catalyse a heterogeneous reaction at the interface of water-insoluble and water-soluble systems, stability and catalytic activity in organic solvents, nontoxic nature, low level of product inhibition, and ability to synthesize esters from glycerol and long-chain fatty acids in non-aqueous media (Chandra et al., 2020). They are commonly obtained from animals, plants, or recombinant microorganisms. The most commercially used lipases are Novozym 435, Lipozyme RM IM, Lipozyme TL IM etc., These lipases are costly hence their uses are limited to some extent. The plant lipases are cheap and easily available. They are generally more accepted for food and medicinal applications. The plant sources of lipases such as Carica papaya latex are of considerable interest because of their potential industrial applications (Abdelkafi et al., 2011). In the last two decades, investigations have been reported from various seeds, such as castor bean, corn, rapeseed, elm, mustard, palm, and pinus seed (Barros et al., 2010). Besides plant seeds and plant latexes, rice bran also shows significant lipolytic activity during its storage time, which was reported to be minimized under temperature -20°C and with the presence of hydrochloric acid (Singh & Sogi, 2016). It is therefore imperative that exploration of environment-friendly and less expensive source of lipase is required for Oleochemicals industry market. To satisfy the special demands, we unveiled for the first time the novel lipase from Wrightia tinctoria latex. Wrightia tinctoria R. belongs to family Apocynaceae commonly called as Sweet Indrajao, Dyer's Oleander. "Jaundice curative tree" in India. The juice of the tender leaves is used efficaciously in jaundice, psoriasis and other skin diseases. Similarly, it is an important medicinal plant with diverse pharmacological spectrum. Few novel chemical constituents isolated from and showed anti-cancer, anti-HIV, and anti-diabetic (type 2 diabetic) properties too. For the first time we are explored the presence of novel lipase from the latex of Wrightia tinctoria. In this study, the enzyme activity of lipase was characterized and developed kinetic model.

MATERIALS AND METHODS

Extraction and processing of latex

Fresh latex is extracted from **Wrightia tinctoria** in a sterile container or Eppendorf tubes in approximately 1:1 dilution (0.5 ml of solvent and approximately 0.5ml of latex were collected due to the challenging nature of latex) with suitable extract solvents such as methanol extract, isopropanol or 1x phosphate buffer saline. The obtained crude extract is centrifuged at 8000rpm for 10 min at - 4°C. The supernatant thus obtained and stored for further analysis.

Lipase Assay

The spectrophotometric assay was used for the estimation of lipase activity by measuring the hydrolysis of p-nitrophenyl palmitate at a wavelength of 410 nm according to the method of (Okunwaye et al., 2019). The reaction mixture briefly consists of 2.5 ml of 0.1 M Tris-Cl buffer, pH 8.2, and 2.5 ml of p-nitrophenyl palmitate (420 mM) substrate solution, the reaction was initiated by adding 1ml of appropriately diluted crude latex preparation from WTL. The protein content from crude preparation was determined by Bradford dye binding method (Kruger et al., 2009).

Influence of pH and temperature on Lipase Activity. The lipase activity was measured at pH ranging from pH 7 to pH 10 using pNP-palmitate as the substrate. The substrate emulsion was prepared in different buffer solutions. Similarly, the determination of optimum temperature for lipolytic activity, lipase was assayed at temperatures ranging between 30-80°C in a temperature-controlled water bath and the residual activity of WTL was determined as earlier described.

Selection of Organic Solvent. The lipase activity, with different organic solvents were investigated, i.e., acetonitrile, methanol, isopropanol, ethanol, DMSO, acetone (ranging from 2 to 10% (v/v)). Then, the reaction mixture was prepared in Tris-HCl buffer (50 mM, pH 9.0). Lipase activity was measured according to the standard procedure described above.

Determination of Lipase Activity in the Presence of Emulsifier Agents. The WTL activity was measured in aqueous/organic biphasic emulsions i.e., coconut oil, PEG 6000, Triton X-100, SDS, CTAB, Tween-20 in Tris-buffer system pH 9.0.

Kinetic Measurements and Enzyme Kinetics and Data Handling. The kinetic parameters for WTL were determined at optimal conditions for measurement of enzyme activity. The rate of pNP-palmitate hydrolysis of lipase from Wrightia tinctoria was described using the mono-substrate Michaelis— Menten equation and Hill equation respectively. The mathematical model was estimated by nonlinear regression analysis using the least-squares method implemented in Graph Pad Prism 8.4). They were estimated by fitting the model to the experimental data. The calculated data were compared with the experimental data, recalculated in the optimization routine and fitted again until a minimal error between experimental and integrated values obtained.

Influence of Emulsifier Agents. In order to prepare a stable emulsion (aqueous/organic biphasic reaction system), different emulsifiers were investigated, i.e., (PEG 8000, Triton X-100, SDS, CTAB, Tween-20). The substrate stock solution (20 mM) was prepared in acetonitrile.

Effects of Monovalent, Divalent, and Trivalent metals

The effects of various monovalent (Na1+ and K1+), divalent (Ca2+, Mg2+, Mn2+, Ni2+, Cu2+, Co2+, and Zn2+), and trivalent (Al3+, Cr3+, and Fe3+) ions on lipase activity were determined. The crude latex preparation was incubated with the tested ions at two concentrations (5 and 10 mM) for 15 min at room temperature. Subsequently, the residual

lipase activity was measured by adding the substrate at the optimum pH and temperature for each enzyme. The enzyme reaction mixture without any ion was used as the control, and the relative lipase activity was calculated compared to that of the control.

Effects of selected Inhibitors

The effects of different potential inhibitors, including 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 µM 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF) hydrochloride (also called as Pefabloc, PFB), and 1 mM ethylenediaminetetraacetic acid (EDTA), were investigated. The crude latex preparation was incubated with the tested selected inhibitor for 15 min at room temperature. The residual lipase activity was then assayed by adding the substrate at the optimum pH and temperature for each enzyme. The reaction mixture without any inhibitor was used as the control, and its lipase activity was defined as 100%. The relative lipase activity was estimated compared to that of the control, i.e., as the ratio between the activity of the problem sample and the activity of the control sample and expressed as a percentage.

RESULTS AND DISCUSSION

Latex is a sticky emulsion produced by specialized cells called laticifers, and it is a crucial part of a plant's defense system against herbivory and pathogens. W. tinctorial latex collected from the aerial part of the plant and was analysed for its lipolytic activity. The enzyme assay revealed W. tinctorial latex to have significant lipase activity, capable of degrading fatty acids. The fresh latex contained a high amount of water, protein and low lipid. High protein content could be linked to the presence of various enzymes and the high-water content may explain for its flowability. This result was in accordance with (Macalood et al., 2013). However, the latex tends to solidify quickly in the air, and this may entrap lipase and affect the lipolytic activity of the latex. To define the optimal condition for measuring initial lipase activity, pNPpalmitate was chosen as a standard substrate. (Figure -1).

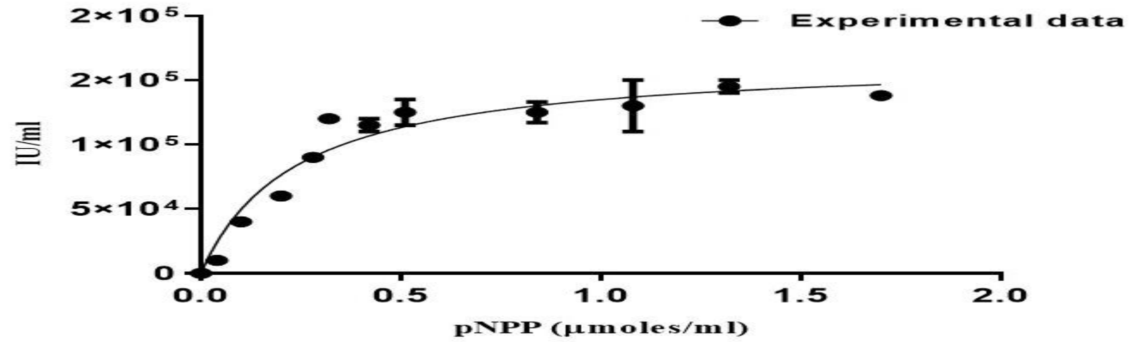

Figure 1. Effect of the pNP-palmitate concentration on the WTL **(Wrightia tinctoria)**

It is important to examine the effect of substrate concentration on lipase activity because the reaction may slow down due to substrate depletion. Furthermore, a high concentration of the substrate could lead to the substrate inhibition of the enzyme increase in lipase activity was measured at (50 µM) substrate concentration. To define the optimal pH value for lipase activity, different buffer solutions were prepared in the pH range of 6—10 and an ionic strength of 50 mM. and found at pH 8-9 to be the optimum for the WTL activity. Similar results were observed by (Zheng et al., 2011) (Figure -2)

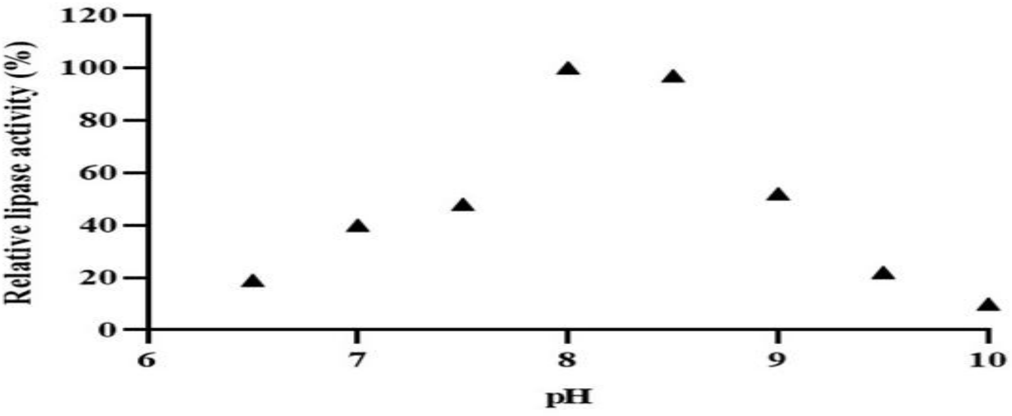

Figure 2. Effects of pH on the activity on the activities of lipases from Wrightia tinctoria.

As shown in Figure 2, the lipase from WTL active (≥50% relative activity) from pH 7.5 to 9.0, with an optimum pH at 8.0 confers these lipases suitable ingredients in detergent manufacture, bioremediation, and flavour synthesis (Priyanka et al., 2020). The activity significantly decreased as the pH was decreased from 8.5 to 9.5. This decrease in lipase activity may be ascribed to a decreased saturation of the enzymes with the substrate due to a decreased affinity. Furthermore, changes in solution pH alter the ionization of the functional groups of the enzyme and substrate, as well as the conformation of the enzyme structure in the solution due to the breaking or formation of ionic interactions, thereby decreasing enzyme activity (Harvinda et al., 2023). Temperature is an important factor influencing the activity and application of lipases in industrial processes (Li et al., 2014). The activity of the WTL significantly increased with temperature and reached the optimum level at 40 °C (Figure 3). This result may be ascribed to the fact that an increase in temperature increases the kinetic energy contained within the enzyme and substrate molecules; consequently, they move faster, and the substrate collides successfully with the active site of the lipases frequently (Harvinda, et al., **2023**). Although most yeast lipases exhibit maximum activities at temperatures ranging from 28 °C to 40 °C, the lipases from T. asteroides , C. curvata, and Kurtzmanomyces sp. have optimum temperatures of 50 °C, 60 °C, and 75 °C, respectively (Romo-Silva et al., 2024). The above results indicate that the lipases from WTL have heat stable.

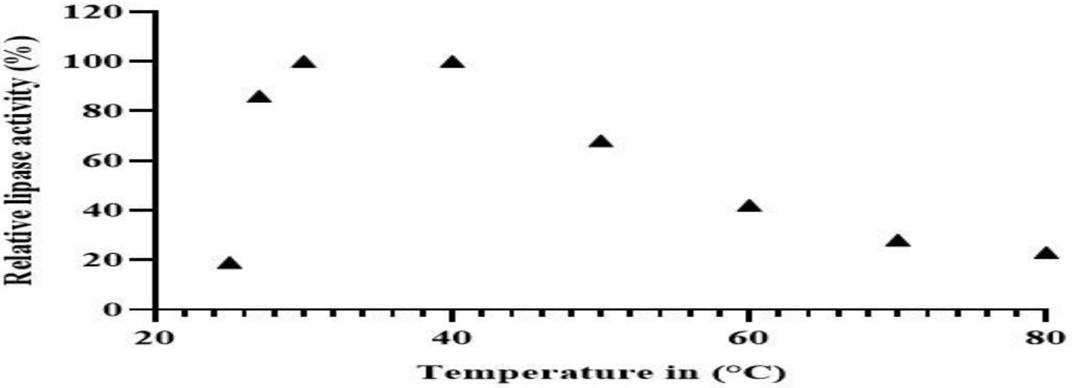

Figure 3. Effects of temperature on the activity on the activities of lipases from Wrightia tinctoria.

EDTA, PMSF, and PFB strongly inhibited the activities of the WTL lipases in the following order: PMSF> EDTA> PFB. (Figure 4). The inhibition by the metal chelator EDTA suggests that the conformation of the WTL was modulated by metal cations and the lipases could be metalloproteins/ metal-activated enzymes (Zhao et al., 2021). The lipase from Candida viswanathii was also inhibited by EDTA, whereas the lipase from T. asteroides was not (de Almeida et al., 2018). Meanwhile, the lipase activity inhibition by PMSF and PFB indicates that both enzymes are serine hydrolases whose serine nucleophilic residue in the active site is crucial for enzymatic catalysis. The lipases from C. viswanathii and Y. lipolytica are inhibited by PMSF, whereas the lipase from C. rugosa DSM-2031 is completely inhibited by PFB (Romo-Silva et al., 2024).

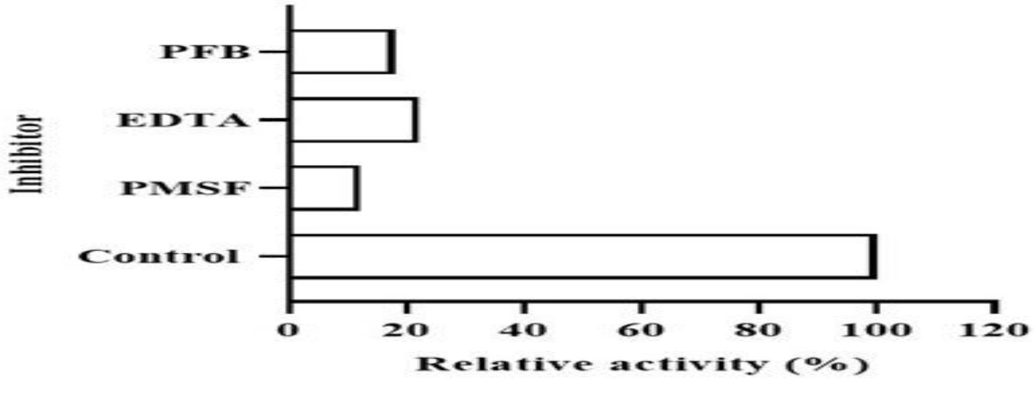

Figure 4. Effects of selected inhibitors on the activities of lipases from Wrightia tinctoria. PMSF, phenyl methyl sulfonyl fluoride; PFB, pefabloc (4-(2-amino ethyl) benzene sulfonyl fluoride (AEBSF) hydrochloride); EDTA, ethylenediaminetetraacetic acid.

The effects of monovalent (Na1+ and K1+), divalent (Ca2+, Mg2+, Co2+, Mn2+, Cu2+, Ni2+, and Zn2+), and trivalent (Fe3+, Al3+, and Cr3+) metal cations at low (5 mM) concentration on lipase activity was analysed (Figure 5). The monovalent cations had negligible effects on lipase activity, whereas most of the divalent and trivalent exerted a concentration-dependent inhibitory or stimulatory effect on lipase activity. Fe3+, Al3+, and Zn2+ ions moderately inhibited the activities of WTL. However, the strongest inhibition was caused by Ni2+, Cu2+, Cr3+, and Co2+ ions. This result may be due to an alteration in the conformation of lipases, as previously reported for other lipases (Romo-Silva et al., 2024). In this context, the inhibitory effects on lipase activity may be attributed to the reduction of the enzyme's key cysteine residues that are oxidized by the metal cations, which could alter enzyme structural conformation. However, WTL was significantly improved in the presence of Ca2+ and Mg2+ ions, these findings indicate that the lipase from WTL found to be the metalloenzyme dependent on Ca2+, and dependent on Mg2+. Ca2+ and Mg2+ activate the lipases from some yeast species, such as C. rugosa (DSM-2031) and Y. lipolytic, whereas Mg2+ activates the lipase from C. viswanathii. This enhanced lipase activity has been attributed to the improved cross-linking of the lipase polypeptide chain with Ca2+/Mg2+ bridges, increasing the stability and rigidity of the metal ion–enzyme complex. Furthermore, Ca2+ ions may play other roles in lipase action, such as removing fatty acids as insoluble Ca2+ salts and directing lipase activation from accumulation at the water–oil interface (Romo-Silva et al., 2024). Lipases tolerant to organic solvents have received considerable attention in various industrial applications. Therefore, stability and activity in organic solvents are considered novel attributes of a lipase. The activity and stability of lipases in organic solvents depend not only on the nature of the enzymes but also on the concentration and properties of the organic solvents. The inhibition of enzyme activity by hydrophilic organic solvents has been attributed to their ability to strip off tightly bound essential water required for lipase activity and partition deeper into the lipase active site, leading to structural conformational changes and activity loss of enzymes. (Romo-Silva et al., 2024).

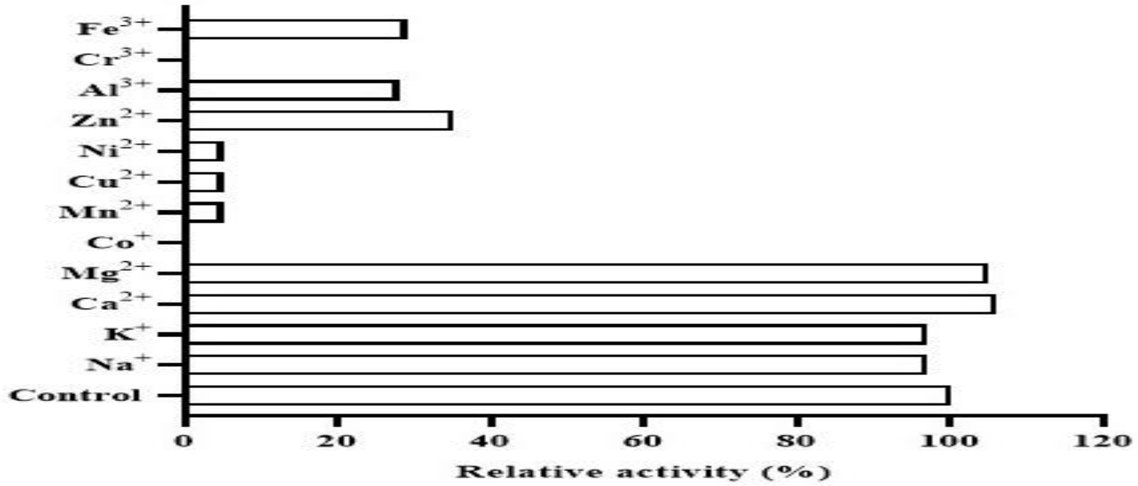

Figure 5. Effects of monovalent, divalent, and trivalent metal cations on the activities of lipases from **Wrightia tinctoria.**

Lipases tolerant to organic solvents have received considerable attention in various industrial applications, such as the syntheses of biodiesel, organic chemicals, catalysts, valuable peptides, fatty acid esters, oligosaccharide derivatives, and other compounds (Joseph et al., 2013). The inhibition of enzyme activity by hydrophilic organic solvents has been attributed to their ability to strip off tightly bound essential water required for lipase activity and partition deeper into the lipase active site, leading to structural conformational changes and activity loss of enzymes (Oliveira et al., 2014). By contrast, hydrophobic organic solvents probably cause changes in the equilibrium between the closed and open conformation of enzymes and the solubility of substrates and reaction products [80]. Lipases lose their activity after adding organic solvents at concentrations higher than 10–20% (v/v) (Borkar et al., 2009). Therefore, in the present work, we investigated the effects of several organic solvents on the lipase activity. The results obtained are shown in (Figure-6).

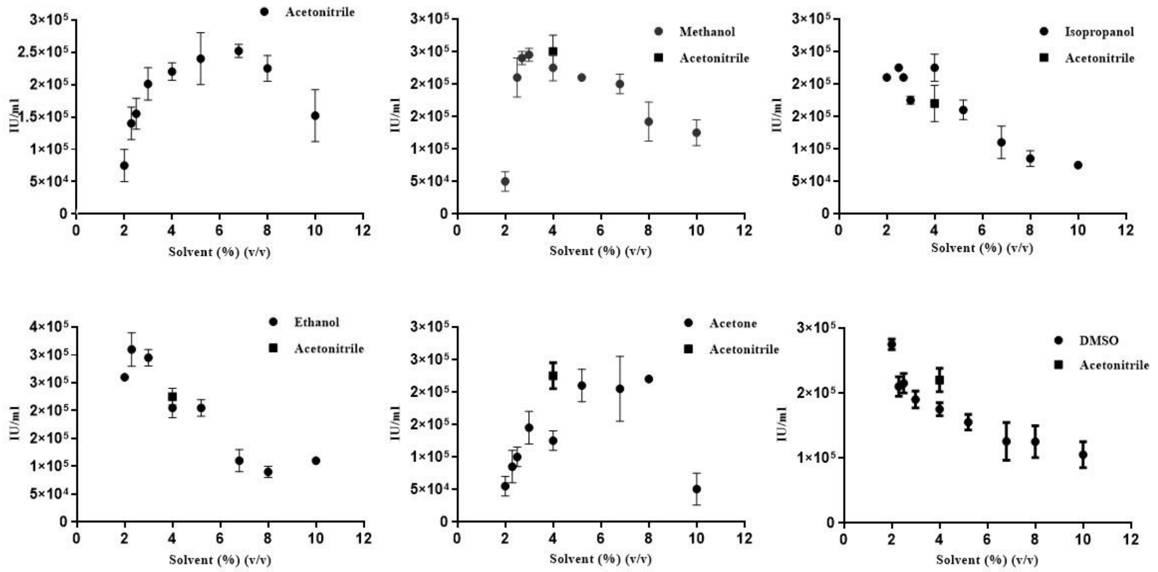

Figure 6. Influence of the organic solvents on the WTL activity with different volume ratio of organic phase (2—10%) in the buffer solution. Acetonitrile (with 4% in reaction medium) was used for comparison with other organic solvents. Isopropanol has been extensively employed as a solvent for assay. However, the rate of pNP-palmitate hydrolysis was lower with 4% of isopropanol in the reaction mixture in comparison with acetonitrile. Similar data were recorded for the most of the organic solvents used for this assay, with methanol as the exception. In the case of methanol, lipase activity was higher than in experiment with acetonitrile for 4% volume ratio. However, it is known that lipase catalyses both hydrolysis and synthesis of esters, so the lipase could catalyse the esterification reaction between released p-nitrophenol and alcohol. In order to avoid the reverse reaction, acetonitrile is the optimal choice for a solvent of the assay. Also, methanol has a higher relative polarity (0.762) than acetonitrile (0.460), and it is known that highly polar solvent can inhibit the enzyme due to removal of the essential water layer surrounding the enzyme surface. Rosset et al. highlight the fact that high alcohol concentrations in the reaction mixture led to the protein unfolding and denaturation of lipase. (Rosset et al., 2019). Since, the biphasic system results comprises an immiscible organic phase in water, the presence of emulsifier in the reaction mixture allows the solubilization of hydrophobic substrate which further leads to proper mass transfer and higher hydrolytic activity. Moreover, the emulsifier may trigger the opening of the lipase catalytic site by lid movement and cause the conformation changes from the closed to open form. Previous research proved the sensitivity of lipase against the emulsifier due to its ability to disrupt the structure of proteins. For this reason, various emulsifiers in different concentrations were tested for their effect on enzyme activity (PEG 6000, Triton X-100, SDS, CTAB, Tween-20)**.** Therefore, stability and activity in organic solvents are considered novel attributes of a lipase. The lipase activity in Table 2 is presented as relative activity, it was observed that the hydrolysis rate of the substrate was significantly higher when coconut oil was applied in comparison with PEG 6000 (reduction of lipase activity about 70%). The minimum presence of the other tested emulsifiers in the reaction medium leads to complete inhibition of lipase. (Table-1). The results are in agreement with previous reports from other authors. Mesa et al. explained the behaviour and catalytic performance of the WTL in the presence of the surfactant Triton X-100 at 25 °C and different pH values (Mesa et al., 2018). In this study, results also show optimal activity at pH 9 and complete inhibition of WTL in the presence of Triton X-100. Kanwar et al. reported that SDS, as the anionic detergent, acts as an agent for inhibition of the residual activity of lipase (Kanwar et al, 2005).

Table-1 Influence of the Emulsifier on the lipase from Wrightia tinctoria.

Emulsifier (w/v) (%)	**0.3**	**0.4**	**0.5**
Coconut oil	97	100	70

PEG 6000	27	32	24
Triton-X-100	0	0	0
SDS	0	0	0
CTAB	0	0	0
Tween 20	0	0	0

In order to determine the kinetic parameters. We conducted for Michaelis—Menten enzyme kinetic. According to the data, substrate saturation or inhibition was not detected. From the following results it can be seen that at the lower concentration, kinetics under the defined system follow a sigmoidal curve. These results are directly related to a smaller lipase—substrate interaction between two phases and depend on the quality of the interface. This behaviour of WTL was also observed by other authors (Palacios et al., 2014). The author proposes that the hydrolytic activity of lipase is not dependent on the actual structure of the substrate but on the "interfacial quality" of a surface composed of that particular substrate. Rates of lipase catalysed reactions are governed by the binding of lipase to the substrate interface, which is a cooperative process. The process includes conformational change in the enzyme, a structural change in the substrate interface. On the other hand, Marangoni proposed the Hill model for description of lipase kinetics (Marangoni et al., 1999). The author proposes that the
hydrolytic activity of lipase is not dependent on the actual structure of the substrate but on the "interfacial quality" of a surface composed of that particular substrate. Rates of lipase catalyzed reactions are governed by the binding of lipase to the substrate interface, which is a cooperative process. The process includes conformational change in the enzyme, a structural change in the substrate interface, and a penetration of the lipase into that substrate interface. In this case, Hill constant n represents the number of substrate molecules associated with catalyst per catalytic cycle and it is an index of the cooperativity of the process. In the case where $n > 1$, positively cooperative binding occurs; for $n < 1$, negatively cooperative binding is observed, and if $n = 1$ binding is noncooperative (completely independent — Michaelis—Menten model). Based on this assumption, kinetic parameters were estimated by using the Hill model. Obtained results are presented in (Table 2) and a good agreement of the proposed model and experimental data is achieved (Figure 7).

Table-2: Kinetic Parameters Obtained from Michaelis—Menten and Hill Model

Optimized test conditions				
	Michaelis—Menten kinetics	Hill kinetics	Model selection	
Km (mM)	0.214 ± 0.056	0.260 ± 0.02	2.17	2.47
Vmax(U/ml)	168621 ± 13546	145628 ± 7827		
n (—)	—	2.37 ± 0.547		

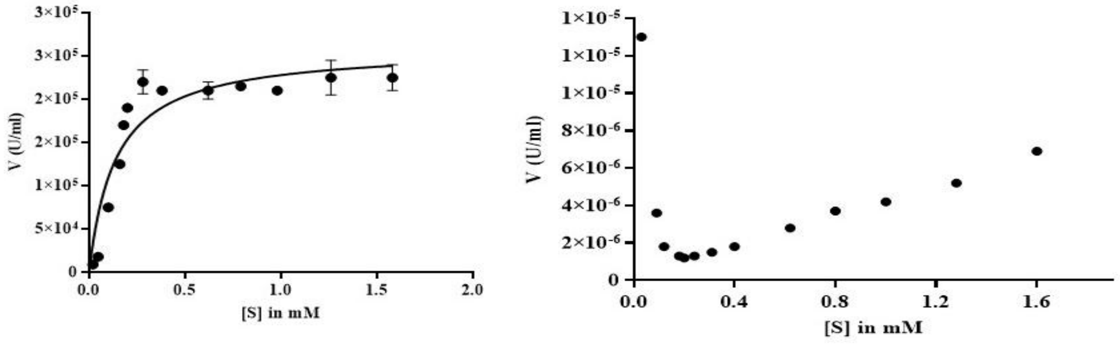

Figure 7. (a) Effect of the pNP-palmitate concentration on the WTL initial reaction rate — Michaelis—Menten and Hill kinetics and the (b) Hanes-Woolf plot.

The Hill coefficient obtained from the experiment with initial test conditions and the one obtained from optimized test conditions indicates cooperative binding. On the other hand, the coefficient obtained for optimized test conditions is higher (˜2 fold), indicating that the optimized cooperation process, mentioned by Marangoni, was improved by the assay. The same effect can be noticed from maximal reaction rates that increased after test optimization. The Hill model describes pNP-palmitate hydrolysis better than the Michaelis— Menten model.

CONCLUSION

Exploration of plant latexes as source of lipase have gained significant importance in almost every field of life due to their numerous applications particularly due to low cost, easy acceptance with high economic feasibility and its unique characteristics. The present research would emphasis, a novel source of lipase from WT. It implies the potential for lipolysis of polyunsaturated triglycerides. The enzyme was found to be stable even at higher temperatures, and thermostability and very much necessary requirement in industries. After the optimal conditions were
defined, kinetic parameters for hydrolysis of pNP-palmitate
were estimated using the Michaelis-Menten and Hill model. Although obtained results indicated that both models could be used for kinetic parameters estimation, the Hill model was selected as more appropriate based on model selection criterion for analysis

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