

## Degradation of Castor Oil and Lipase Production by *Pseudomonas aeruginosa*

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**Abstract:** Many Enzymes produced by *Pseudomonas aeruginosa* have been used in commercial scales, lipase is an example. *P. aeruginosa* has been investigated intensively during past decays regarding its medical and industrial importance. It will establish in different wastes treatment processes such as oil waste bioremediation. In this study, two strains of *P. aeruginosa* were used to produce lipase from Castor oil aiming to control the different castor oil wastes and technical lipase production. Both strains are able to grow on media consists of 20 g/l Castor oil and 3 g/l Yeast extract at 37°C. The lipase enzyme activity at 37, 60 and 75°C in presence of 0.4, 0.8, 2 and 2.8 mg/ml *p*-nitrophenyl palmitate were investigated. The  $V_{max}$  and  $K_m$  using of both Hanes and Lineweaver-Burk plots were determined. Lipase produced from the two strains revealed mesophilic and thermophilic activities. The maximum activity at 37°C was 1121.00 and 470.82 Units/ml, while at 75°C was 358.72 and 147.18 Unit/ml for strain 1 and strain 2, respectively. We recommends for using *P. aeruginosa* lipases for castor oil waste control and for the production of mesophilic and thermophilic lipases under mesophilic conditions.

**Key words:** Castor oil % *Pseudomonas aeruginosa* % Kinetic % Hanes plot % Lineweaver-Burk

### INTRODUCTION

The castor oil from *Ricinus communis* represent a potential bioproduct and used in many technical and medical applications. During castor oil extraction process and refining as well as its use in different industrial applications like bio-diesel production, different types of wastes elevated. Using different lipases for castor oil wastes control has been described by authors such as lipases from ground oats, castor bean itself, *Aspergillus oryzae*, *Pseudomonas aeruginosa* KKA-5, *Pseudomonas fluorescens* NS2W, *A. oryzae*, *Geotrichum candidum*, *Candida rugosa*, *Pseudomonas cepacia* [1-7].

Different studies describe an efficient low-cost method for biodegradation of castor oil has been reported. As an example, an efficient method for crude castor oil bioremediation was described by Godoya et al [8]. Lipases have been used for degrading castor oil either for production of new products or for technical lipases production. A method for bioconversion of castor oil to ricinoleic acid was described Goswami et al [9]. Lipase was

one of the most technical enzymes which represent 1 billion USD in 1999 [10]. Part of these enzymes was the thermostable enzymes which were better suited for harsh industrial processes and constitute more than 65% of the global market [10-12]. Enzymes have many applications especially in paper industry, detergent, drugs, degradation of different wastes, textile, food, pharmaceutical, leather, degumming of silk goods, manufacture of liquid glue, cosmetics, meat tenderization, cheese production, growth promoters etc [10,13]. Thermostable enzymes can be produced by both of thermophilic and mesophilic microbes [14]. Some reports described Psychrophiles as a source of some stable enzymes but using thermophiles reduce the risk of contamination by common mesophiles. Pseudomonads a general name referred to a part of a large, heterogeneous and ubiquitous (Being present everywhere at once) group of microorganisms [15,16]. The big genetic material of the *P. aeruginosa* support the idea of using this microbes in biotechnological applications. The diversity of *P. aeruginosa* different strains and that each strain should be

study as a single case were highlighted [17,18]. Protease, lipase, uricase and asparaginase were important enzymes produced by *P. aeruginosa* as well as many other products like PHA and alginate which used in many biotechnological applications [10,19-27]. There were an increasing interest in *P. aeruginosa* lipase and protease [19] *P. aeruginosa* has been reported early for its ability to produce lipase [25]. Lipase was characterized by its ability to hydrolysis long chain triglycerides [26]. The commercial applications of lipase includes dish washing detergents, clearing of drains clogged by lipids in food processing or domestic/industrial effluent treatment plants [27]. Further, it was used in liquid leather cleaner [28], a bleaching composition [29], decomposition of lipid contaminants in dry-cleaning solvents and removal of dirt/cattle manure from domestic animals by lipases and cellulases [30], contact lens cleaning and washing, degreasing and water reconditioning by using lipases along with oxidoreductases, which allows for smaller amounts of surfactants and operation at low temperatures [31], degradation of organic wastes on the surface of exhaust pipes, toilet bowls, etc. [32]. The specificity of lipase caused tremendous interest among scientist and industrialists specially those dealing with producing new chemical components from fats and oil degradation [33]. In this study two, *P. aeruginosa* strains have been isolated from the Egyptian ecosystem were used for extracellular lipase production by growing them on castor oil. The results of lipases activities were promising.

## MATERIALS AND METHODS

***P. aeruginosa* Strains:** Two different strains of *P. aeruginosa* were isolated from the Egyptian ecosystem in our lab., through the past five years from different location were screened for their ability to grow on castor oil as the main carbon source. The strains were maintained routinely by cultivating them on LB media [34].

**Castor Oil Degradation Rate:** Pure castor oil has been bought from local Pharmacy and was produced by MEPACo-Egypt. The castor oil degradation rate was calculated by determining the time needed for complete degradation. The degradation rate/day has been determined for each experiment.

**Production of Lipases:** *P. aeruginosa* strains were grown on media contain castor oil 20 g/L and yeast extract 3 g/L. Tap water has been used. 250 ml flasks each contain

100 ml media as above were overnight incubated at shaker incubator (Innova 4230 - New Brunswick Scientific) at 37°C, pH 7 and 150 rpm. The cells free supernatant collected by centrifugation at 4000 rpm at 4°C for 30 min. and stored at -20°C for further studies.

**Lipase Assays:** Extracellular lipase activity was assayed according to a method adapted by [35-In press]. The substrate mixture was prepared by adding 200  $\mu$ l Tween-20 to 40 mg *p*-nitrophenyl palmitate dissolved in 10 ml DMSO. The crude enzyme activity was determined by adding 40  $\mu$ l from the supernatant to 500  $\mu$ l from 50 mM Tris HCl, pH 8.0. The enzyme reaction was started by adding different volumes of substrate which contain 0.4, 0.8, 2 and 2.8 mg/ml *p*-nitrophenyl palmitate. The activities for each substrate concentration has been measured at different temperature represent 37, 60 and 75°C. The different activities were determined by the rate of *p*-nitrophenol production (*p*NP) which measured at 405 nm spectrophotometrically (PerkinElmer-UV/VIS Spectrometer Lambda). The increase in absorbance against times is measured at different time intervals. The *p*NP extinction coefficient under the conditions described was 14500 L mol cm<sup>-1</sup>. Lipolytic activity was determined, using substrate free blanks as control. One unit (U) was defined as the amount of enzyme catalyzing the liberation of 1  $\mu$ mol *p*-nitrophenol/min under the given conditions [33].

**Protein Determination:** Protein analysis of the different supernatants was determined spectrophotometry at 280 nm. The protein different concentrations were derived from Bovine serum Albumin standard curve.

**Kinetic Study:** Hyperbolic Regression Software 3.95 was used for determining the  $K_m$  and  $V_{max}$  from Lineweaver-Burk plots. The kinetic parameters for release of *p*-nitrophenol at different substrate and temperature (37, 60 and 75°C) were determined. The apparent kinetic parameters were estimated from Hanes and Lineweaver-Burk plots and  $K_m$  and  $V_{max}$  were calculated for each experiment by non-linear regression analysis. Starting values of  $K_m$  and  $V_{max}$  are obtained by linear regression fitting of a Hanes plot. These values are used to seed the non-linear regression, which effectively fits the data directly to the best hyperbola. This approach is to be preferred to methods, which fit to linear transformations of the data. A Taylor series of partial derivatives of  $K_m$  and  $V_{max}$  is constructed and their errors are estimated by multivariate, linear regression [36,37].

**RESULTS**

**Castor Oil Degradation Rate:** The castor oil degradation rates were 5.7 and 3.8 g/L/day for strains 1 and 2, respectively.

**Lipase Activity:** The *in vivo* enzyme activities and kinetics were determined spectrophotometrically (PerkinElmer-UV/VIS Spectrometer Lambda) at 405 nm by monitoring the liberation of *p*NP against time. The activity against different substrate concentrations under experimental different conditions were plotted using Hanes and Lineweaver-Burk plot model as in Figures 1-12. The  $K_m$  and  $V_{max}$  were calculated for each enzyme and summarized as in table strain 1 revealed the maximum activity that represents 1121.00 Units/ml, while strain 2 revealed 470.82 Units/ml activity at 37°C. Meanwhile, there was an activity at 75°C that represent 358.72 and 147.18 Units/ml for strains 1 and 2, respectively as in Table 1. The different  $V_{max}$  and  $K_m$ , which represent the velocity of the enzymes in presence of different substrate concentrations have been summarized in Table 2. The enzyme activity was calculated as Units/ml and the  $V_{max}$  and  $K_m$  for the different experiments have been clearly showed the activity at different conditions (Figs. 13 and 14).

**DISCUSSION**

The castor bean consists of approximately 50% oil. Castor oil has a special characteristics regarding its viscosity, heat pressure stability, low freezing point and its ability to form waxy substance after chemical modifications [8]. Recently, castor oil was a promising candidate for biodiesel production. The Petrobras Research Center has been developing a biodiesel production process from castor oil [37]. During the processes part of the castor oil byproduct were relished either during the extraction process or during the biotransformation. Isolating efficient microbial strains for castor oil degradation will lead to efficient control for these wastes. Production of bioproducts from castor oil degradation like lipases will add biotechnological and economical values for the overall process. Lipases, which were produced spontaneously during the microbial degradation of castor oil, was the most promising candidate. For matching the main two targets, castor oil degradation and lipase production, *P. aeruginosa* strains isolated in our Lab. have been investigated. Media consists of castor oil, yeast extract and tap water were used during the degradation process. For monitoring the lipase activities, two main strategies have been used;

Table 1: Lipase activities of different experiment as Units/ml

		Substrate mg/ml			
		0.4	0.8	2	2.8
Temperature	<i>P. aeruginosa</i>	Specific activity <i>p</i> -nitrophenyl palmitate μmol/ml/min			
37°C	Strain 1	175.55	389.53	725.98	1121.00
	Strain 2	157.85	200.10	491.10	470.82
60°C	Strain 1	133.91	193.39	288.26	288.26*
	Strain 2	19.61*	55.21	77.94*	77.94
75°C	Strain 1	70.07	94.56	234.88	358.72
	Strain 2	27.07	54.91	76.87	147.18

Table 2:  $V_{max}$  and  $K_m$  Hanes and Lineweaver-Burk plots for lipase activities at different temperature

Strains	Kinetic parameter	Hanes Plot	Lineweaver-Burk	Hanes Plot	Lineweaver-Burk	Hanes Plot	Lineweaver-Burk
		37°C		60°C		75°C	
Strain 1	$V_{max}$	5.349e <sup>03</sup>	9.368e <sup>03</sup>	0.127	367.1000	0.148	135.100
	$K_m$	11.320	20.550	0.744	0.6821	2.088	1.757
Strain 2	$V_{max}$	870.500	680.300	0.072	374.0000	0.044	340.900
	$K_m$	2.102	1.420	1.877	0.7208	0.962	6.145

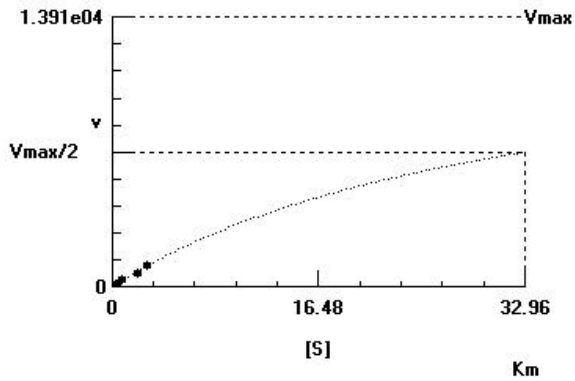


Fig. 1: Hanes plot of strain 1 lipase at 37°C

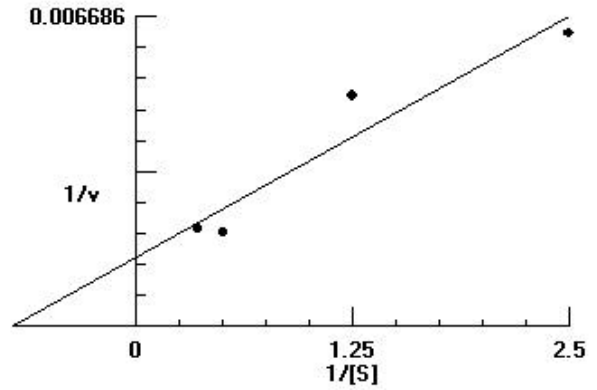


Fig. 4: Lineweaver-Burk plot of strain 2 lipase at 37°C

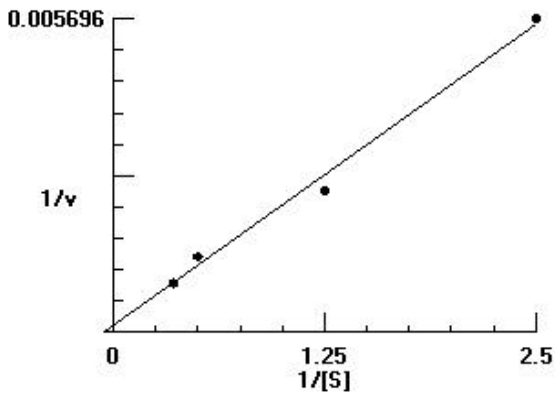


Fig. 2: Lineweaver-Burk plot of strain 1 lipase at 37°C

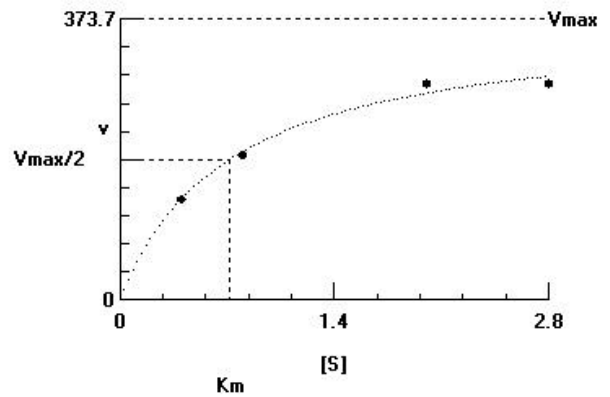


Fig. 5: Hanes plot of strain 1 lipase at 60°C

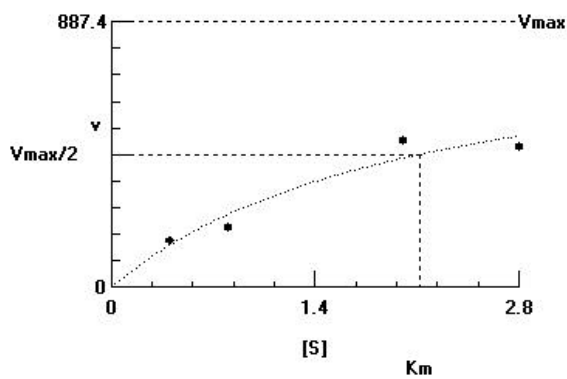


Fig. 3: Hanes plot of strain 2 lipase at 37°C

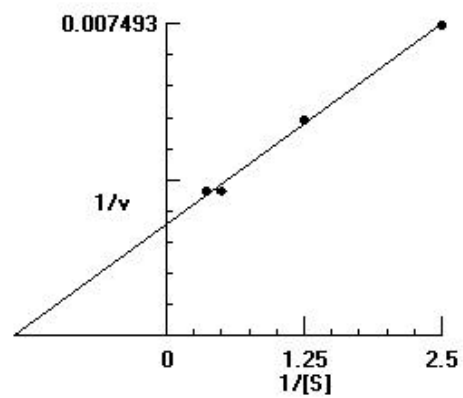


Fig. 6: Lineweaver-Burk plot of strain 1 lipase at 60°C

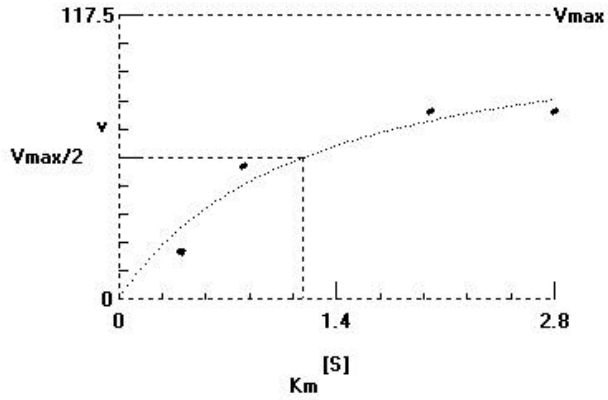


Fig. 7: Hanes plot of strain 2 lipase at 60°C

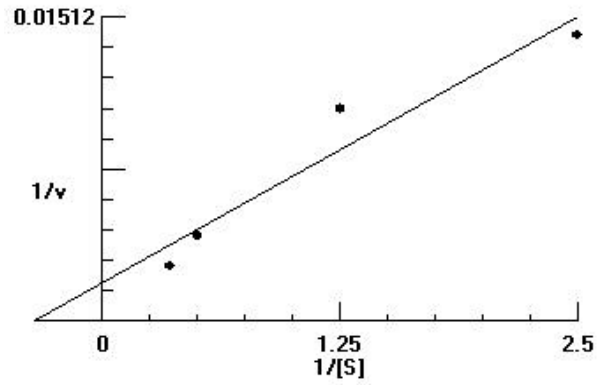


Fig. 10: Lineweaver-Burk plot of strain 1 lipase at 75°C

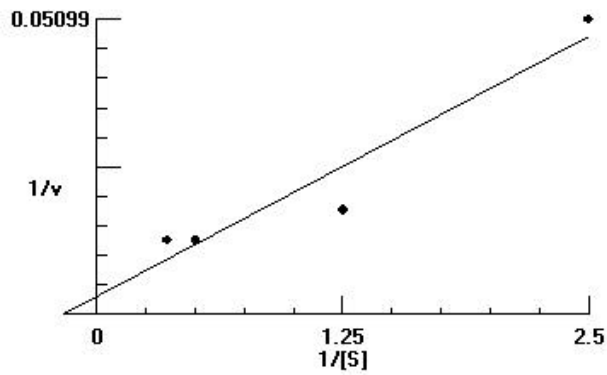


Fig. 8: Lineweaver-Burk plot of strain 2 lipase at 60°C

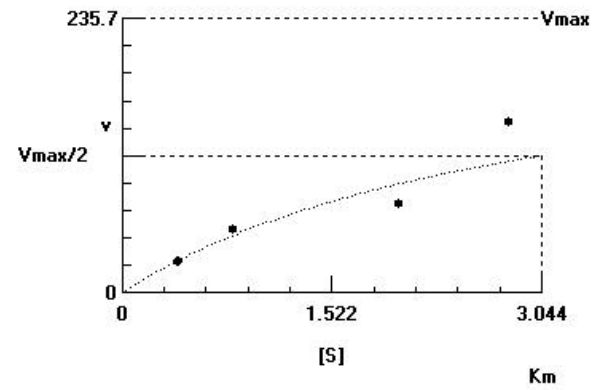


Fig. 11: Hanes plot of strain 2 lipase at 75°C

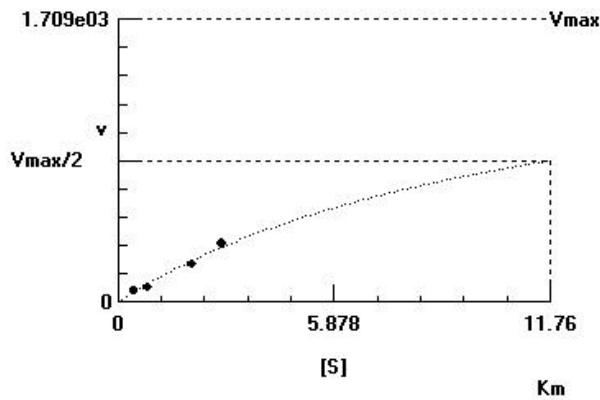


Fig. 9: Hanes plot of strain 1 lipase at 75°C

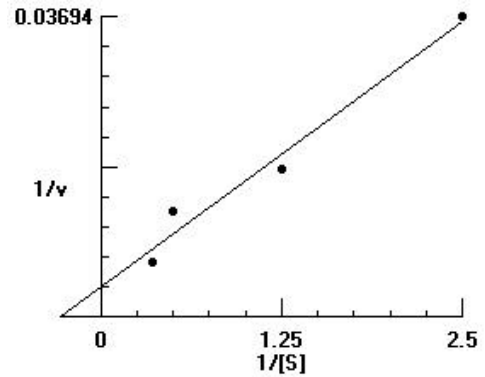


Fig. 12: Lineweaver-Burk plot of strain 2 lipase at 75°C

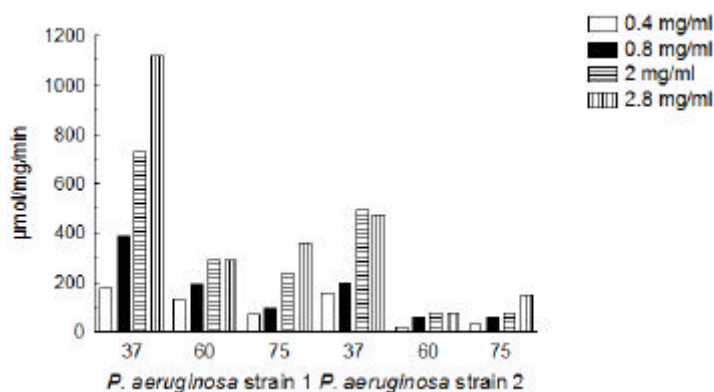


Fig. 13: Enzyme activity as µmol/mg/ml of strains 1 and 2 at different temperature using different substrate concentration

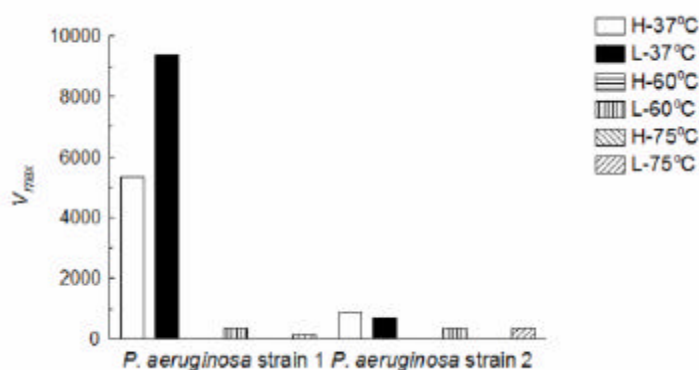


Fig. 14:  $V_{max}$  of strains 1 and 2 at different temperature H-Hanes plot, L-Lineweaver Burk plot

the first based on determining the lipase activities under different temperature and the second based on using different substrate concentrations aiming to kinetics analysis of the data. The enzyme reaction was started by adding different volumes of substrate which represent 0.4, 0.8, 2 and 2.8 mg/ml *p*-nitrophenyl palmitate. The activity for each substrate concentration has been measured at different temperatures (37, 60 and 75°C). *p*-nitrophenyl palmitate that used during various enzyme activities determination was not the main original substrate (the castor oil) for lipase production in this study. Anyhow, using *p*-nitrophenyl palmitate was recommended for its long fatty acid chains and its wide range of specificity. The maximum activity level at 37°C was 1121.00 and 470.82 Units/ml, while the maximum activity at 75°C was 358.72 and 147.18 Unit/ml for strains 1 and 2, respectively. The activity level of strain 1 at 37°C indicate that producing lipase during the degradation of castor oil was economically interested. The lipase enzyme revealed mesophilic and thermophilic activities although the enzymes have been produced in mesophilic conditions. This was agree with the fact that thermophilic enzymes were able to be produced under mesophilic conditions. The  $V_{max}$  and  $K_m$  for each enzymatic reaction

has been calculated. The hyperbolic regression software has been used. This program were able to analysis the enzyme kinetics data. The velocity ( $V$ ), if enzyme catalysed reaction was hyperbolically related to the substrate concentration ( $[S]$ ) through the Michaelis - Menten equation:  $V = V_{max} [S] / (K_m + [S])$ .

The  $V_{max}$  and  $K_m$  were kinetic constants. The data were fitted to the best possible hyperbole. This was achieved by non-linear regression analysis. The Hans plot equation is  $[S]/v = K_m/V_{max} + [S]/V_{max}$  and for Lienweaver-Burk plot is  $1/V = (K_m/V_{max} \cdot (1/[S])) + 1/V_{max}$ . Strain 1 at 37°C revealed the highest  $V_{max}$  which agree with the enzyme activity data. Lipase enzyme produced by strain 1 was more efficient in castor oil degradation as well as *p*-nitrophenyl palmitate than strain 2. This agree with previous investigation, which clearly prove the biodiversity for *P. aeruginosa* strains and that each strains should be studied as a single case [17, 18]. More studies should be done for characterizing both of lipases from *P. aeruginosa* strain 1 and 2 regarding their substrate specificity. One limitation in this study that the castor oil being used is highly pure which was not correctly represent the native crude wastes of caster oil. Meanwhile, it enable to isolate *P. aeruginosa* strains

produced lipase enzyme specific for castor oil. From all the above data, we recommend for using *P. aeruginosa* lipase for castor oil degradation and for the production of mesophilic and thermophilic lipases under mesophilic conditions.

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