



## HIGH-THROUGHPUT SCREENING METHOD FOR ALKALINE LIPASES USING TRIOLEIN AS SUBSTRATE

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**Introduction.** Lipases (EC 3.1.1.3) are soluble enzymes that can act on natural insoluble triacylglycerols (TAG) (1). They are present in many bacteria, fungi, plants, and animals, and have been used in numerous applications such as food, cosmetics, detergents, and pharmaceutical industries (2). Most lipases have been reported to be optimally active at neutral pH (3), but it will be useful to have alkaline lipases for biocatalysis and industrial applications. The success of their screening depends on reliable, appropriate, and sensitive assays to detect a true lipase activity. High-throughput screening (HTS) methods for lipases are usually performed using non appropriate synthetic chromogenic substrates (4); new HTS methods employing natural substrates of lipases are necessary.

In this work we propose a HTS method for alkaline lipases using triolein (an insoluble TAG), wherein the lipolytic activity can be measured spectrophotometrically due to the fatty acid liberation and the color change of a pH indicator.

**Methods.** Microtiter plates wells were coated with triolein as described by Verger et al. (5) and left to equilibrate at 37°C for at least 10 min with 200  $\mu$ L of mixture reaction containing 19 parts of assay buffer (2.5 mM CHES buffer pH 9.2, 150 mM NaCl, 6 mM CaCl<sub>2</sub>, and 3 mg/mL  $\beta$ -CD) mixed with 1 part of thymol blue solution (10 mM) as pH indicator. 2-10  $\mu$ L of an adequate dilution of commercial *Thermomyces lanuginosus* lipase (TLL, Lipex® from Novozymes) was added to each well. The absorbance at 600 nm was recorded continuously at regular time intervals (30 s) using a microtiter plate-scanning spectrophotometer. A blank without enzyme was done. A standard curve with oleic acid was prepared at the same conditions of reaction. The  $\lambda$  Max was determined by thymol blue spectra in the assay conditions. One unit (U) was defined as one  $\mu$ mol of oleic acid produced by min in the assay conditions.

**Results.** Two  $\lambda$  Max values (435 and 600 nm wavelength) were detected for thymol blue (Fig. 1);  $\lambda=600$  nm was chosen because in the addition of oleic acid, the difference in the optical density was greater than  $\lambda=435$  nm. A standard curve relating the absorbance at 600 nm with oleic acid concentration was performed obtaining  $R^2=0.99$  and it was useful to quantify the enzymatic activity (Fig. 2a). In the enzymatic assays, the initial hydrolysis rate of triolein was proportional to enzyme concentration (Fig. 2) obtaining values of  $793\pm 100$  U/mL for TLL (12 fold less compared to pH-stat method).

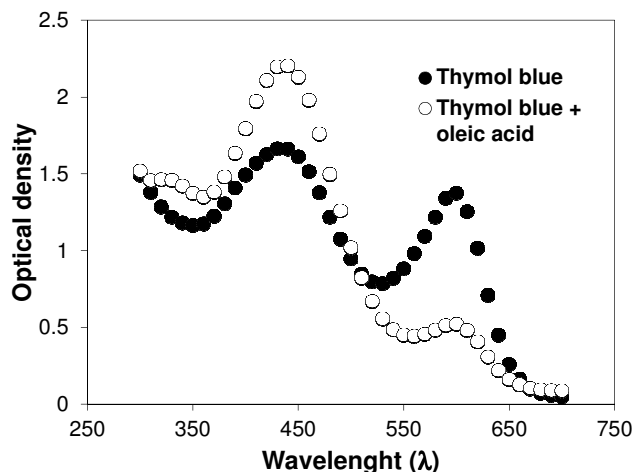


Fig.1 Spectra of thymol blue in the absence or presence of oleic acid.

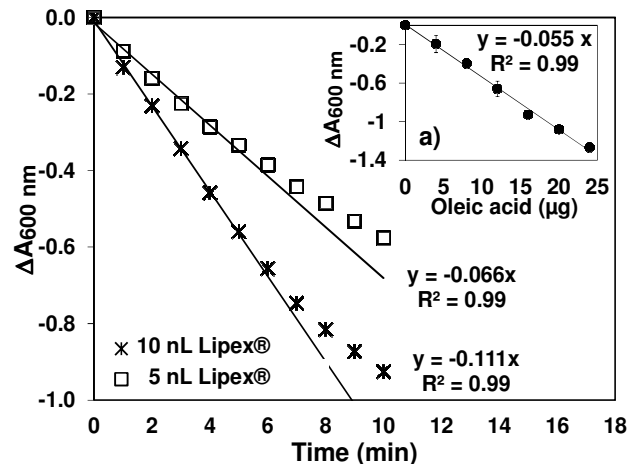


Fig. 2 Effect of enzyme amount on the reaction rate. a) Standard curve of oleic acid.

**Conclusions.** The proposed HTS method was useful to measure the lipolysis of an insoluble long chain TAG at pH 9.2 quickly. This method could be useful for the screening of alkaline lipases as TLL.

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