# Accurately Determining Esterase Activity *via* the Isosbestic Point of *p*-Nitrophenol

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Esterase is an important enzyme for ester hydrolysis or synthesis. Its activity, however, has not been accurately ascertained due to a lack of accurate protocols. In this study, the isosbestic point of *p*-nitrophenol was found and used as the marker for its activity. The methodology avoided decomposition of the substrate, chromophore agents, and pH changes. The esterase activity was determined accurately and rapidly in a complex solution. In this protocol system, organic solvents were used for dissolving substrates, which influenced activity determination to some extent. Among the solvents tested, methanol exerted the least inhibitory influence. The results indicated that this modified method has potential to be applied for esterase activity determination on a large scale and in real time.

*Key words: Esterase activity; UV-VIS; p-Nitrophenol; Isosbestic point*

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## INTRODUCTION

Esterases (EC 3.1.1.1) represent a diverse group of enzymes that catalyze the cleavage of ester bonds and are widely distributed in animals, plants, and microorganisms (Nam *et al.* 2009). These enzymes are commonly categorized according to their corresponding substrates and are known as carboxylesterase, sulfatase, and phosphatase (Tirawongsaroj *et al.* 2008; Calero-Rueda *et al.* 2009; Chimtong *et al.* 2014). There are many reports on esterases that discuss distribution, quantitation, targeted synthesis, production, purification, and molecular biology. The main difference between esterases and lipases is in the interfacial activation and substrate specificity (Panda and Gowrishankar 2005). In general, esterases hydrolyze short carbon water-soluble chain ester substrates (< 12) in homogeneous conditions. Lipases, alternatively, prefer long-chain triglycerides because the active site is covered by a hydrophobic domain and catalysis is heterogeneous (Gupta *et al.* 2004).

The applications of esterases are widespread among a number of disciplines, such as pulp and paper (Topakas *et al.* 2007), chemical synthesis, cosmetics (Jeon *et al.* 2009), pitch control for fiber recycling (Gutiérrez *et al.* 2001), bleaching (Gangwar *et al.* 2016) and mechanical pulp manufacture (Kontkanen *et al.* 2006; Torres *et al.* 2008), and pharmaceuticals (Panda and Gowrishankar 2005). In more sophisticated applications, they are also used for the resolution of racemic mixtures of compounds to produce pure enantiomers by esterification or interesterification reactions (Panda and Gowrishankar 2005). Both esterases and lipases can produce enantiomeric excesses of up to 98% by transesterification (Gupta and Kazlauskas 1993; Ozaki *et al.* 1995). Esterases are also employed in wine, alcohol, fruit juice, beer, and cheese production. Milk fat, for example, can be significantly hydrolyzed by esterases and lipases from *Lactobacillus casei* CL96 for

flavor enhancement in cheese-related products (Choi and Lee 2001). However, given their widespread applicability, there is a pressing need for esterases to be produced efficiently and characterized thoroughly.

Their enzyme activity is the single most important parameter that is obtained by specific assays. Various methods for esterase activity determination have been reported, such as inter alia, titration (Gilham and Lehner 2005; Schmidt and Bornscheuer 2005), high performance liquid chromatography (HPLC) (Faulds and Williamson 1995; O'Neill *et al.* 1996; Barbe and Dubourdieu 1998; Yue *et al.* 2009), and gas chromatography (GC) (Borneman *et al.* 1990). All ascertain enzyme activity through the determination of acid or other hydrolytes after enzyme action. However, there are shortcomings with these methods. Titration lacks sensitivity and reliability, whereas GC is often expensive, requires long testing times, and uses toxic organic solvents. These methods are generally regarded as lacking the needed accuracy and rapidity (Mastihuba *et al.* 2002). A spectral method that overcomes the latter problems has already been established (Mccallum *et al.* 1991; Calero-Rueda *et al.* 2002). This method requires a simple ultraviolet-visible (UV-Vis) spectrophotometer in which *p*-nitrophenyl esters with aliphatic acyl chains of various lengths or carboxyl fluorescent type esters are used as substrates. Esterase activity can be calculated by detecting the amount of *p*-nitrophenol (PNP) that is hydrolyzed. PNP has two characteristic absorption peaks that are adversely affected by and change with solution pH. Therefore, its accuracy and reliability are questionable, and hence, its assay of esterase activity is limited.

In this study, a characteristic isosbestic point for PNP that is proportional to its concentration after hydrolysis of the substrate was found. The modified method used the acetate salt of PNP as the substrate to rapidly and accurately determine esterase activity.

## EXPERIMENTAL

### Materials and Chemicals

Feruloyl esterase (E.C. 3.1.1.73) from recombined *Escherichia coli* was donated by Professor Yuhuan Liu from Sun Yat-Sen University. The protein content was 0.3 mg/L. Three other esterases from porcine pancreas, *Candida*, and *Pseudomonas* sp. were purchased from Aladdin (Shanghai, China), as was the substrate *p*-nitrophenyl acetate. Other chemical reagents used were of analytical grade.

The preparation of the substrate solution was done by dissolving 0.0180 g of *p*-nitrophenyl acetate in methanol, and then diluting the solution with methanol to 200 mL. The solution was diluted step-wise to the required concentration.

A sodium phosphate buffer with a concentration of 0.1 mol/L was used to maintain pH. In addition, individual pHs were adjusted to appropriate values by 0.1 mol/L of HCl or 0.1 mol/L of NaOH.

### Modified Determination Method of *p*-Nitrophenol

The PNP absorption spectra were obtained at different pH values by UV-Vis (Agilent 8453, San Francisco, USA), over a pH range of 4 to 11.

The UV absorption values were measured under different PNP concentrations and pH values. The concentrations were 4 mg/L, 8 mg/L, 10 mg/L, 12 mg/L, and 15 mg/L, and pH ranged from 4 to 11. A calibration curve of PNP at 347 nm was obtained.

### Esterase Activity Determination Using *p*-Nitrophenyl Acetate as Substrate

The substrate used to determine esterase activity was *p*-nitrophenyl acetate. Esterase activity was calculated according to PNP content in the solution after esterase hydrolysis. Esterase activity was expressed as the amount of esterase to 1 μmol PNP released per min. Incubation mixtures contained 1 mL of enzyme solution in 3 mL of 0.1 M sodium phosphate buffer. The reaction was initiated by adding 1 mL of the substrate. After incubation, the mixtures were immediately scanned by UV-Vis at 347 nm. Deionized water was used as a control. The activity of feruloyl esterase from recombined *Escherichia coli* and other three esterases from different sources were determined using this method. Three replicates were done per assay.

### Condition Optimization of the Esterase Activity Determination

Methanol, ethanol, and acetone were selected as the solvents for promoting enzyme solubility. The effect of the organic reagent on activity was also investigated. Percentages of organic reagent were varied. The percentages were 0.5%, 1%, 2%, 4%, 6%, and 10%. Temperature and pH were the most important factors affecting enzyme stability, and were also investigated. The pH value of the incubation ranged from 4 to 11 using 0.1 M sodium phosphate as the buffer. To adjust the pH, 0.1 mol/L of HCl or 0.1 mol/L of NaOH was used. The method of esterase activity determination was described previously. The mixture was incubated for 5 min at 55 °C. Additionally, the effect of temperature was considered. The experiments were carried out from 30 °C to 70 °C at a pH of 6.5. The relative activity was obtained in different conditions.

### Application of the Modified Method for Esterase Activity Determination

A modified method was used to simulate esterase activity of zymotic fluid by the addition of protein or carbohydrates. Casein, glucose, and maltose were selected. The casein concentrations were 0 to 0.6 mg/L, while the glucose and maltose concentrations were 0 to 6 g/L. In this case, the mixture was incubated for 5 min at 55 °C and pH values remained at 6.5. The mixture which contained protein or carbohydrates without esterase was set as control experiment.

## RESULTS AND DISCUSSION

### Isosbestic Point for PNP Quantification

In general, PNP is determined by analysis of its UV-Vis spectrum according to its characteristic absorption peak (Chern and Chien 2002). However, it is severely affected by solution pH, as evidenced by the different absorption in a pH range from 3 to 10, which is a result of different ionizations (Gao *et al.* 2011). For an adequate measurement, a pH adjustment procedure is required that, in general, can be error prone, time consuming, and not suitable for rapid measurements (Tian *et al.* 2007).

The UV-Vis absorption spectra of PNP at different pH values were measured, as shown in Fig. 1. The absorption spectra of PNP varied greatly from pH 4 to 11. The two main peaks were at 317 nm and 400 nm, which was consistent with the literature. The peak at 317 nm appeared when the pH was lower than 4, and the peak at 400 nm appeared when the pH was higher than 9, which was due to ionization differences (Fig. 2). The two states co-exist when pH is neutral, but change as a function of pH.

However, the spectra, as shown in Fig. 1, had an isosbestic point at 347 nm that was first utilized in this study. The optical density (OD) at 347 nm was not affected by pH and only related to the concentration. It provided a theoretical basis for a new stable protocol for esterase activity.

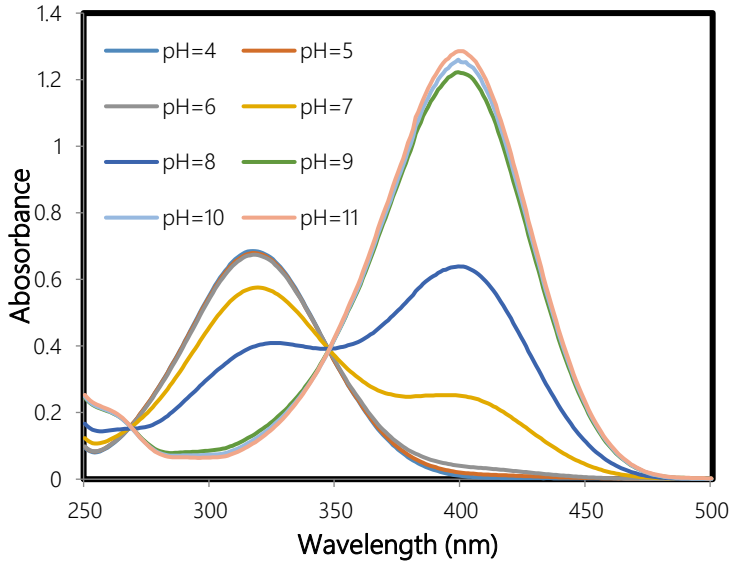


**Fig. 1.** Ultraviolet absorption spectrum of PNP at various pH values. The concentration of PNP is 10 mg/L. The pH values of the solutions ranged from 4 to 11.

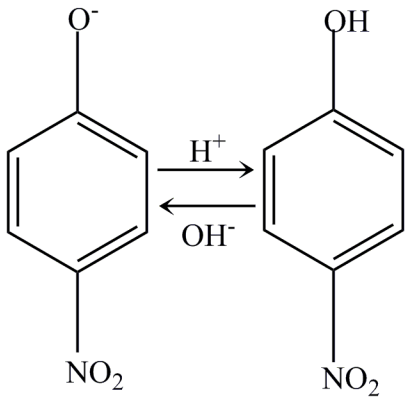


**Fig. 2.** Two states of PNP in different conditions

## Developing a New Calibration Method

To obtain the relationship between the isosbestic point and concentration, the absorption spectra of PNP at different concentrations and pH were investigated. As shown in Fig. 3, the spectra of PNP regularly changed with concentration under different pH values. Under the same concentration and different pH values, two characteristic peaks were observed, which was consistent with what was described earlier.

To further illustrate the relationship between the isosbestic point and concentration, the data were fitted. A calibration curve of PNP at 347 nm was obtained, and is shown in Fig. 4. The $R^2$ was 0.9995, which meant there was a high level of fit.

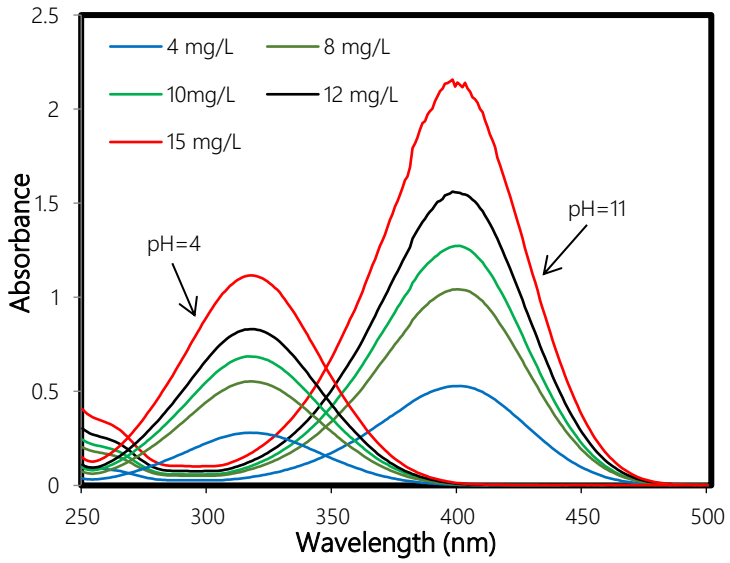


**Fig. 3.** UV-Vis absorption spectra of PNP at different pH values and concentrations. The concentration of PNP solutions are 4 mg/L, 8 mg/L, 10mg/L, 12 mg/L, and 15 mg/L, and pH values are 4 and 11. The OD value at 347 nm did not change with the pH value of the solution, but related to the concentration.

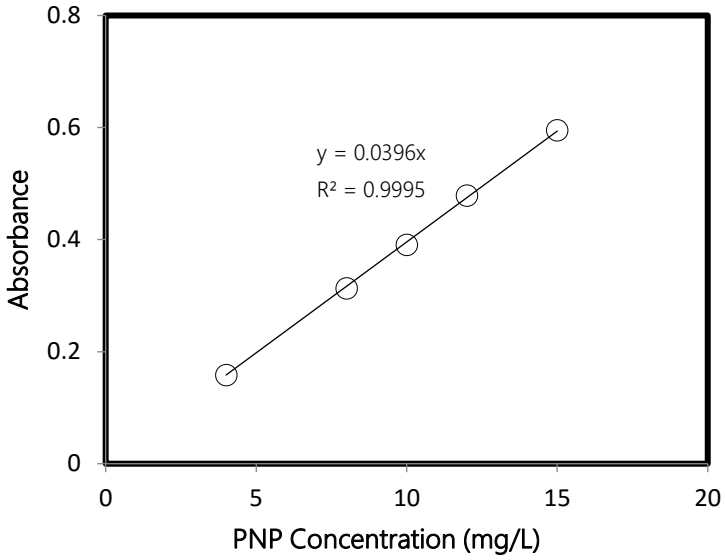


**Fig. 4.** Calibration curve of PNP at 347 nm

## Accuracy, Reproducibility, and Validity

A simple external standard calibration of absorbance at 347 nm was accomplished with a series of standard PNP solutions (2 to 18 mg/L), generating a standard calibration curve that followed Eq. 1,

$$A = 0.0427(\pm 0.0014) \times C - 0.0286(\pm 0.0056) \quad (1)$$

where $A$ represents the absorbance at 347 nm, and $C$ is the concentration of PNP solution (mg/L). For Eq. 1, n was 5 and $R^2$ was 0.9992. The limit of quantitation of the present method was 0.39 mg/L. Also, as shown in Fig. 3, absorbance values were decreased from 1.3 to 0.4 when changing from 400 nm to 347 nm. This results in a 3-fold drop in sensitivity.

So, using the same method, we calculated the limit of quantitation at 400 nm. The result was 0.35 mg/L. This means that the limit of quantitation of PNP at 347nm is almost equal with those of at 400 nm.

To investigate the validity and reliability of the method, recovery was tested. A set of PNP standard solutions were prepared, and their concentrations were 2 mg/L, 6 mg/L, 10 mg/L, 14 mg/L, and 18 mg/L. The buffer solution, without PNP, was measured as a reference. Thus, the contribution from the added PNP was determined by Eq. 1.

Table 1 shows a comparison of the experimental data with the actual amount added. The high recovery obtained indicated that the present method was a reliable and suitable method for the determination of PNP.

**Table 1.** Method Validation

| # | Added (mg/L) | Measured value (mg/L) | | | Recovery (%)* |
|---|---|---|---|---|---|
| 1 | 2 | 2.11 | 2.09 | 2.07 | 104.5 ± 0.8 |
| 2 | 6 | 5.98 | 6.01 | 6.04 | 100.2 ± 0.5 |
| 3 | 10 | 10.06 | 10.02 | 9.98 | 100.2 ± 0.4 |
| 4 | 14 | 14.43 | 14.31 | 14.18 | 102.2 ± 0.9 |
| 5 | 18 | 17.55 | 17.77 | 17.98 | 98.7 ± 1.2 |
| *Values represent mean ± standard deviation of three replicates | | | | | |

## Modified Activity Assay Procedure

When *p*-nitrophenyl acetate was used as the substrate for esterase activity measurement with this method, the concentration of PNP in solution was calculated by Eq. 2,

$$C = 23.5A + 0.67 \quad (2)$$

$$\Delta C = 23.5 \times \Delta A \quad (3)$$

$$\text{Activity of esterase} = (\Delta Ca \times V) / \Delta t \times 2N$$

$$= 0.17 \frac{\Delta A}{\Delta t} \times V \times 2N \quad (4)$$

The change of PNP concentration ($\Delta C$, mg/L) was obtained from Eq. 3, where $\Delta A$ is the increase in absorbance at 347 nm. The activity of esterase, which is the amount of product from the reaction (μmol/min×mL), was calculated by Eq. 4. In Eq. 4, $Ca$ is the concentration (μmol/L), $V$ is the volume of measurement solution (L), and $N$ is the enzyme solution diluted multiples. A factor of 2 was used because one part of the enzyme solution was mixed with one equal part of substrate solution.

The specific esterase activity measurement was done by preparing incubation mixtures that contained 1 mL of the diluted enzyme solution in 3 mL of 0.1 M sodium phosphate buffer. The incubation temperature was 55 °C for 5 min at pH 6.5. The reaction was initiated by adding 1 mL of the substrate solution (10 mg/L). After incubation, the mixtures were immediately scanned by UV-Vis at 347 nm, and the buffer was used as a reference. The esterase activity was calculated according to Eq. 4. Three replicates were done per assay.

To test the validation of this method, three other commercial esterases were selected. The results are shown in Table 2. The relative standard deviation (RSD) of this method was 0.82% to 2.31%, which was less than the RSD for titration (4.90%) (Vorderwülbecke *et al.* 1992) and was similar with the results measured at 400 nm (2.03%

to 2.70). Also, the data from Table 2 indicated that the method was suitable for commercial esterases.

**Table 2.** Application of Method for Esterases from Commercial Sources

| Sample No. | Source | Activity | |
|---|---|---|---|
| | | Measured at 347 nm | Measured at 400 nm |
| 1 | Recombined *E. coli* | 150.1 ± 1.2 (U/mL) | 172.6 ± 3.5 (U/mL) |
| 2 | Porcine pancreas | 301.5 ± 3.6 (U/g) | 334.6 ± 5.8 (U/g) |
| 3 | *Candida* | 110.8 ± 1.1 (U/g) | 121.9 ± 3.2 (U/g) |
| 4 | *Pseudomonas* sp. | 240.8 ± 5.6 (U/g) | 255.3 ± 6.9 (U/g) |
| *Values represent mean ± standard deviation of three replicates | | | |

## Co-solvent for Promoting Substrate Solubility

Substrate dissolution was a key factor for favorable enzymatic reaction. In the past, some studies used $CH_2Cl_2$ as the solvent for the *p*-nitrophenyl ester. However, that may affect enzyme stability because of toxicity and water immiscibility (Lehner and Verger 1997). Other dispersing systems have been used, such as detergents, lipids, bile acids, *etc*., that denatured the enzyme, which resulted in loss of activity (Gilham and Lehner 2005).

Methanol, ethanol, and acetone were used to dissolve the substrate. The three organic solvents had no absorbance at 347 nm, and had no influence on the measurement of PNP.

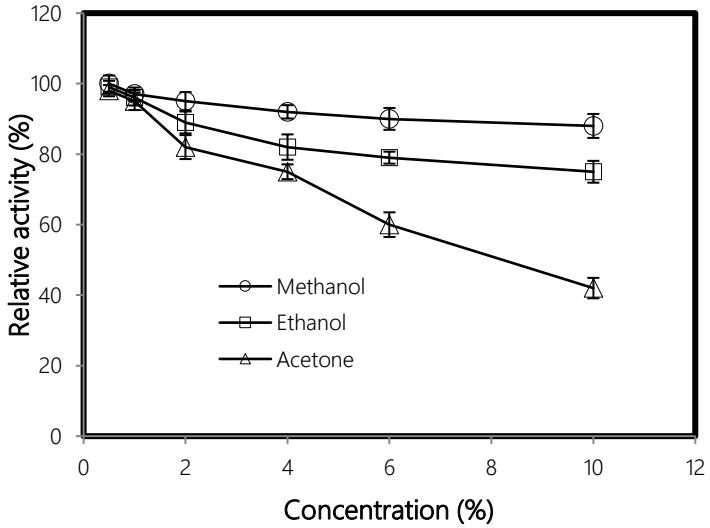


**Fig. 5.** Effect of organic solvents on esterase activity. The organic reagents used were methanol, ethanol, and acetone. The concentrations of the organic reagent used were 0.5%, 1%, 2%, 4%, 6%, and 10%. The temperature and pH were 55°C and 6.5, respectively.

As shown in Fig. 5, methanol had little impact, while acetone had a major impact on the esterase activity. At low concentrations (< 1%), the effects of the three organic solvents were minor, as evidenced by the 95% enzyme activity. However, as the concentration of the solvent was increased, the influence of acetone became more obvious, which was likely due to enzyme protein denaturation. When ethanol and acetone concentration were higher than 6%, more 20% relative activity lose was measured. While

high concentrations of methanol only had minor effects on enzyme activity (90%, respectively). This means that methanol is more suitable as co-solvent for substrate than the other two organic solvents.

In addition to enzyme denaturation caused by acetone, Gomes *et al.* (2011) reported that it accentuated the color of the reaction mixture, which affected visual acuity. Also, the calibration curves of PNP were prepared in the presence and absence of acetone, which may affect the accuracy (Gomes *et al.* 2011). Thus, methanol can be used as the substrate solvent for the esterase activity measurements.

## Optimization of Esterase Activity Determination

Temperature and pH are the two most important factors in the study of enzymology (Angelis *et al.* 2010). This method was applied to measure esterase activity from recombined *E. coli* using *p*-nitrophenyl acetate as the substrate. The effects of pH and temperature on enzyme activity were investigated, and the data are shown in Fig. 6 below.

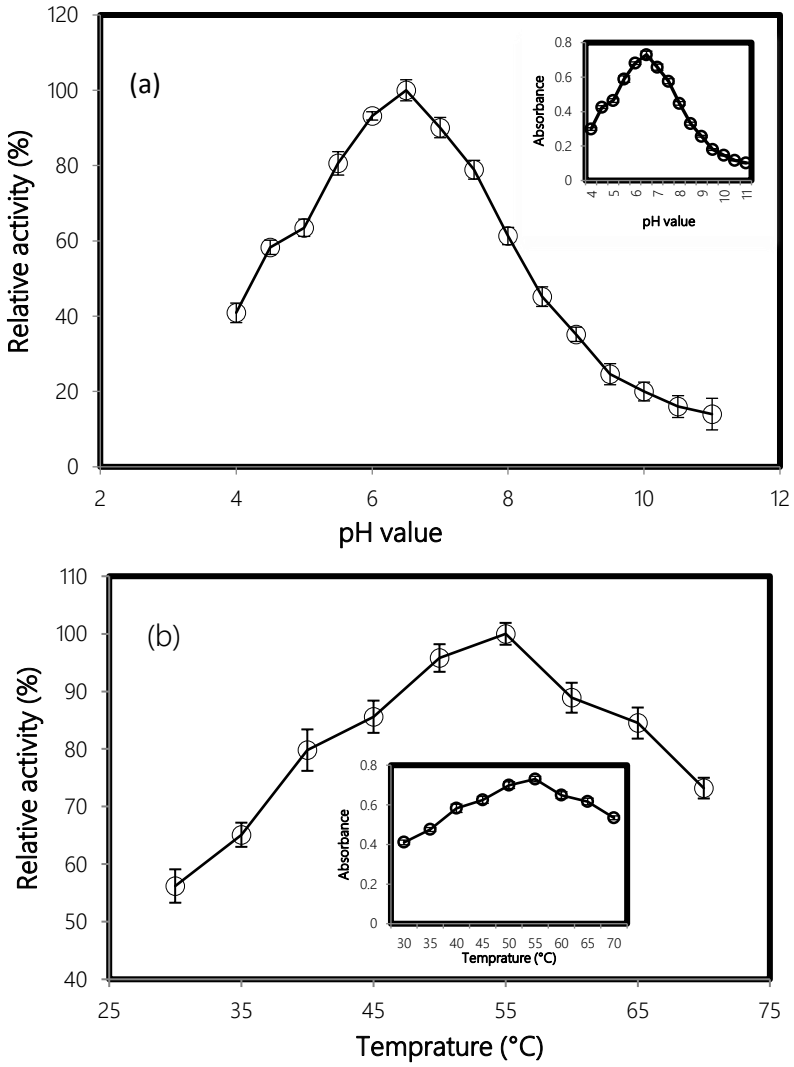


**Fig. 6.** The variation of esterase activity under different pH and temperature. (a) pH value of the incubation mixture, which ranged from 4 to 11. The mixture was incubated for 5 min at 55 °C. (b) Experiments carried out with the incubation mixture temperature ranging from 30 °C to 70 °C. The pH value remained at 6.5.

The absorbance at the isosbestic point was in direct proportion to PNP concentration at pH ranging from 4 to 11 and at 55 °C. The esterase activity obtained was greatly influenced by pH and had a relatively narrow pH tolerance range. The optimal pH was 6.5 for esterase from recombined *E. coli*.

As shown in Fig. 6, the influence of temperature on esterase activity was tested. The enzyme activity ranged from 30 °C to 70 °C with a pH of 6.5, and the optimal temperature was 55 °C.

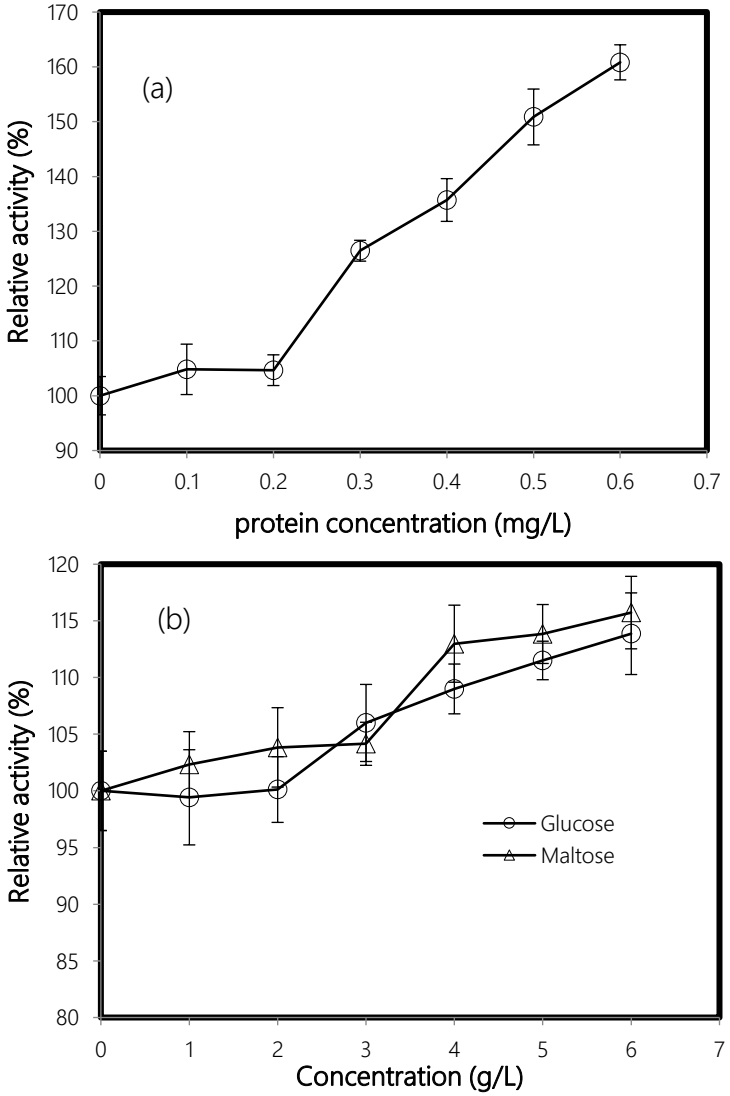


**Fig. 7.** Effect of protein (a) and carbohydrates (b) on esterase activity. The mixture was incubated for 5 min at 55 °C. The pH value remained at 6.5.

## Application of the Modified Method for Esterase Activity Determination

A positive impact on the cultivation and screening of enzymes can be obtained if the activity could be determined quickly and in real time (Manzo *et al*. 2013). Other impurities, such as protein and carbohydrates, in a zymotic fluid can influence the accuracy of enzyme activity determination. The modified method was applied to the esterase activity determination of a simulated zymotic fluid that had protein (casein) or carbohydrates (glucose and maltose) added in.

As shown in Fig. 7a, the esterase activity increased after adding protein. A noticeable effect on the esterase activity occurred when the casein concentration was more than 0.2 mg/L. There was a slight influence on the esterase activity determination when the casein concentration was low. It has been suggested that the *p*-nitrophenyl ester can be hydrolyzed by non-specific esterases, non-enzymatic proteins, or proteases, such as serum albumin or insulin (De Caro *et al.* 1986), which are often found in biological samples. Also, it has been previously reported that the hydrolysis rates of aspirin were greatly enhanced in the presence of human serum albumin (HAS) which confirmed the esterase-like activity of HAS (Moradi N *et al.* 2015). A low protein concentration had minor effect on the esterase activity determination using the present method.

Figure 7b shows the effect of two carbohydrates, glucose and maltose, on the esterase activity determination. Glucose and maltose are common carbohydrates found in fermentation assays. The data showed that the two carbohydrates both interfered with the esterase activity when the concentration of glucose and maltose were greater than 2 g/L and 3 g/L, respectively. This was because the visual detection of the samples was affected by the glucose and maltose addition. This may be associated with the dielectric constant of solution. Dielectric constant decreased with increasing glucose contents in the water system (Park *et al.* 2004). Overall, less than 20% relative activity was lost due to the presence of the two carbohydrates. This means carbohydrates had little effect on the activity determination using this method. Compared with protein, the influence of carbohydrates on the esterase activity determination using the present method was not remarkable. Therefore, the present method was suitable for the esterase activity determination of zymotic fluid when protein and carbohydrate concentrations were low.

## CONCLUSIONS

1. A modified spectrophotometric method was described to determine esterase activity towards *p*-nitrophenol based on a new calibration method using the isosbestic point at 347 nm. Absorbance was determined at 347 nm as concentration increased linearly from 2 mg/L to 18 mg/L. The calibration method was highly accurate and reproducible. The recovery was 98% to 104%.
2. Using *p*-nitrophenyl acetate as the substrate, the method was used to determine esterase activity from recombinant *E. coli*, as well as three commercial esterases from different sources. The RSD for this method was less than for traditional methods. The modified method was fast, accurate, and required no pH adjustment. The whole experiment time was less than 10 min. The co-solvent was methanol, which had a minimal influence on esterase activity determination. The optimal pH and temperature from recombined *E. coli* using this method were 6.5 and 55 °C, respectively.
3. The esterase activity of simulative zymotic fluid with protein or carbohydrates was determined using the modified method. It was found that the modified method was suitable for simulative zymotic fluid when the protein and carbohydrate concentrations were low, less than 0.2 mg/L and 2 g/L, respectively. It allowed for continuous recording of the changes in esterase activity under fermentation, and had positive ramifications on enzyme screening.

## ACKNOWLEDGMENTS

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