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# EMPLOYING LIPASE OF *CANDIDA ANTARCTICA (CALB*) AS CATALYST IN THE ACETYLATION OF *PARA*-AMINOPHENOL IN AQUEOUS AND WATER-FREE MEDIUM

# PEMANFAATAN LIPASE DARI *CANDIDA ANTARCTICA (CALB*) SEBAGAI KATALIS PADA ASETILASI PARA-AMINOFENOL DALAM MEDIUM BERAIR DAN BEBAS AIR

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## ABSTRACT

*Candida antarctica lipase B (CaLB) is one of lipase classes enzymes that has many advantages to be used in the process of synthesizing organic compounds. In this study, some experiments were conducted to examine the ability of CaLB as a catalyst in the para-aminophenol (PAP) acetylation to produce paracetamol as the result. Two types of research have been carried out, the first one is to utilize CaLB to catalyze acetylation of PAP in a water-free reaction medium, and the second one is to use CaLB as catalyst in aqueous medium through oxidative amidation reaction. Reaction in water free system was held in ethyl catalyst acetate as solvent that also act as the acyl donor, while in the aqueous medium, acetylacetone was used as acyl donor and ethyl acetate as source to produce peracid that will be used as oxidator. Analysis was done by HPLC and TLC densitometric to follow the amount of paracetamol produced. The results of CaLB-catalyzed acylation in water free system showed that the enzyme could accept PAF and ethyl acetate as a substrate in a nucleophilic substitution reaction, resulting in paracetamol as a product. However, the yield from the acylation of PAP is still not satisfactory. In the reaction in aqueous medium, CaLB has been proven to show its activity to catalyze the acylation of PAP with acetylacetone, as well as the reaction of peracid formation from ethyl acetate. The results show that this strategy can work well and give better yields than the other reaction in water-free medium.*

***Keywords:*** *CaLB; para-aminophenol; acetylation; oxidative amidation.*

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## ABSTRAK

Candida antarctica lipase B (CaLB) adalah enzim jenis lipase yang dilaporkan memiliki banyak kelebihan sebagai katalis dalam sintesis senyawa organik. Dalam penelitian ini, dilakukan percobaan untuk menguji kemampuan CaLB dalam mengkatalisis asetilasi para-aminofenol (PAF) untuk menghasilkan parasetamol. Dua macam penelitian yang telah dilakukan, yaitu memanfaatkan CaLB untuk mengkatalisis reaksi asetilasi PAF dalam media reaksi bebas air, serta peranan CaLB sebagai katalis dalam media berair melalui reaksi amidasi oksidatif. Reaksi dalam sistem bebas air dilakukan dengan menggunakan etil asetat sebagai pelarut sekaligus sebagai donor asil, sedangkan reaksi dalam media berair menggunakan asetilaseton dan etil asetat. Analisis terhadap produk parasetamol dilakukan menggunakan HPLC dan KLT densitometri. Hasil asetilasi dalam sistem bebas air menunjukkan bahwa CaLB dapat menerima PAF dan etil asetat sebagai substrat dalam reaksi substitusi nukleofilik, menghasilkan produk parasetamol. Rendemen hasil reaksi dalam medium bebas air lebih kecil dibandingkan hasil reaksi dalam medium berair. Menunjukkan bahwa CaLB memiliki aktivitas dalam mengkatalisis asilasi PAF menggunakan asetilaseton dan pembentukan peracid dari etil asetat. Kesimpulan dari penelitian ini menunjukkan bahwa enzim lipase CaLB dapat digunakan dalam asetilasi PAF melalui reaksi amidasi oksidatif, dan memberikan hasil yang lebih baik dibanding reaksi asetilasi dalam media bebas air.


**Kata kunci:** *CaLB; asetilasi; para-aminofenol; amidasi oksidatif.*

## INTRODUCTION

Paracetamol is a common analgesic and antipyretic drug that is widely used in medical practices (Katzung, 2012). Synthesis of paracetamol is still interesting topics for research, since the high need for paracetamol production provokes the efforts to increase synthesis efficiency as regard to possible economic advantage. At the other hand, the emergence of new strategies in drug synthesis has increased the possibilities for new improvement in paracetamol synthesis using the most current strategy (Adams, 2019; Magadum, 2018)

Synthesis of paracetamol involves several reaction steps that ended by the formation of amide bond through acylation of *para*-aminophenol (PAP) using acetic anhydride as the acylating agent. However, as the main ingredient in the reaction, acetic anhydride still shows some drawbacks. This compound is known to be unstable and easily hydrolyzed in the presence of water traces. Furthermore, at the end of the reaction a large amount of acetic acid will be produced as waste, which then requires further treatment. Regarding this, enzymatic reactions then appear as an interesting alternative to compete the common procedure (Adams, 2019). Enzymatic synthesis is known to have more advantages over conventional chemical synthesis, as the reaction is highly specific, giving less by-products, and saves energy for it is mostly held at room temperature. (Van Damme, 2005)

One of most popular enzyme applied in nowadays chemical synthesis is *lipase.* Lipase (triacylglycerol hydrolase) is an enzyme which in nature hydrolyses triglycerides to fatty acid and glycerol. Some lipases have been reported to have high catalytic activity and stability in organic solvents. In particular CaLB (lipase B from *Candida antarctica*), which seems to prefer anhydrous condition and has been widely used in many studies (Dakin, 1905; Schmidt,1998). When amine or alcohol was used as nucleophile in a water-free system, the reaction will turn to an amine- or alcohol- acylation reaction, resulting amide or ester as a product. CaLB has been reported to be successful in catalyzing many acetylation of secondary aromatic amines with good conversion rates. Accordingly, CaLB is often used in the resolution of chiral amines, as it has a specific binding site for amines and having ability to identify the enantiomer in racemic mixture (Sheldon, 1993; Lau, R., 2007; Ismail, H., 2008). Hence, it is interesting to study the ability of CaLB in catalyzing the formation of amides in PAP, as the member of primary aromatic amine.

In this research, two kinds of CaLB-catalyzed enzymatic acylation of APA were studied. In the first study CaLB was employed to catalyze PAP acylation in water-free condition, while in the second the potency of CaLB was studied in aqueous medium through an oxidative amidation reactions with PAP.

## CaLB as catalyst for the acylation of PAP in a water-free condition

CaLB (*Candida Antarctica* Lipase B) is currently widely used as biocatalyst in various organic synthesis reactions. This superiority is related to CaLB's ability in maintaining its catalytic activity in organic solvent media. Accordingly, CaLB often been used to catalyze esterification and amidation reaction in a water-free conditions. CaLB is a member of serine hydrolase group, which has a catalytic system consisting of 3 amino acids on the 'catalytic triad' (amino acids ser-100, Asp-187 and His 224) and an *'oxyanion hole'* system to stabilize the negative charge of oxygen carbonyl (Martinelle, 1995).

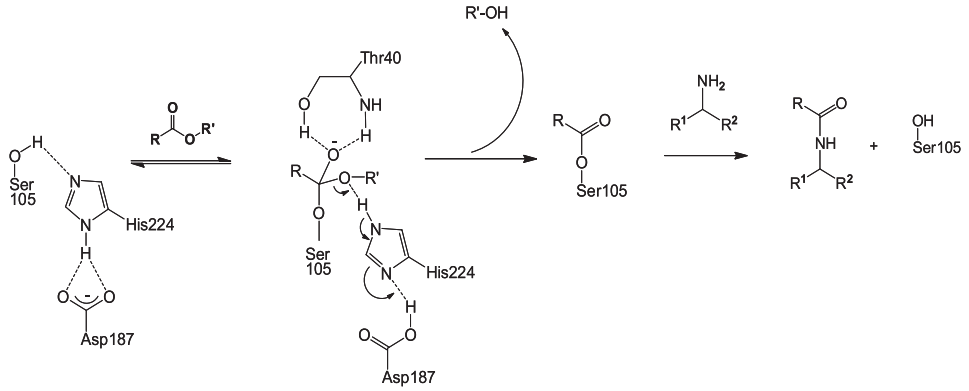


Figure 1
Reaction mechanism of CaLB catalysis in acylation of amines
Source: Martinelle *et.al.* (1995)

The catalytic triad of lipases consists of a serine residue that is activated by histidine and aspartate residues. The reaction mechanism is shown in figure 1. First, the carbonyl ester bound to the OH-serine group to form an acyl-enzyme intermediate, the negative charge on the carbonyl oxygen is stabilized by the oxyanion hole. In the second step, the enzyme-acyl intermediate eliminate alcohol to form serinate ester, which then reacted to a nucleophile to produce the product and releasing the enzyme. In the case of hydrolysis, the nucleophilic attack is carried out by water molecule. In the case for amine acylation, water molecule is replaced by amine group, affording the corresponding amide as the product (Van Rantwijk, 2004).

## CaLB as a catalyst for oxidative amidation in aqueous medium.

Beside accepting molecule with -OH and NH- groups as nucleophiles, CaLB also can accept hydroperoxides as substrate to react with carboxylic acid to form peroxycarboxylic (peracids) (Bjorkling, 1992). The advantage of this reaction is that formation of peracids happen under mild reaction condition. In the presence of an unsaturated molecule, this peracids can rapidly oxidize double bonds in a molecule to form epoxides (Svedendahl, 2008). The reaction mechanism of the CaLB-catalyzed epoxide formation is presented in figure 2.

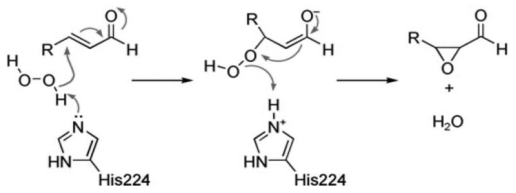


Figure 2
Reaction mechanism of CaLB
in peracid and epoxide formation
Source: Svedendahl *et.al.* (2008)

Based in this, Zhang, *et. al*. (2017) has published a report on a study of the ability of CaLB to catalyze the formation of amide from aniline in a water system. The reactions were done by employing CaLB in the forma-

tion of peracid as oxidizing agent to produce epoxides. Along with it, CaLB was also playing role in the coupling reaction between primary amine and acetyl acetone, resulting in an enaminone (Zhang, 2017). These two reactions were combined to eventually produce amide as the final product. The overall reaction scheme is shown in the figure 3.

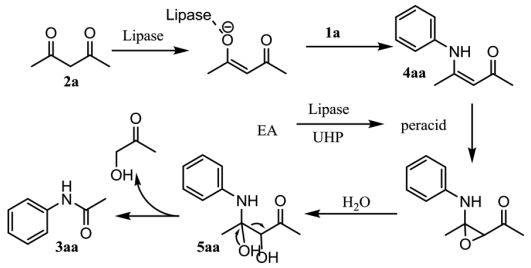


Figure 3
Proposed reaction mechanism of oxidative acylation of aromatic amines
Source: Zhang *et.al* (2017)

A number of aniline derivatives have been studied to be acylated with acyl donor derived from acetyl acetone in the presence of CaLB, resulting in a good yield of amide compounds (64.3-96.2%). Here CaLB catalyze the formation of *enaminone* (4aa), which was further oxidized by peracid to form epoxide. Peracid itself was the result of the perhydrolysis reaction of ethyl acetate, which was also catalyzed by CaLB. Further, the epoxide undergoes hydrolysis and re-arrangement reaction in the presence of water to produce amide and released *hydroxy ketone* as by product. (Zhang, et.al., 2017).

The aim of this experiment is to study the possibility of using CaLB for the synthesis of paracetamol from *p*-aminophenol (PAP) as an aniline derivative. Here, CaLB was employed to catalyze the peracid formation from ethyl acetate in the presence of urea *hydrogen peroxide* (UHP) as oxidizing agent. Along with it CaLB was also in charge of catalyzing the amidation of PAF, using acetyl acetone as the source of acyl donor to form enaminone which will be further oxidized and hydrolyzed leading to paracetamol as the amide form of PAP.

## Method

### Materials

Lipase B *Candida antartica immobilizied on Immobead 150* ((7488U/g) is from *Sigma Aldrich*, standard paracetamol was obtained from *Konimex Pharmaceutical Industry*, 4-aminophenol was obtained from Sigma Aldrich UK Ltd (PA), TLC Plate (*silicagel* $GF_{254}$) and other chemicals were also purchased from Sigma-Aldrich: acetylacetone (PA), pyridine (PA), chloroform (PA), acetone (PA), ammonium hydroxide 25% (PA), and methanol (PA). Urea Hydrogen Peroxide (UHP) was freshly prepared by dissolving urea in 30% hydrogen peroxide (molar ratio 2:3) at 40-50$^{o}$C and cooled down to get precipitated UHP. Quantitative analysis was executed on HPLC-*Shimadzu* and Scanner Densitometer *Win CATS Camag*. Enzymatic assay for CaLB was executed through *Triacetin test* for lipase in the pH of 7.4 at 37$^{o}$C (Sigmaaldrich, 1997).

### Procedure

a. Enzymatic acylation of *p*-aminophenol with ethyl acetate as acyl donor
   An amount of *p*-aminophenol (PAP, 0.4 mmol) was dissolved in 5 mL of ethyl acetate. CaLB immobead 150 (150 U), and molecular sieves of 4A (100 mg) were added to the reaction mixture. The reaction was then incubated at 50, 60 or 70 °C under stirring for 24 h. A sample (100 µL) was taken every 1 h to follow the reaction over time. The conversion was monitored by HPLC *HITACHI La Chrome Elite* - Serial nr 5472137, to measure the yield.

b. Oxidative Amidation of *p*-aminophenol using acteylacetone as acyl donor
   A mixture of *p*-aminophenol (PAP, 1 mmol); acetyl acetone (2 mmol); CaLB immobead 150 (150 U); ethyl acetate (0,2 mL); and UHP (1,2 mmol; 1,4 mmol; or 1,6 mmol) dissolved in aquadest (2 mL) in 5 mL flacon. Reaction mixture was stirred (200 rpm) at room temperature for 1 hour. After completing the reaction, the product was isolated by extraction from ethyl acetate. Sample was analyzed by TLC on Silica gel $GF_{254}$ and mixture of

chloroform-acetone-ammonium hydroxide 25% (16:4:0,2) as eluen (Pyka, Budzisz, Ma, & Do, 2011). Detection was executed under UV 254 nm. Quantitative analysis was done using *scanner densitometer win CATS Camag* equipped with UV-Vis 254 nm.

## RESULTS AND DISCUSION

a. Enzymatic acylation of *p*-aminophenol with ethyl acetate as acyl donor

The acylation of PAP was carried out in ethyl acetate as the solvent, wherein the ethyl also acts as acyl donor for the formation of paracetamol. CaLB has been reported to be able to maintain its activity in ethyl acetate as solvent (Van Rantwijk, 2004). The system is strictly maintained to keep it free of traces of water, to avoid re-hydrolysis reaction of the formed amide. For this purpose, molecular sieves of 4A was used to absorb water traces and keeping the system dry.

In the first work, the study on CaLB activity to catalyze the acetylation of PAF was first investigated by looking at the effect of temperature and system acidity on the yield of paracetamol produced. The result is shown in table 1 and figure 1.

Table 1.
The effect of temperature and pH on paracetamol yield in the CaLB-catalyzed acylation of PAP using ethyl acetate

| Treatment (pH adjustment) | pH | Temperature (oC) | Product | |
|---|---|---|---|---|
| | | | Paracetamol (mg) | Yield (%) |
| No adjustment | 6 | 50 | 2.3 | 4.1 |
| | | 60 | 6.5 | 11.8 |
| | | 70 | 9.4 | 17.1 |
| Pyridine | 7 | 50 | 2 | 3.6 |
| | | 60 | 6.9 | 12.6 |
| | | 70 | 10.4 | 18.8 |
| Triethylamine | 9 | 50 | 2.5 | 4.5 |
| | | 60 | 5.5 | 10 |
| | | 70 | 10.9 | 19.8 |
| Acetic aicid | 3 | 50 | 5.6 | 10.2 |
| | | 60 | 11.5 | 20.8 |
| | | 70 | 19.2 | 34.9 |

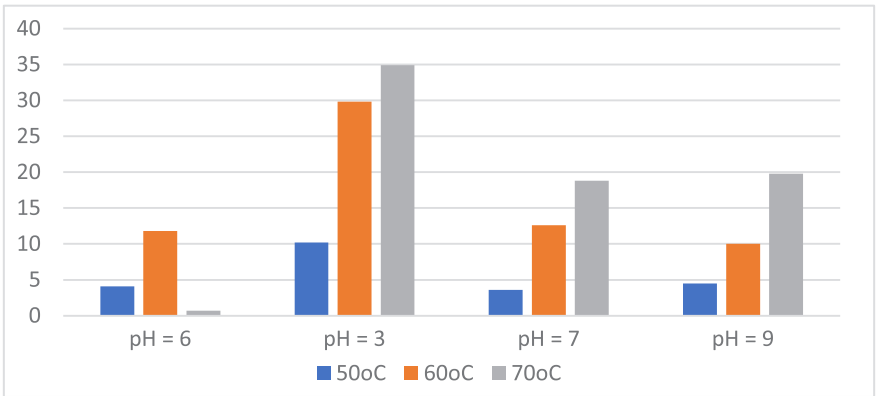


Figure 4
The effect of temperature and pH on paracetamol yield in the CaLB-catalyzed acylation of PAP using ethyl acetate
Source: Lande (2018)

The effect of temperature was clearly observed, as higher temperature resulting in higher yield of paracetamol. Higher temperature increases the effectiveness of intermolecular collisions, provides greater possibility of substrates to bind the catalysts and permit higher yields. On the other hand, rising temperature in certain temperature range, might give effect on the flexibility of the enzymes conformation to be more receptive to the substrates. Although the optimum temperature for CaLB is known as 37- 40$^{o}$C, here CaLB proved its ability to maintain the activity at elevated temperature (Anderson, et. al., 1998). It was shown that the reaction at 70$^{o}$C it gave higher yield (17.1%; 18.8%; 19.8%; and 34.9% for the reaction in pH of 6, 7, 9, and 3 respectively).

It has been reported that CaLB's activity in water was influenced by the pH of the medium, where the optimum pH for immobilized CaLB would be around 7.0 - 8.0 (Djossou et.al, 2016). In this study, we tried to investigate CaLB activity to catalyze PAP acylation in the water-free system, in the condition of slightly base and slightly acid by adding pyridine, triethyl amine, or acetic acid to adjust the pH to 7, 9, and 3. The reaction medium without any adjustment eventually already somewhat acid, with the pH of 6. This might be caused by the chemical property of ethyl acetate which is a weak acid. The result showed that increasing medium alkalinity by addition of pyridine or triethylamine, was slightly increased the yield of-paracetamol.

It is interesting to see the fact that CaLB-catalyzed acylation of PAP gave significantly high results when the reaction condition was acid, as it is known that CaLB's optimum pH in water medium is neutral to alkaline (Anderson, 1998). So here we suspected that a reaction other than that of lipase also occured. Further investigation to check the possibility of non-enzymatic reaction involvement, was done by executing a reaction between PAP and ethyl acetate in the presence of acetic acid (pH 4) at 60$^{o}$C, without CaLB. The result revealed that after 5 hours of reaction, paracetamol was found as the acylation product with the yield 9.9%. This proved that non-enzymatic reaction indeed also occurred, and acetic acid acted as catalyst to facilitate the acylation. However, it is interesting to know that the combination of enzymatic and non-enzymatic acylation might increase the reaction rate, resulting a higher amount of paracetamol produced.

Therefore, the work was continued by performing PAP acylation in a condition where CaLB was combined with acetic acid to catalyze the reaction. The reaction was held at 70$^{o}$C, with addition of acetic acid to pH 3, and ethyl acetate was used as both reaction medium and acyl donor. The paracetamol produced was monitored during the time of reaction for 120 hours. The results are shown in the table 2 below:

Table 2
The progress of paracetamol produced in 120 h

| Time (h) | pH | Product | | |
|---|---|---|---|---|
| | | Paracetamol (mg) | Paracetamol (mg/mL) | Conversion (%) |
| 2 | 4 | 5.6 | 0.12 | 2.1 |
| 4 | 4 | 12.3 | 0.25 | 4.5 |
| 6 | 5 | 13.2 | 0.27 | 4.8 |
| 24 | 5 | 56.2 | 1.22 | 20.5 |
| 48 | 5 | 90.7 | 2.00 | 33.0 |
| 72 | 5 | 106.6 | 2.37 | 38.8 |
| 96 | 5 | 110.2 | 2.57 | 39.8 |
| 120 | 5 | 110.6 | 2.71 | 40.0 |

(Lande, 2018)

The success of employing CaLB as catalyst in the acylation of PAF particularly in the amide bond forming, would relate to the form of CaLB active site and the form of the substrate. Pleiss (1998) revealed the active site of CaLB is anchored at the bottom of the funnel shape binding pocket (Pleiss, 1998; Ema, 2004). The shape of the binding site therefore requires suitable substrate molecular structure to be fit there. Study by Kazlauskas (1997) concluded that various aromatic amines that fit with the binding site of CaLB, are having nitrogen atom with a distance of one carbon atom to the aromatic group (Hoff, 1996; Kazlauskas, 1997). Hence, the nitrogen atom in PAP which is directly attached to the aromatic ring might not be a good substrate for CaLB, resulting a low yield of paracetamol produced.

b. CaLB as a catalyst for oxidative amidation in aqueous medium.

Another breakthrough to use lipase for the acylation of aromatic amines was done by Zhang's, through the oxidative amidation reaction (Zhang, et. al, 2017). According to the proposed mechanism, it is known that the role of CaLB in the course of reaction is to catalyze the enolization reaction of acetylacetone, as well as the perhydrolysis of ethyl acetate to form peracid. It is predicted that the binding site of CaLB involved in this reaction is more suitable for substrates in the form of ethyl acetate or acetyl acetone. Hence, it is expected that the result from this oxidation amidation reaction will be better than the other work on enzymatic acylation with CaLB. The suspected mechanism reaction for oxidative amidation of PAP, based on mechanism proposed by Zhang et.al (2017), is shown in figure 5, below.

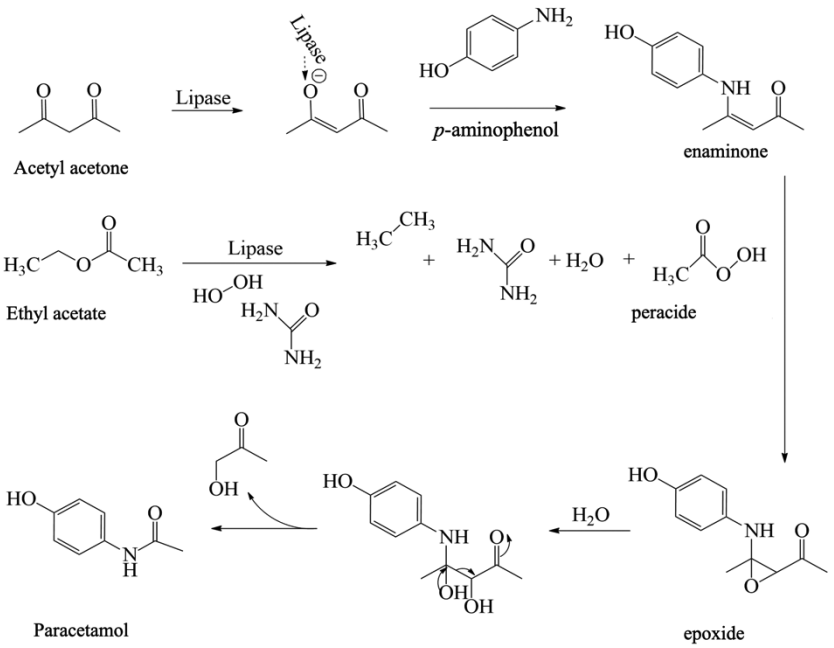


Figure 5
Proposed-reaction mechanism for the oxidative amidation in the synthesis of paracetamol, based on Zhang's reaction for aniline
Source: Indriyawati (2019)

The overall mechanism for this strategy is therefore involving several reactions, a) enzymatic acylation of PAP by acetyl acetone, b) enzymatic formation of peracid from ethyl acetate, c) oxidation of enaminon to form epoxide, d) hydrolysis of epoxide, and e) rearrangement to liberate *hydroxyacetone* and amide. There are two steps that are facilitated

by the work of CaLB as catalyst, and three others are non-enzymatic chemical reactions. Accordingly, final result of this strategy will be affected by both enzymatic and non-enzymatic reactions, where every component in the reaction medium might influence both enzymatic and non-enzymatic reaction.

In this study the oxidative amidation was applied to PAP as the amine-bearing compound, and ethyl acetate as the source to form peracide, acetylacetone for acyl donor, and lipase together with UHP as oxidizing agent to for peracide. Experiments were done with variation on the UHP amount, medium pH and time of reaction. The results are shown in table 3.

Table 3
Performance of CaLB in the oxidative amidation of p-aminophenol

| Treatment | volume | PH | Reaction Time (h) | Paracetamol Product (mg/mL) | yield (%) |
|---|---|---|---|---|---|
| UHP (mmol) | 1.2 | 4 | 1 | 11.28 | 18.7 |
| | 1.4 | 4 | 1 | 14.91 | 24.7 |
| | 1.6 | 4 | 1 | 16.29 | 27 |
| Pyridine (µL) | 0 | 4 | 1 | 13.64 | 22.6 |
| | 60 | 5 | 1 | 19.70 | 32.6 |
| | 100 | 6 | 1 | 34.48 | 57.1 |
| | 140 | 7 | 1 | 29.39 | 48.7 |
| | 200 | 8 | 1 | 30.03 | 49.7 |
| | 300 | 9 | 1 | 12.47 | 20.6 |
| | 600 | 10 | 1 | 6.57 | 10.9 |
| Pyridine (µL) | 100 | 6 | 0.5 | 4.15 | 6.9 |
| | 100 | 6 | 1 | 32.79 | 54.3 |
| | 100 | 6 | 2 | 10.25 | 17 |

Source: Indriyawati (2019)

Results in table 3 revealed that the best yield of paracetamol was around 50%, with the highest of 57.1%. This is remarkably higher compared to CaLB's work on the amine acylation in organic solvent. The first treatment with variation on the amount of UHP showed that increasing the amount of UHP has increased the paracetamol produced. Urea Hydrogen Peroxide (UHP) is an oxidizing agent used to generate peracid from ethyl acetate under the catalyst of CaLB. Since peracid will be used to oxidize the enaminon, increasing the amount of peracid caused increase of the product, too. Study on the effect of pH has shown that rising the pH from 3 to 6 increased the yield of product, and at higher pH the yield was decreased, giving pH 6 as the optimum condition. It is known that lipase prefer a neutral to alkaline medium condition for its best performance, hence it is presumed that the other non-enzymatic reaction (hydrolysis dan re-arrangement) that work better in acidic condition, played important role in increasing the reaction rate.

An advantage of PAP acylation through oxidative amidation was the short reaction duration compared to the other reaction in aqueous medium. The results showed that good yield of product have been obtained within 1 hour of reaction. It is also seen that the yield decreased after 2 hours of reaction, revealing that some of the amides were further hydrolyzed by water in the presence of the enzyme. Here it has been proven that the competition between acylation and hydrolysis reaction become the drawback of this enzymatic strategy which is done in aqueous medium. So, more research is still to be done, in order to find the most suitable reaction

conditions to supress the hydrolysis of the paracetamol produced.

## CONCLUSION

Enzymatic acetylation of *p*-aminophenol by employing CaLB as the catalyst, both in water-free organic solvent and in aqueous medium has been proven to give paracetamol as final product. Enzymatic acetylation in dry ethyl acetate resulting in 40 % yield of product, and enzymatic oxidative amidation in water with acetyl acetone as acyl donor giving 57% yield of paracetamol. Enzymatic oxidative amidation appears as a promising method to be developed. Selection of suitable lipases for primary aromatic amine substrates needs to be done in order to obtain optimal acetylation results.

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