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Study of Enzymatic Hydrolysis Reaction l-Menthyl acetate using Lipase Enzyme from *Candida antarctica* Recombined *Aspergillus oryzae* by In Vitro and In Silico

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Abstract

Research about the hydrolysis of l-menthyl acetate catalyzed by lipase enzyme from Candida antarctica recombined Aspergillus oryzae has been observed by in vitro and in silico. The reaction by in vitro method performed with variations time and solvent. The result obtained that lipase enzyme from Candida antarctica recombined Aspergillus oryzae were able to catalyze the hydrolysis reaction of l-menthyl acetate to produce l-menthol. The best result is observed at 16 hours with ethanol solvent, which give % conversion about 13,76%. The research followed by in silico using the docking method, observed that mechanism between amino acid residues to macromolecules as well as the influence of solvents on such interactions. The residues that involved in the reaction are Glu, Thr, Asp, Gln, Ser, His. The ethanol solvent shows a good interaction with macromolecules by forming hydrogen bonds.

Keywords*—l-menthyl acetate, l-menthol, lipase enzyme from Candida antarctica recombined Aspergillus oryzae, docking*

1. INTRODUCTION

Menthol is a monoterpen compound that widely used not only in pharmacy, but also cosmetic, factory, and food industry. Menthol gives a cooling sensation and has a fresh fragrant. Nature menthol could be extracted from its natural plant like *Menthe piperita* dan *Menthe arvensis* [1]. In another research, menthol was synthesized by hydrogenation thymol, unfortunately this reaction give 8 isomers menthol [2]. Based on study of Brookes, 1987 they found a new method to form l-menthol by stereospecific hydrolysis use biocatalyst [3].

Nowadays reaction involving biocatalyst become a concern for scientist due the using of biocatalyst fulfill the green chemistry concept [4]. Biocatalyst is more selective for chemoselectivity, regioselectivity, stereoselectivity, and enantioselectivity. No need an extreme condition to perform the reaction [5]. In this research lipase enzyme from *Candida antarctica* lipase B (CALB) used as a biocatalyst for hydrolysis l-menthyl acetate. CALB has been used as a catalyst for various reactions such as ester, tioeseter, peptide, epokside, halide alkyl reaction. Moreover, CALB has a high enantioselectivity and shows a good stability in organic solvent [6]. Lipase enzyme could release alcohol compound through hydrolysis reaction [7]. CALB has active site Ser, His, and Asp/Glu. It gives an access to the substrate [6].

Based on Beviliqua, 2016 there is an addition of Na_2CO_3 solution acted as an aqueous phase. It provides water for the hydrolysis reaction and increase the conversion of substrate [8]. In the previous study hydrolysis of l-menthyl acetate still reported by *in vitro* method and the

reaction mechanism wasn't perform with *in silico* approach. Hence the research need to be tested by *in silico* to know the interaction of active site of enzyme and compound.

Docking process predict the ligand conformation and orientation (posing) with target binding site. Docking aims to obtain an appropriate prediction of complex structure at the equilibrium condition [9]. After docking is complete, docking analysis is shown to know docking poses and energy values [10]. This study was conducted to know the factors involved in the enzymatic reaction of hydrolysis l-menthyl acetate and the interaction between active site and its macromolecule.

2. MATERIALS AND METHODS

The material used for *in vitro* method is lipase enzyme from *Candida antarctica* immobead 150 recombined *Aspergillus oryzae*, l-menthyl acetate were purchased from Sigma-Aldrich, ethanol, n-hexane, solution of Na_2CO_3. The material used for in silico experiment are macromlekul CALB 3D from *pdb.org* with code 4ZVJ, ligand l-menthyl acetate, ethanol, n-hexane and l-menthol from *Chemspider Search* and *Share Chemistry* (*www.chemspider.com*).

Hydrolysis l-menthyl acetate using lipase enzyme from *Candida antarctica* B by in vitro method

The hydrolysis reaction were perfomed in ethanol 17 mL, with 5 mL of l-menthyl acetate, 3 mL of Na_2CO_3 solution, and 0,15 g of lipase enzyme from *Candida antarctica* B. This reaction was shaken at 200 rpm and temperature at 50^{o}C. This reaction was taken every 8, 16, and 24 hours. The same reaction is carried out on the n-hexane solvent.

Analysis method

Starting material l-menthyl acetate was analyzed first using gas chromatography spectra mass and FT-IR. The product of hydrolysis reaction was analyzed using GC-MS and FT-IR as well. The product was injected in CG-MS shimadzu QP2010S equipped with kapiler coloumn of restrex rtx-5. The injector was set at 310^{o}C, the carrier gas was He, the initial coloumn was 60 – 215^{o}C at 50 mL/minutes. From this analyzing we were able to determine the percentage area of the product.

Data analysis

Based on profile analysis through GC-MS characterization, we were able to know the percentage of conversion of the product through this formula:

$$\%\ Conversion = \frac{\%\ \text{initial substrate} - \%\text{final substrate}}{\%\ \text{initial substrate}} \times 100\%$$

In silico method of l-menthyl acetate compound and macromolecule CALB
Ligand Preparation and macromolecule

The various ligand l-menthyl acetate, ethanol, and l-menthol was obtained from *chemspider.com* (.mol). Continued by ligand optimization using hyperchem software to obtain the stabilization of ligand. The optimization ligand must be converted to .pdb existence using open babel gui, then observed by AutoDockTools and iGEMDOCK tools. For macromolecule CALB begin with downloading that compound through *pdb.org* with access code 4ZVJ. Then optimize this macromolecule using *Discovery Studio Visualizer 2016* to remove water and

ligand that linked in active site of macromolecule. Macromolecule saved in .pdb existence viewed by AutoDockTools and iGEMDOCK tools.

Docking process

The interaction between macromolecule l-menthyl acetate and active site of enzyme lipase CALB shown by running trough AutoDockTools-1.5.6 software begin with the preparation of ligand coordinates and macromolecules, continued by running autogrid to determine the specified position and size of the grid box. Then running grid using the program cmd. On docking multiligands, l-menthyl acetate ligand and l-mentol compounds interact with the macromolecule CALB using iGEMDOCK. In addition the ethanol and n-hexane solvents would act as a ligand that interacts with the substrate and CALB macromolecules. From the iGEMDOCK program, the results were obtained as best poses, analysis results, and interaction tables. From the analysis can be known the value of ΔG°, so it can be used to determine the value of Kd. From that result can be known the strength of interaction between macromolecules with ligands.

3. RESULT

The result analysis of in vitro test
Analysis of substrate (starting material l-menthyl acetate)

Substrate of l-menthyl acetate was firstly analyzed using the KGSM instrument and FT-IR to know the purity of substrate that will be used. Characterization results through KG-SM data chromatograms arranged in **Fig. 1**.

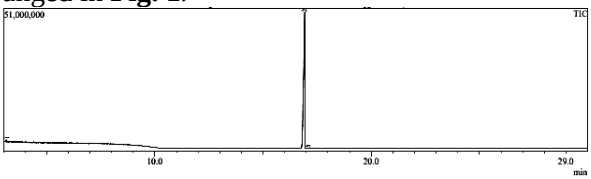

FIGURE 1. Chromatogram GC-MS of substrate l-menthyl acetate

Based on the result of GC-MS can be seen that the substrate of l-menthyl acetate shows a good purities that is 99,27%. Then the analysis using the FT-IR shown in **Fig. 2**. Analysis using FT-IR aims to know the group contained in the substrate of l-menthyl acetate

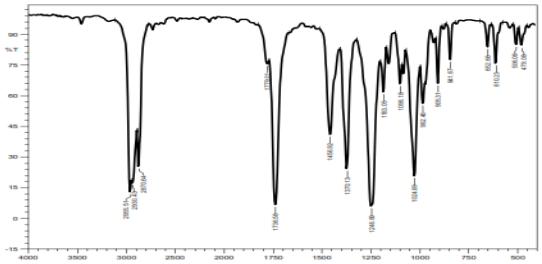

FIGURE 2. Spectra FT-IR of substrate l-menthyl acetate

This spectra allows to know the absorption at the wave number 2955.51cm^{-1} which indicates the existence -CH sp3. Then there is the absorption at 1736.58 cm^{-1} shows the presence of C=O ester group. Also found the absorption at 1246.6 cm^{-1} indicates the presence of C-O esters.

Effect of time course and solvent for hydrolysis reaction of l-menthyl acetate

The effect of the time reaction of hydrolysis of l-menthyl acetate was observed for 24 hours. At the 8th, 16th, and 24th hours the products were taken and analyzed with GC-MS instrumentation. Results analysis through GC-MS instrumentation aims to know product l-menthol that has been formed.

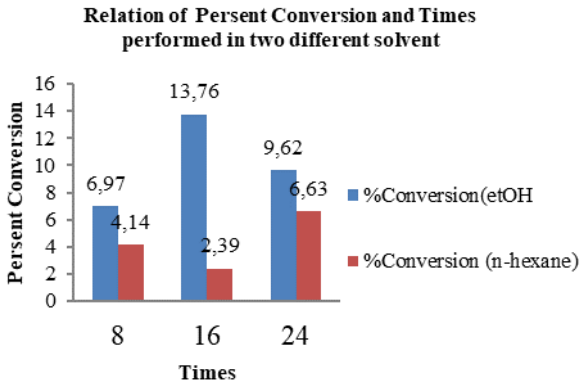

FIGURE 3. Graph of Relation of Percent Conversion and Times performed in two different solvent

In the hydrolysis of l-menthyl acetate, the solvent type plays an important role in the ongoing reaction. Because the reaction involves the lipase enzyme it must be known that the lipase is more active when reacted in an organic water-solvent compared to homogeneous solutions. n-hexane solvent is an organic solvent non polar with a polarity of 1.89 (εr). Meanwhile the polarity of ethanol is 24.3 (εr). Based on the **Fig. 3** it can be seen that the best conversion is obtained on ethanol solvent in which l-mentil acetate has been converted to 13.76% at 16h.

Then product of hydrolysis was analyzed using FT-IR aims to know the group contained in the substrate of l-menthyl acetate.

Based on **Fig. 4** it shows an absorption in the wave number 3631.1 cm-1 supposedly contains the -OH group. The -OH group on the hydrolysis reaction using ethanol solvent has a higher intensity than the n-hexane solvent. The intensity of l-menthyl acetate groups decreased with increasing polarity of solvent

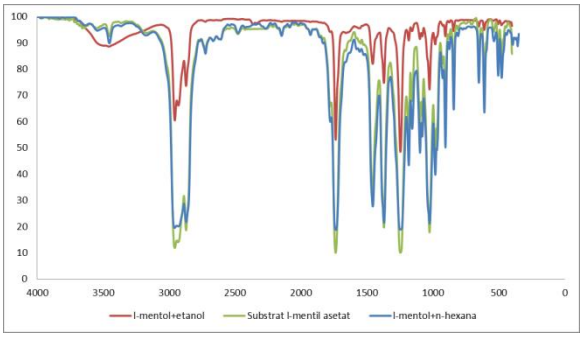

FIGURE 4. Overlay of FT-IR spectra hydrolysis product using n-hexane and ethanol solvent at 24 hour

The result analysis of in silico test

Interaction between ligand l-menthyl acetate with macromolecules CALB was analyzed by docking method and reported in 2D through *Discovery Studio Visualizer 2016*.

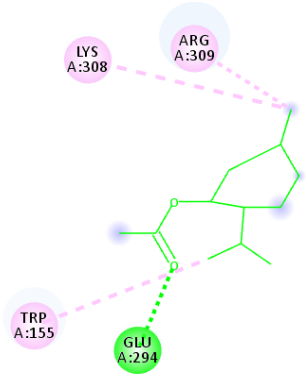

FIGURE 5. Interaction between ligand of l-menthyl acetate with macromolecule CALB

In **fig. 5** shown the interaction between ligand of l-menthyl acetate with macromolecule CALB. Amino acid residue Glu 294 linked to ligands l- menthyl acetate through hydrogen bonding. For interaction occurs in multiligand, l-menthyl acetate and l-menthol acted as a ligand that interact with macromecule CALB shows in **fig. 6**

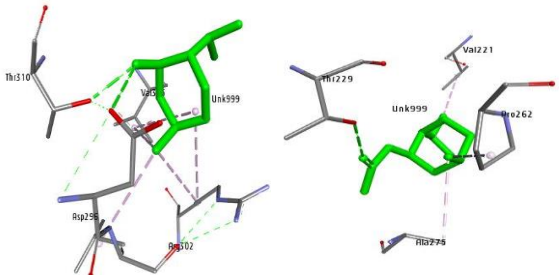

FIGURE 6. Interaction between ligand of l-menthyl acetate and l-menthol with macromolecule CALB

Fig. 6 shows the interaction between ligand l-menthyl acetate with amino acid residue Thr229 through a hydrogen bond. In the other side the interaction of macromolecules and ligand l-menthol takes place between the amino acid residues Asp296 and Thr130 through hydrogen bond as well.

TABLE 1. Energy data of docked product ligand of l-menthyl acetate and l-menthol with macromolecule.

Ligand	Energy Total	Kd
l-menthyl acetate	-57.986	1.097
l-menthol	-62.4603	1.102

Based on the docking results, the most stable conformation was selected. The stability of complex compounds expressed by the constanta dissociation, Kd. Kd of l-menthyl acetate ligand is 1.097, whereas Kd of l-menthol ligand is 1,102 (**Table 1**). The Kd value of the l-menthol ligand is bigger than l-menthyl acetate ligand. Therefore l-mentol is more easily removed from the active site of the enzyme and binds to another ligand compared with l-mentil acetate which has a lower Kd value. To determine the effect of solvent on the reaction of hydrolysis l-menthyl acetate then tested in silico. Solvent used is a non-polar organic solvent (n-hexane) and a polar solvent (ethanol).

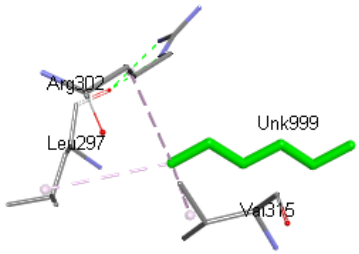

FIGURE 7. Interaction between ligand of n-hexane and macromolecule CALB

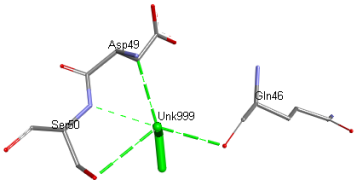

Figure 8. Interaction between ligand of ethanol and macromolecule CALB

Based on the results of in silico test shown in **Fig. 7**, the interaction between amino acid residues and n-hexane is not shows the presence of hydrogen bonds. On the other hand the **Fig. 8** indicate an interaction between ethanol and acid amino residues from macromolecules CALB. Interaction occurs between acid amino residues is known to give the catalytic site of the enzyme Gln46, Ser50, and Asp49 linked to macromolecule CALB.

Overview Analysis of the In-vitro and In-silico Mechanism of Hydrolysis Reaction

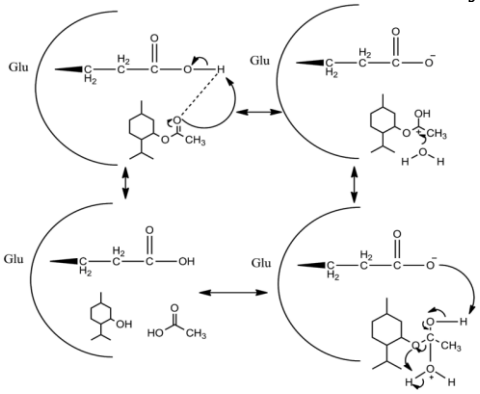

FIGURE 9. The mechanism of the amino acid residue Glu with l-menthyl acetate

The reaction mechanism of **Fig. 9** refers to the results of the *in silico* test on **Fig. 5**. There is hydrogen interactions between ligands and amino acid residues. Though bond hydrogen classified as a weak bond however hydrogen bonds play an important role for studying molecular interaction design. Hydrogen bonds are formed from electrostatic interactions between H atoms which attached to atoms with high electronegativity and the other atoms having other high electronegativity as well. Hydrogen bonding occurs intermolecularly.

The amino acid residue of Glu294 contains the R group of $CH_3CH_2COO^-$ is acidic and has negative charged. The H atom attached to the group will bind to the group of O atoms on the acetic acid by the hydrogen bond. Then the C atom of acetate undergoes a carbocation phase so it requires an O atom of water to be neutral. Then obtain the l-mentol and acetate compounds of the hydrolysis reaction as the Glu residue is reformed.

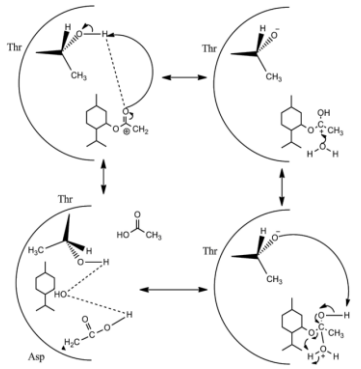

FIGURE 10. The mechanism of the amino acid residue with l-menthyl acetate and l-menthol

The reaction occur in **Fig. 10** begins with the binding of the O atoms of l-menthyl acetate to the H atom of the amino acid residue Thr229 through the hydrogen bond. The amino acid residue Thr 229 is an aliphatic hydroxy amino acid. With a noncharged of polar R group. Then l-menthol and acetic acid will be released by re-formation of amino acid residue Thr 229. Ligand l-menthol binds to the amino acid residues Thr and Asp through hydrogen bonds. O atom of l-menthol binds to the H atom of Thr and Asp. This mechanism was shown through **Fig. 10**

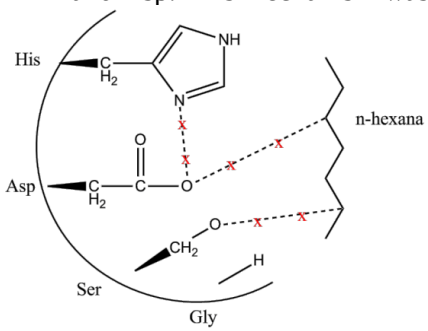

FIGURE 11. The mechanism of the amino acid residue and n-hexane

The interaction between solvent n-hexane and macromolecule represented by **Fig. 11**, shows no hydrogen bonds formed between the amino acid residues and the substrate. This matter probably due n-hexane is a non-polar organic solvent which is hydrophobic. Meanwhile the substrate is more soluble in hydrophilic solvent. So the substrate is not easily soluble in n-hexane solvent. n-hexane solvent is difficult to interact with the catalytic site of enzyme. So the enzyme can not catalyze the hydrolysis reaction optimally.

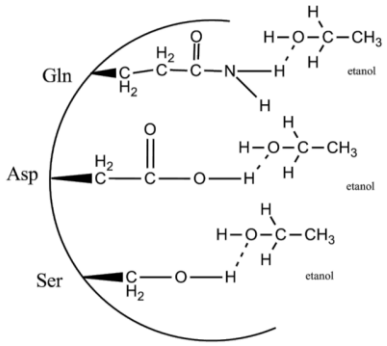

FIGURE 12. The mechanism of the amino acid residue and ethanol

While from **Fig. 12** shows the presence of interaction between the amino acid residues to the ethanol solvent through hydrogen bond. Amino acid residues Gln46, Ser50, and Asp49 give the catalytic site of enzymes. The amino acid residue Gln46 (Glutamine) contain uncharged polar

R-group, NH_2. H atom of NH_2 bind to the O atom of ethanol. While the Asp group has a negative charged R group, $CH_2CH_2COO^-$. H atom attach to the O atom from ethanol to form an O-H hydrogen bond. Same goes in Ser where the H atom of the -OH group will bind to the O atoms of ethanol form a hydrogen bond. Based on the mechanisms in **Fig. 11** can be observed that ethanol solvent plays an important role in the hydrolysis of l-menthyl acetate. Accordance to the by Sharma [11], hydrophilic solvent has good interaction with enzyme molecules. Enzyme shows a good rate because the polar solvent can form the environment hydrophilic agents that support the hydrolysis reaction.

4. CONCLUSION

Based on the research that has been done, it can be concluded that:

1. The highest conversion percentage of hydrolysis of l-menthyl acetate to l-mentol was produced at 16 hours with ethanol solvent is 13.76%
2. Based on the results of in silico test showed that there is an interaction between the amino acid residues Glu, Thr, Asp, Gln, and Ser against macromolecules.
3. Through the in-silico test, ethanol solvent gives a good interaction with macromolecules through hydrogen bonding

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