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Hydrolysis of Waste Cooking Oil Using *Rhizopus oryzae* to Produce Free Fatty Acids

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ABSTRACT

Waste cooking oil is waste produced from palm oil after it has been heated and fried at high temperatures, and it can pollute the environment. One effort to reuse waste cooking oil is by a fermentation process that produces free fatty acids with the help of Rhizopus oryzae as the biocatalyst. Variations in initial substrate concentration ranged from 10 g/L to 70 g/L, followed by varied types of nitrogen sources, namely malt extract, beef extract, $(NH_2)2CO$, NH_4Cl, and $(NH_4)2SO_4$ at a concentration of 70 g/L to determine free fatty acid concentration. Fermentation was carried out for 7 days. The analysis carried out included the concentration of free fatty acids, biomass, and $Y_{P/X}$ value. The optimum initial used cooking oil substrate was obtained at a concentration of 30 g/L with a YP/X value of 13.63%, a free fatty acid concentration of 2.13 g/L and a dry cell weight of 15.48 g/L. The best nitrogen source is beef extract with a $Y_{p/x}$ value of 11.78%, a free fatty acid concentration of 2.02 g/L and a dry cell weight of 17.0 g/L.

INTRODUCTION

Waste cooking oil is a palm coconut oil that has undergone a series of heating and frying processes at high temperatures, especially for food processing [1]. According to the International Council on Clean Transportation (ICCT) survey of 2018, Indonesia has the potential to produce 1.638 million L of crude oil per year [2]. Waste cooking oil which is discharged into the environment can lead to environmental pollution. Waste cooking oil can be used to produce fatty acids and glycerol through chemical and enzymatic hydrolysis processes. Fatty acids are organic acids whose use is quite widespread, especially as a component of body care products, medicines, and food. The advantage of the enzymatic hydrolysis process is it does not involve concentrated acid substances, so it is more environmentally friendly [3].

The waste cooking oil to be used as the raw material of the enzymatic hydrolysis process must undergo a pre-treatment process first to reduce the content of heavy metals. One method that can reduce the content of metals in waste cooking oil is by the adsorption method using active carbon adsorbents.

Enzymatic hydrolysis of waste cooking oil to produce fatty acids and glycerol can use the enzyme lipase biocatalyst. One of the enzyme-producing microorganisms is *Rhizopus oryzae*. *Rhizopus oryzae* is a type of filamentary fungus that can utilize some types of simple substrates such as glucose as well as complex ones such as amylum and triglycerides as one of its nutrient sources.

Sahara et al research reports that in solid fermentation results were obtained with the addition 7 mL of the inoculum of *Rhizopus oryzae* with the best fermenting time of 3 days, free fatty acids were obtained through the process of solidification of oil of 35.94% w/w [6].

The activity of the enzyme lipase derived from microorganisms in addition to being affected by operating conditions is also influenced by the type and composition of the fermentation medium [7]. The fermentation medium should contain the nutrient components of macronutrients and micro-nutrients necessary for cell growth, metabolite formation and sufficient energy for cell biosynthesis and maintenance. The macronutrients that are needed in large quantities and are a limiting substrate in the

fermentation process are nitrogen and carbon.

Nitrogen can be given in the form of organic nitrogen and inorganic nitrogen. Commonly used sources of organic nitrogen are peptone, beef extract, malt extract and yeast extract. The most common sources of inorganic nitrogen are Urea, $(NH_4)_2SO_4$, and NH_4Cl. Organic nitrogen is more expensive than inorganic nitrogen but provides higher conditions for microbial growth compared to inorganic nitrogen. *Rhizopus oryzae* is a filamentary fungus that can take advantage of various sources of nutrients, so it does not exclude the possibility of using inorganic nitrogen in its metabolism. The selection of the optimal type and quantity of inorganic nitrogen sources is expected to produce the best fermentation performance [8].

Nitrogen in cells is a component of cell protein and nucleic acid. Nitrogen is also used by microorganisms as energy for biosynthesis and cell maintenance [9]. Sources of nitrogen used as a source of energy contain micronutrients such as elements of metal, minerals, or vitamins. Metal elements such as Mg^{2+} and Fe^{2+} are co-factors, chemical compounds in non-protein groups that can determine catalytic activity. Enzyme activators can increase enzyme catalytic activity by binding enzymes to the substrate, which will induce the formation of enzyme binding sites within the microorganism. In addition to being enzyme activators, minerals and vitamins are also often found in organic nitrogen used as growth substances for organisms [9].

Carbon will be converted and used for cell biosynthesis and product biosynthesis during the fermentation process. Carbon is commonly obtained from glucose, lactose, and polysaccharides. The rate of microbial growth is affected by the kind and concentration of carbon sources. Each microbe has a specific ability to utilize carbon sources. If the carbon source concentration is too high, it will be toxic, but if it is too low, the activity of microorganisms in the fermentation process will be less than optimal. Carbon, nitrogen, and other nutrients will be transformed into biomass, metabolite products, energy, and CO^2 during the fermentation process [8].

In this study, triglycerides are used as a source of carbon for the enzymatic hydrolysis process using *Rhizopus oryzae* to produce biomass products and metabolite products such as fatty acids and glycerol. The research was conducted on various variations in the concentration of waste cooking oil and variation in the types of organic and inorganic nitrogen sources.

METHODS

The following equipment was used: Erlenmeyer 250 mL, Whatman No. 1, magnetic stirrer, glass beaker 100 mL, reaction tube, incubator shaker, and autoclave.

The following substances were used: 500 mL waste cooking oil, culture *Rhizopus oryzae,* potato dextrose agar (PDA) powder, activated carbon, peptone, sodium nitrate, potassium dihydrogen phosphate, $MgSO_4$, glucose, beef extract, malt extracts, urea, $(NH_4)_2SO_4$, NH_4Cl, ethanol 96%, KOH 0,1 N, polyvinyl alcohol 0.3 g.

Prior to the use of the fermentation process, the waste cooking oil is first pre-treatment by adsorption using an active carbon adsorbent with a composition of 10% of the weight of the oil. The PDA medium for preparing the microbial stock is made from 4 g of PDA powder and 1 g of bacteriological so that it is dissolved with 100 ml of aquades and then sterilized. The inoculum media is made with the composition of peptone 70 g/L, sodium nitrate 1 g/L, potassium dihydrogen phosphate 1 g/L, magnesium sulphate 0,5 g/L, and glucose 10 g/L.

The fermentation process is carried out on a production medium volume of 100 mL with the composition of the materials such as peptone 70 g/L, sodium nitrate 1 g/L, potassium dihydrogen phosphate 1 g/L, and magnesium sulphate 0,5 g/l. To determine the optimal substrate concentration, substrates added with variations in the initial concentration of olive oil in the range of 10 g/L to 70 g/L. Variation of nitrogen sources is carried out by replacing peptone as an organic nitrogen source in the form of beef extract, malt extracts, urea, as well as inorganic source of Nitrogen namely $(NH_4)_2SO_4$, NH_4Cl with constant composition of other materials. The inoculum added to each fermentation process is 15 mL. The fermentation process lasts for 7 days at a temperature of 32^oC and an agitation rate of 150 rpm.

The product was analysed using the SNI 3714:2013 titration method to obtain

free fatty acid concentrations and the gravimetric method to obtain dry cell weight to produce biomass concentration and the product yield value against biomass ($Y_{P/X}$).

RESULT AND DISCUSSION

Effects of Variation in Initial Substrate Concentration

Waste cooking oil as a substrate in the medium causes *Rhizopus oryzae* to produce the enzyme lipase, resulting in free fatty acid products. This study's findings demonstrate the effect of the concentration of the initial waste cooking oil on the change of concentrations of free fatty acids (FFA) and biomass products, as shown in **Figure 1** and **Figure 2**.

Figure 1 and **Figure 2** shows that the concentration of free fatty acids (g/L) and

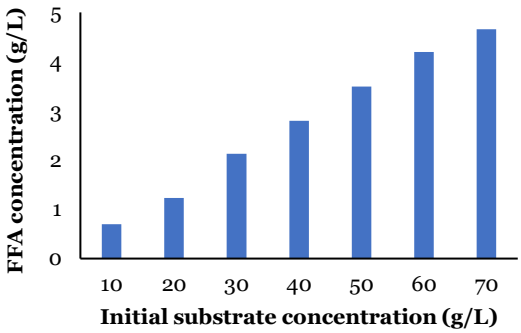

Figure 1. The Effects of Initial Waste Cooking Oil Concentration on the Concentration of Free Fatty Acids

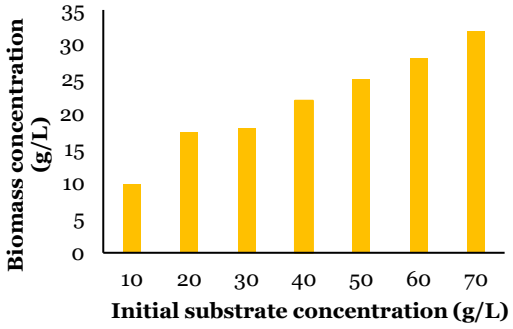

Figure 2. The Effects of Initial Waste Cooking Oil Concentration on The Concentration of Biomass

biomass (g/L) increases as the initial concentrations of waste cooking oil as substrate increase. This can be explained by the fact that *Rhizopus oryzae* produces an enzyme lipase that can break the ester bond on the triglycerides of waste cooking oil into fatty acids. The highest concentration of free fatty acids was achieved at 4.66 g/L at a 70 g/L substrate concentration, while the lowest level of free acid concentration was obtained at 0.7 g/L at a 10 g/L substrate concentration. The study conducted by Wulan et al, obtained a concentration of fatty acids of 17.4 g/L [11]. It can be said that using enzymes to produce concentrations of fatty acids is better than using fungi, but the deficiencies when using the enzyme are relatively more expensive and if seen the conversion results are not much different from using the fungus.

The results of the research showed that the pattern of product formation on the fermentation of waste cooking oil is classified as a growth-associated product. The rate of product formation will be directly relative to growth. *Rhizopus oryzae* produces the enzyme lipase, which is an intracellular and extra-cellular enzyme. The extracellular lipase enzyme is excreted from within the *Rhizopus oryzae* cell wall and then distributed onto the fermentation medium so that it can react with organic compounds. Triglycerides in waste cooking oil are complex organic compounds, then extra-cellular enzymes will break down triglycerides into simple compounds, then will be absorbed by the cells as a nutrient source of carbon in growth.

Product yield relative to biomass ($Y_{P/X}$) on various concentrations of waste cooking oil substrate is presented in **Table 1**.

Table 1. $Y_{p/x}$ Value on Various Initial Substrate Concentration

Initial substrate concentration (g/L)	$Y_{P/X}$ (%)
10	6,94
20	8,55
30	13,64
40	12,68
50	13,89
60	13,92
70	14,55

Table 1 demonstrates that $Y_{P/X}$ values increase significantly in the initial substrate concentration range of 10 - 30 g/L (from 6.94% to 13.64%), but YP/X values tend to be steady in the initial substrate concentration range of 40 - 70 g/L (from 12.68% to 14.55%). That is, utilizing an initial substrate concentration of 40 - 70 g/L in the fermentation process of waste

cooking oil has no effect on the $Y_{P/X}$ value. The study's findings indicate that 30 g/L is the optimum concentration in the initial waste cooking oil substrates that *Rhizopus oryzae* can metabolize to convert triglycerides to fatty acids.

Rhizopus oryzae can use a maximum concentration of 200 g/L of glucose as a substrate [13], but in this study utilizing waste cooking oil as a substrate, it was only able to metabolize at a concentration of 30 g/L. This is possible since the carbon chain in waste cooking oil is more complex and has stronger bonds than glucose.

Effects of Variation in Nitrogen Source

The type of nitrogen source used can alter the fermentation of waste cooking oil into free fatty acids. The better the type of nitrogen source employed, the better the growth of *Rhizopus oryzae* cells will be. When the cells of the *Rhizope oryzae* grow well, an extra-cellular lipase enzyme is generated, which can be used to convert triglycerides to fatty acids. By comparing the $Y_{P/X}$ values produced at the optimum concentration of substrate, the best type of nitrogen supply can be established.

Figure 3 depicts the findings of free fatty acid production during the fermentation process employing the nitrogen sources such as beef extract, malt extract, urea, NH_4Cl, and $(NH_4)_2SO_4$ showed a difference with the highest concentration of free fats produced by beef extract of 2,02 g/L and the lowest concentrations of free fat acids obtained NH_4Cl and $(NH_4)_2SO_4$ with a value of 0,51 g/L. NH_4Cl and $(NH_4)_2SO_4$ will produce acidic conditions because the ions in ammonium will release free acids that can affect fermentation. Furthermore, ammonium ions at room temperature have the same properties as non-essential heavy metals of Hg (amalgam) so these two sources of nitrogen are less capable of metabolizing *Rhizopus oryzae* in biomass growth and product formation.

Figure 4 shows that organic sources of nitrogen (malt extract, beef extracts, urea) produce higher biomass concentrations (18.61 g/L; 17 g / L; 11.91 g / l) compared to inorganic sources (NH_4Cl and $(NH_4)_2SO_4$) with the same biomass concentration of 10.70 g /L. Organic sources of nitrogen are more supportive of the development of biomass than inorganic sources [8]. Organic sources of nitrogen will supply cell growth factors such as amino acids and proteins needed to carry out cell metabolism and enzyme synthesis.

The beef extract has an advantage over malt extract and urea because beef extract contains vitamins, minerals, and coenzymes. The coenzyme in beef extract can be highly needed by *Rhizopus oryzae* in stimulating the lipase enzyme performance so that the coenzyme will bind the substrate and produce better products [16]. Malt extracts itself has a supportive protein that comes from nuts and more contains carbohydrates, whereas in this study malt extract is only able to be used by *Rhizopus oryzae* as a nutrient for reproduction and is less good in promoting the formation of products [17].

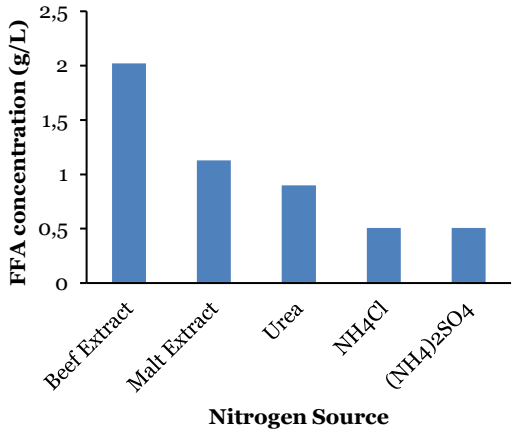

Figure 3. The Effects of Nitrogen Source Types on Free Fatty Acid Concentration

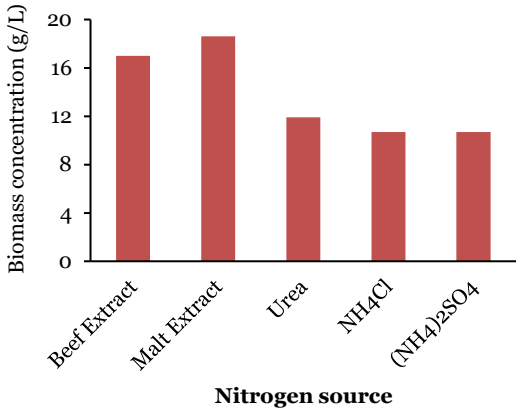

Figure 4. The Effects of Nitrogen Source Types on Biomass Concentration

Product yield relative to biomass ($Y_{P/X}$) on various nitrogen source types is presented in **Table 2**.

Table 2. The Effects of Nitrogen Source

Types on $Y_{p/s}$ Value

Nitrogen Source	$Y_{P/X}$ (%)
Beef Extract	11,78
Malt Extract	5,98
Urea	7,40
NH_4Cl	4,58
$(NH_4)_2SO_4$	4,58

According to **Table 2**, the usage of beef extract as a nitrogen source yielded the greatest $Y_{P/X}$ value of 11.78% when compared to other sources such as malt extract, $(NH_2)_2CO$, NH_4Cl, and $(NH_4)_2SO_4$. The results suggest that the fermentation process employing a nitrogen source beef extract is superior to the use of alternative nitrogen sources, as evidenced by yield values and the formation of fatty acids. The fermentation procedure with different organic nitrogen sources produced superior outcomes than inorganic nitrogen sources in the study. Because organic nitrogen contains supporting components such as minerals and vitamins.

Identification of Free Fatty Acids and Glycerol in Fermented Products

The results of identification of free fatty acids and glycerol in the fermentation products using Gas Chromatography–Mass Spectroscopy (GC-MS) are presented in **Figure 5**. According to the chromatogram, the free fatty acids obtained from the fermentation process of waste cooking oil are saturated fatty acids in the form of palmitic acid and unsaturated fatty acids in the form of oleic acid, as well as glycerol by-products.

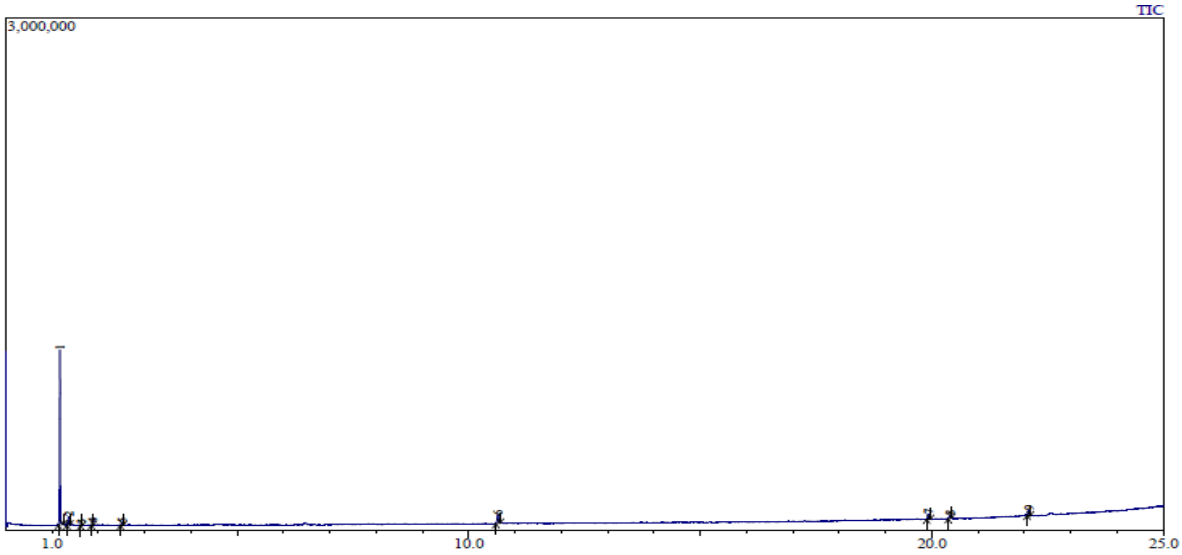

Figure 4. Chromatogram of Fermentation Product Identification Using GC-MS

CONCLUSION

This study has successfully performed the process of hydrolysis of waste cooking oil with *Rhizopus oryzae* as a biocatalyst for the release of free fatty acids. The optimal waste cooking oil as the substrate is obtained at a concentration of 30 g/L with a $Y_{P/X}$ value of 13.63%, free fatty acid concentration is 2.13 g/L and dry cell weight is 15.48 g/L. The best source of nitrogen is beef extract with an $Y_{P/X}$ value of 11.78%, which can produce free fatty acids of 2.02 g/L and a dry cell weight of 17.0 g/L.

Further research is needed to find out the influence of nitrogen source concentration and agitation rate on the enzymatic hydrolysis process using *Rhizopus oryzae*. The sufficiently high biomass acquisition potential in this study can be directed by the application of *Rhizopus oryzae* to waste processing containing triglycerides to produce fungal biomass as a single-cell protein producer for livestock feed.

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