Whole Cell Hydrolysis of Sardine (Sardinella lemuru) Oil Waste using Mucor circinelloides NRRL 1405 Immobilized in Poly-urethane Foam

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Abstract

Sardine (Sardinella lemuru) fish canning industries produce oil waste that can be potentially reused because of its omega-3 content. However, the majority of the omega-3 content are still bound to triacylglyceride form. The objective of this study is to determine optimum pH and temperature of hydrolysis process using lipase of whole-cell biocatalyst Mucor circinelloides immobilized in Poly-Urethane Foam (PUF). The optimum hydrolysis products were obtained at pH 7 and temperature 35 °C. The hydrolysis product at the optimum condition was fractionated using Thin Layer Chromatography (TLC). For further analysis, every fraction was extracted and esterified using Fatty Acid Methyl Ester (FAME) method. Fatty acid methyl ester were analyzed using Gas Chromatography (GC) to determine the omega-3 content. After hydolysis, GC result showed an increase in omega-3 (EPA and DHA) content as much as 12.56 % compared to the crude oil. However, total lipid was reduced, presumably by lipid consumption activity of fungus during the hydrolysis process, resulting in the lost of more than 80 % of total lipid in oil waste.

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Keywords : Fish oil; Mucor circinelloides; omega-3

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1. Introduction

*Sardinella lemuru* canning industries regularly produce a high volume of fish oil as a waste. The end product only weighs (50 to 60) % of the initial fish mass as the raw material, while the rest become waste\(^1\). In fact, lipid from Sarden fish oil contains a significant amount of mono-unsaturated fatty acid (MUFA) and poly-unsaturated fatty acid (PUFA)\(^2\). This means the fish oil waste should be potential as a source of the health beneficial omega-3. As a food supplement, the price of omega-3 in the market is pretty high\(^3\)–\(^5\). The recovery of omega-3 from the fish oil waste should then be a good alternative to produce omega-3 supplement with a lower production cost.

The major part of omega-3 in fish oil exists as the triacylglyceride form (TAG), so it needs to be hydrolyzed in order to get the free fatty acids. The omega-3 free fatty acids can then be separated from the other no-omega-3 free fatty acids. Microbial hydrolysis, for the sake of environmental friendliness, is favored over the corresponding chemical processes. Enzymatic hydrolysis process using whole cell of fungi is also expected to be cheaper than the one using the pure enzyme. Andrate et al.\(^6\) showed that *Mucor circinelloides* exhibited a lipase activity higher than many other fungi species. Furthermore, the poly-urethane foam (PUF) immobilized form of *M. circinelloides* showed (2.1 to 4.3) times higher activity than the free enzyme did\(^7\).

In this work the optimum pH and temperature will be determined for the hydrolysis process of fish oil waste using PUF-immobilized *M. circinelloides*. The hydrolysis is expected to be able to concentrate the omega-3 from the initial fish oil mixture.

2. Materials and methods

2.1. Basal medium

Medium composition: 5.25 g pepton (Merck); 5.25 g tripton (Bacto\(^\text{TM}\)); 0.15 g NaNO\(_3\) (Merck); 0.15 g KH\(_2\)PO\(_4\) (Merck); 0.075 g MgSO\(_4\)\(_{7}\)H\(_2\)O (Merck); and 4.5 g olive oil (Borges) in 150 mL distilled water.

2.2. Immobilization of Mucor circinelloides using PUF matrix

*Mucor circinelloides* was immobilized by adding a 10\(^6\) spora suspension into the mixture of 100 cubes PUF dan 150 mL basal medium which had been sterilized previously (121 °C and 1 atm for 15 min. The mixture was incubated for 72 h at 30 °C and 170 rpm. Immobilized biomass was then separated from the medium by filtration and washed by sterile distilled water. Immobilized cell was stored at 4 °C.

2.3. Determination of optimum hydrolysis time

Hydrolysis was done for a mixture of 25 mL fish oil waste 0.1 M buffer pH 7 and 20 % (w/w) PUF-immobilized *M. circinelloides*. The mixture was incubated at 35 °C with 170 rpm agitation speed and sampling was done daily by taking out 5 mL of mixture each time for determination of total acid number and saponification value according to Adamczak et al.\(^7\). Sampling was terminated 5 d after the highest value of total acid number.
2.4. Determination of optimum pH

Hydrolysis was done as above by varying the pH as follows: 4, 5, 6, and 7. The mixture was incubated at 35 °C for 72 h with 170 rpm agitation speed and sampling was done at the beginning and the end of hydrolysis time for determination of total acid number and saponification value.

2.5. Determination of optimum hydrolysis temperature

Hydrolysis was done as above by varying the temperature as follows: (25, 30, 35, and 40) °C. The mixture was incubated at 35 °C for 72 h with 170 rpm agitation speed and sampling was done at the beginning and the end of hydrolysis time for determination of total acid number and saponification value.

2.6. Product analysis

Hydrolysis product was separated by Thin Layer Chromatography/TLC using Silica Gel 60 F254 (20 × 20) cm with the eluent of hexane : ether : acetic acid (60 : 40 : 1). 10 μL of the diluted fish oil waste and hydrolysis mixture was spotted on the TLC plate and eluted in the TLC chamber. The dried TLC plate was visualized by I2 vapour for (15 to 20) min and every spot was identified according to Pelizzola et al. Each spot was then extracted using chloroform as a solvent and analyzed by GC (Carbowax/20M, p 30 m and d 250 μm, FID detector) for determination of EPA and DHA contents, compared to the initial fish oil waste.

3. Result and discussion

3.1. Hydrolysis time

According to the result presented in the Figure 1, optimum hydrolysis time was concluded as 3 d.

![Fig. 1. Hydrolysis of fish oil waste by M. circinelloides at 35 °C, pH 7, 170 rpm (shaker), 20 % substrate concentration, 20 % immobilized cell](image-url)

The hydrolysis activity of the lipase represents the fungi activity to break down the complex substrate like lipids into smaller molecules necessary for its metabolism. This activity was known to be effected by the provided substrate concentration, product concentration and the age/life phase of the fungi. The higher the triasilgliserida (TAG) concentration as substrate, the higher the activity of lipase. On the other hand, the higher the fatty acid concentration as the hydrolysis product, the lower the lipase activity. According to Bayizit and Basoglu, fungi will possible tend to accumulate lipids rather than produce lipase to hydrolyze the lipids at their stationary phase.
3.2. Determination of optimum pH

In this work, the lipase activity was found to be optimum at pH 5 which is in line with the result from Nadia et al.\textsuperscript{12} reporting the optimum lipase production from \textit{Mucor racemus} at pH 4 to pH 7. Figure 2 also reveals the fact that the activity of lipase increased at the pH 7, possible because of the presence of lipase isoform\textsuperscript{7} that once reported that \textit{Rhizomucor} also give two values of optimum pH. Bertolini et al.\textsuperscript{13} also proved the existence of two genes coding lipase isolated from four strains of \textit{Geotrichum candidum} fungi which referred to two lipase isoforms from each fungi.

![Graph showing the effect of pH on lipase activity](image1)

\textbf{Fig. 2.} Hydrolysis of fish oil waste by \textit{M. circinelloides} at 35 °C, 170 rpm (shaker), 20 % substrate concentration, 20 % immobilized cell (hydrolysis time = 3 d) and variation of pH.

3.3. Determination of optimum hydrolysis temperature

In terms of temperature, the lipase activity at pH 5 was found to be optimum at 35 °C. The optimum growth of \textit{M. circinelloides} was once reported to be at 30 °C\textsuperscript{15}, but the optimum activity was found at 35° to 40 °C\textsuperscript{12}. The result of this work where the maximum acidity number was observed at 35 °C might be the interplay between the optimum growth and the optimum activity.

![Graph showing the effect of temperature on lipase activity](image2)

\textbf{Fig. 3.} Hydrolysis of fish oil waste by \textit{M. circinelloides} at pH 5, 170 rpm (shaker), 20 % substrate concentration, 20 % immobilized cell (hydrolysis time = 3 d) and variation of temperature.
3.4. Product analysis

The result of TLC showed an increase concentration of free fatty acid component (FFA) in the oil), monoaslyglyceride (MAG), and diacylglyceride (DAG) spots, while a decrease in triasylglyceride (TAG) spot. Overall, the TLC concluded that the hydrolysis indeed took place to a significant level.

![Fig. 4. TLC separation of the hydrolysate of fish oil. (A) Standard TLC/Rickers–Haunerland, (B) TLC of hydrolysate (S) and initial crude fish oil waste (CR)](image)

<table>
<thead>
<tr>
<th>Component</th>
<th>Rf value</th>
<th>Spot intensity after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAG</td>
<td>0.16</td>
<td>significantly decreased</td>
</tr>
<tr>
<td>DAG</td>
<td>0.65 ; 0.69</td>
<td>slightly increased</td>
</tr>
<tr>
<td>MAG</td>
<td>0.90</td>
<td>significantly increased</td>
</tr>
<tr>
<td>FFA</td>
<td>0.51</td>
<td>significantly increased</td>
</tr>
</tbody>
</table>

The further analysis using GC showed a 12.56 % increase of omega-3 (EPA dan DHA) concentration in free fatty acid fraction (Figure 5) while a decrease in the other fractions.
Intracellular lipase *Mucor circinelloides* had been reported before to be specific towards *sn-1, 3* position of the triacylglyceride\(^{16}\). This means the favourable cleavage sites are the position 1 and 3 in the triacylglyceride. The decrease of omega-3 content in the TAG, DAG and MAG fractions could possibly mean that the omega-3 (EPA and DHA) exist in majority at those two positions in the triacylglyceride.

Another important fact was revealed from the saponification values as presented in the Table 1. In all hydrolysis conditions, a severe decrease of saponification value (≥ 80 %) was observed, strongly indicating a lipids consumption activity by the fungi. It is probably urgent to investigate, whether or not the incubation time was too long, and to find the proper time to terminate the hydrolysis before the consumption of lipids by the fungi.

### Table 2. Saponification value before and after hydrolysis

<table>
<thead>
<tr>
<th>Hydrolysis condition</th>
<th>Saponification value (mg KOH/g substrate)</th>
<th>% Decrease of Saponification value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (T = 35 °C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>165.495/8.415</td>
<td>94.915</td>
</tr>
<tr>
<td>5</td>
<td>165.495/25.245</td>
<td>84.746</td>
</tr>
<tr>
<td>6</td>
<td>165.495/5.610</td>
<td>96.610</td>
</tr>
<tr>
<td>7</td>
<td>165.495/21.038</td>
<td>87.288</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>208.271/20.336</td>
<td>90.236</td>
</tr>
<tr>
<td>30</td>
<td>208.271/21.739</td>
<td>89.562</td>
</tr>
<tr>
<td>35</td>
<td>208.271/14.025</td>
<td>93.266</td>
</tr>
<tr>
<td>40</td>
<td>208.271/28.050</td>
<td>86.532</td>
</tr>
</tbody>
</table>

### 4. Conclusion

The whole cell hydrolysis by PUF–immobilized *Mucor circinelloides* NRRL 1405 using fish oil waste was found to optimum at pH 5 and 35 °C. The end result showed a 12.56 % increase of omega-3 (EPA dan DHA) concentration in form of free fatty acids acid in comparison to initial omega-3 concentration the crude fish oil waste. However, more than 80 % of the initial lipids was consumed by the fungi during the incubation time. Further optimization would then be necessary on the hydrolysis conditions, i.e.: substrate:buffer ratio, aeration level, and substrate:cell ratio.

### Reference


