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Research Article

Production of polyunsaturated fatty acids with *Rhizomucor miehei* by submerged fermentation

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Abstract

The *w*-3 and *w*-6 series of polyunsaturated fatty acids (PUFA) have shown tremendous potential for use in food additives and pharmaceuticals for heart and circulatory disorders and cancer as well as inflammatory diseases. The submerged culture is usually used for PUFA production with glucose or glycerol as a carbon source. PUFA production with *Rhizomucor miehei* by submerged fermentation has been studied. *Rhizomucor miehei* showed a greater potentiality than *Rhizopus microsporus* to produce polyunsaturated fatty acids by submerged fermentation. *Rhizomucor miehei* produced linoleic acid of 12,31%, linolenic acid of 5,78%, eicosapentaenoic acid of 0.67% and docosahexaenoic acid 1,10% in the Saboraud Dextrose Broth medium at 30^{o}C for 7 days. Linoleic acid, linolenic acid, eicosapentaenoic acid and docosahexaenoic acid was determined by Gas Chromatography (GC). *Rhizomucor miehei* also showed the potentiality to produce linoleic acid, linolenic acid, eicosapentaenoic acid and docosahexaenoic acid with cane molasses, wheat bran and pollard as medium. The result showed that cane molasses was the most effective as medium for PUFA production than wheat bran and pollard. 15% of cane molasses was the optimum concentration for eicosapentaenoic acid and docosahexaenoic acid production. At the concentration, *Rhizomucor miehei* produced eicosapentaenoic acid and docosahexaenoic acid of 0.86% and 0.82%, respectively.

Keywords: *Rhizomucor miehei*, polyunsaturated fatty acid, submerged fermentation, cane molasses, wheat bran, wheat pollard, Indonesia

Introduction

Polyunsaturated Fatty Acids (PUFA) are long chain fatty acids with two or more of double bounds. PUFA control the expression of certain genes and thus affect some processes including fatty acids biosynthesis and cholesterol transport in the body. The second function is that PUFA serve as precursors of a wide variety of metabolites, such as prostaglandins, leukotrienes and hydroxy-fatty acids, which regulate critical biological functions. The various roles played by PUFA make it apparent that they are required in every organ in the body in order to keep the organ functioning normally. Therefore, it is not surprising that PUFA deficienies lead to abnormalities in the skin, nervous system, immune and inflammatory system, cardiovascular system, endocrine system, kidneys, respiratory and reproductive systems [1]. The *w*-3 and *w*-6 series of PUFA have shown tremendous potential for use in food additives and pharmaceuticals for heart and circulatory disorders and cancer as well as inflammatory diseases [2, 3].

PUFA occur throughout the animal and plant kingdom, although the greatest diversity is encountered in microorganisms especially algae, fungi and bacteria. In microorganisms, the fatty acids are typically present in storage oil and in membrane phospholipids [1]. PUFA yields in agricultural and animal products are generally low and vary by season, climate and geographical location. Marine fish oil, which are the main source of commercial EPA and DHA, have disadvantages of objectionable taste and odors, high cholesterol and small amount of potential toxic impurities that are difficult to remove. Therefore, the quantity and quality of conventional sources of PUFA may encounter problems meeting an increasing market demand [1].

A variety of PUFA have been detected in microorganisms including bacteria (*Bacillus megaterium, Bacillus pumilus* and *Pseudomonas aeruginosa*); yeasts (*Candida curvata, Rhodotorula glutinis, Lipomyces lipofer*); fungi (*Aspergillus terreus, Claviceps purpurea, Mucor ramannianus, Tolyposporium ehrenbergii* and *Mortierella alpina*) [4, 5]. Microorganisms are thought to be very promising lipid producers because of their high growth rate on simple media and the simplicity of their manipulation. *Rhizomucor miehei* and *Rhizopus microsporus* are a group of fungi found to produce linolenic acid, however production of other PUFA have not been studied.

The submerged culture is usually used for PUFA production with glucose or glycerol as a carbon source. In addition, culture media and culture conditions affect the quality and the quantity of PUFA production [2]. Agricultural wastes, such as cane molasses, wheat bran and wheat pollard, contain carbohydrates and other nutrients which can be used as substrate for fermentation. Wheat bran and pollard have been used to produce monascus pigment with fungi *Monascus purpureus* [6].

The objectives of this research were to firstly study PUFA production with *Rhizomucor miehei* and *Rhizopus microsporus* and to then study PUFA production with *Rhizomucor miehei* by submerged fermentation on cane molasses, wheat bran and wheat pollard.

Materials and Methods

Materials

Rhizomucor miehei and *Rhizopus microsporus* were obtained from the Food and Nutrition Collection Culture, Centre for Food and Nutrition Research, Gadjah Mada University,

Yogyakarta. Cane molasses was obtained from Kebonagung Sugar Cane Factory, Malang, East Java. Wheat bran and pollard were obtained from PT Bogasari Flour Mills, Indofood Sukses Makmur, Surabaya, East Java. Chemicals were obtained from a local supplier.

Production of PUFA with Rhizomucor miehei and Rhizopus microsporus

Production of PUFA was undertaken by submerged fermentation on the same medium and conditions of fermentation as shown in Figure 1.

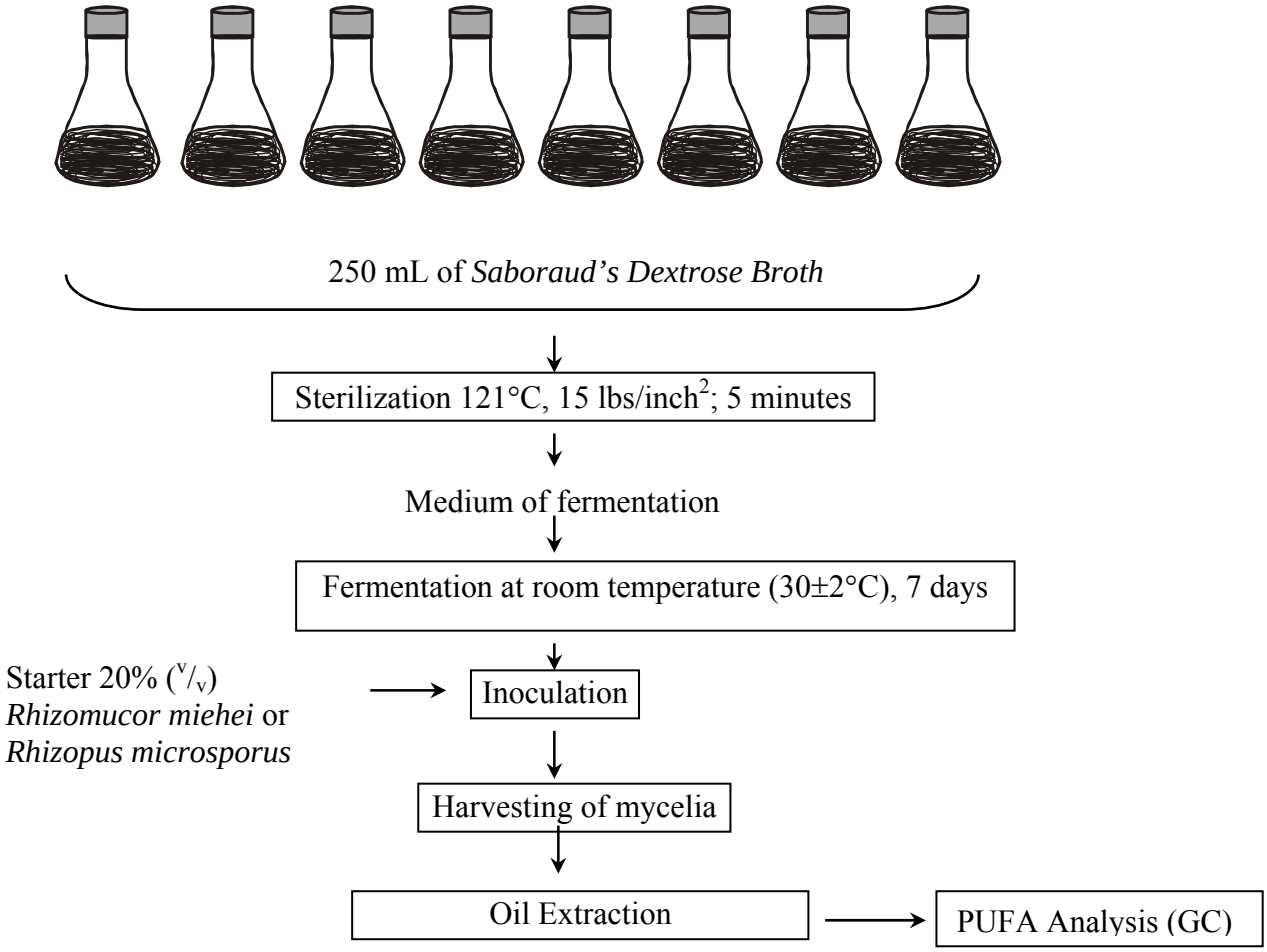


Figure 1. Production of PUFA by Submerged Fermentation.

PUFA production with Rhizomucor miehei on cane molasses, wheat bran and pollard

Rhizomucor miehei was chosen for further research since it showed higher PUFA productivity than *Rhizopus microsporus.* Production of PUFA with *Rhizomucor miehei* was undertaken by submerged fermentation on cane molasses, wheat bran and wheat pollard.

Oil Extraction

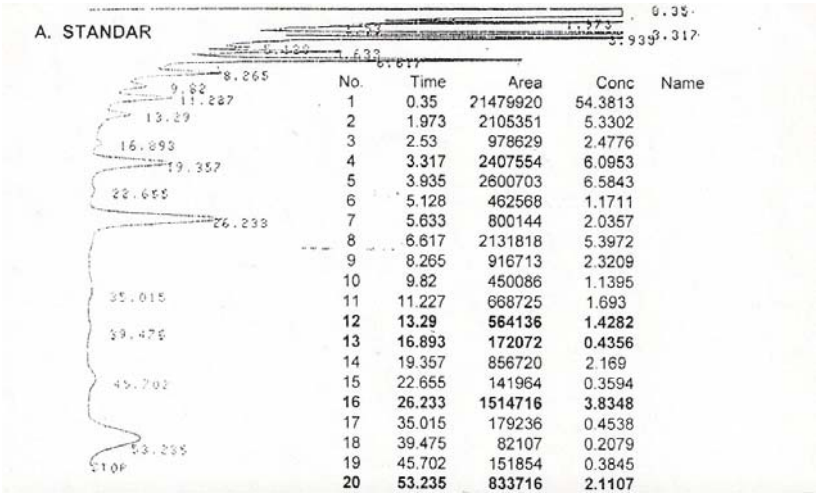
Oil extraction was performed according to Indrati *et al* [7]. Harvested mycelia was freeze dried at -40^{o}C for 24 hours, then crushed manually by mortar. 100 mL of chloroform was added to the crushed mycelia, then sonicated for 10 minutes, filtered with Whatman 40 filter paper. Solvent of the filtrate was evaporated by rotary vaccuum evaporator at 40^{o}C. Residual oil was analysed for PUFA composition using gas chromatography.

PUFA analysis using gas chromatography

Extracted oil was analysed of the PUFA using Gas Chromatography (GC) with standard of mixture of fatty acids containing Linoleic acid, Linolenic Acid, EPA and DHA. Oil was methylated by KOH 2 N in methanol to produce Fatty Acid Methyl Ester (FAME). 1 µL of FAME was injected to GC column with below condition: column (2 m length, 3.3 mm ID, DEGS (*Diethyl Glycol Sucinat*), *Chromosorb* 80 – 100 mesh) with temperature program from 190^{o}C to 250^{o}C and temperature gradient 10^{o}C/min, mobile phase (N_2 with flow rate of 30 mL/min), FID detector with H_2 and O_2 pressure of 1 kg/cm^2 and 1,2 kg/cm^2.

Results and Discussion

The chromatogram of standard PUFA in Figure 2 showed that retention time of linoleic acid, linolenic acid, EPA and DHA were 13.29, 16.89, 26.23 and 53.24 minutes, respectively. Longer fatty acid needs longer retention time, which is related to its solubility in the stationary phase and volatility.



No.	Time	Area	Conc	Name
1	0.35	21479920	54.3813	
2	1.973	2105351	5.3302	
3	2.53	978629	2.4776	
4	3.317	2407554	6.0953	
5	3.935	2600703	6.5843	
6	5.128	462568	1.1711	
7	5.633	800144	2.0357	
8	6.617	2131818	5.3972	
9	8.265	916713	2.3209	
10	9.82	450086	1.1395	
11	11.227	668725	1.693	
12	**13.29**	**564136**	**1.4282**	
13	**16.893**	**172072**	**0.4356**	
14	19.357	856720	2.169	
15	22.655	141964	0.3594	
16	**26.233**	**1514716**	**3.8348**	
17	35.015	179236	0.4538	
18	39.475	82107	0.2079	
19	45.702	151854	0.3845	
20	**53.235**	**833716**	**2.1107**	

Figure 2. Chromatogram of Standard PUFA.
LA = *Linoleic Acid*, LNA = *Linolenic Acid*, EPA = *Eicosa Pentaenoic Acid* and DHA = *Docosa Hexaenoic Acid*

PUFA production with Rhizomucor miehei and Rhizopus microsporus

Table 1 shows the production of PUFA with *Rhizomucor miehei* and *Rhizopus microsporus* using Saboraud's Dextrose Broth at 30^{o}C for 7 days. PUFA productivity of *Rhizomucor miehei* was higher than that of *Rhizopus microsporus*. Linoleic acid and linolenic acid were produced by both fungi. These fatty acids were also produced by other fungi species (shown in Table 1). However, some of the species did not produce EPA and DHA. EPA and DHA were synthesized by fungi through desaturation and elongation of linoleic acid and linolenic acid. The ability of fungi to produce desaturase and elongase enzymes plays an important role in the desaturation and elongation process. Desaturation process of linoleic and linolenic acid occured in both *Rhizomucor miehei* and *Rhizopus microsporus*, however the process in *Rhizomucor miehei* was much higher than that of *Rhizopus microsporus.*

Table 1. Production of PUFA with *Rhizomucor miehei, Rhizopus microsporus* and other fungi.

Fungi	Polyunsaturated Fatty Acid (%)			
	LA	**LNA**	**EPA**	**DHA**
Rhizomucor miehei	12,31	5,78	0,67	1,10
Rhizopus microsporus	2,39	1,44	0,02	-
*Mortierella isabellia**	8	5	-	-
*Mortierella alpina**	13	9	15	25
*Mucor javanicus**	14	31	-	-
*Mucor mucedo**	33	6	10	17
*Aspergillus niger**	46	11	1	-
*Conidiobolus denaesporus**	2	2	9	-
*Entomophthora coronata**	2	1	1	-
*Saprolegnia parasitica**	14	3	10	-

*[8]

Note: LA = *Linoleic Acid*, LNA = *Linolenic Acid*, EPA = *Eicosa Pentaenoic Acid* and DHA = *Docosa Hexaenoic Acid*

PUFA production with Rhizomucor miehei on cane molasses, wheat bran and pollard

The chromatograms in Figures 3, 4 and 5 show the PUFA production with *Rhizomucor miehei* on cane molasses, wheat bran and pollard, respectively. The results showed that cane molasses was the most effective medium for PUFA production than wheat bran and pollard. This might be because cane molasses contained sugars much higher than wheat bran and pollard. According to Vastag *et al* [9], *Rhizomucor miehei* has the ability to use sugars as a carbon source for its growth and triglicerides synthesis. The sugars included glucose and fructose which are found in cane molasses at high concentration, about 55% [10]. Other nutrients are also found in cane molasses, such as nitrogen substances, vitamins and minerals. Wheat bran and pollard contained fibre and starch at high levels.

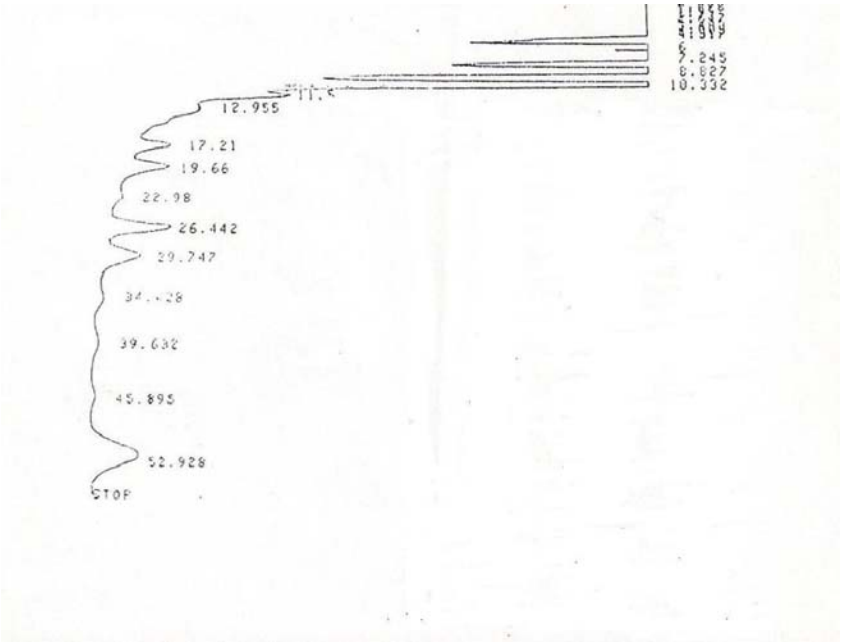


Figure 3. Chromatogram of PUFA of Sample Oil Extracted from *Rhizomucor miehei* on cane molasses.

LA = *Linoleic Acid*, LNA = *Linolenic Acid*, EPA = *Eicosa Pentaenoic Acid* and DHA = *Docosa Hexaenoic Acid*

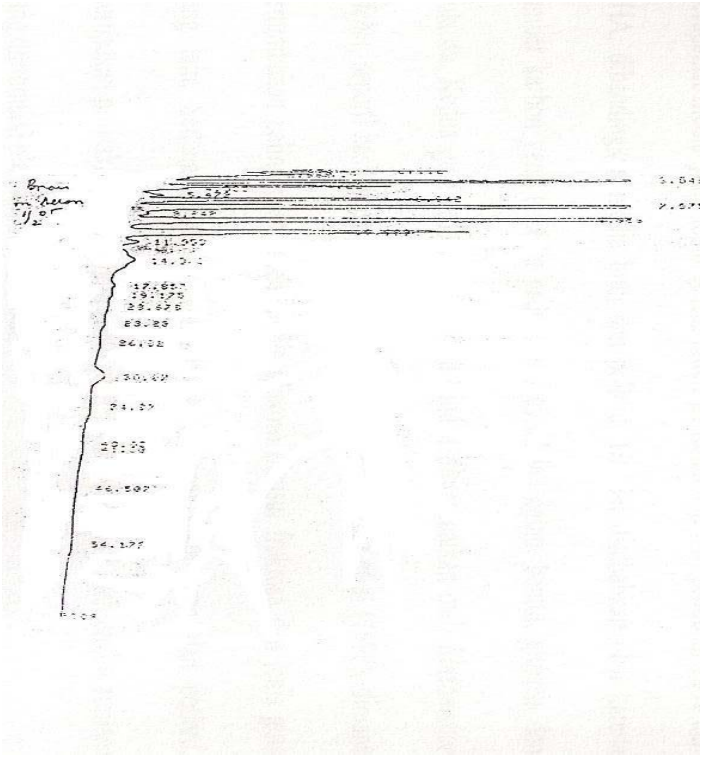


Figure 4. Chromatogram of PUFA of Sample Oil Extracted from *Rhizomucor miehei* on cane molasses.

LA = *Linoleic Acid*, LNA = *Linolenic Acid*, EPA = *Eicosa Pentaenoic Acid* and DHA = *Docosa Hexaenoic Acid*

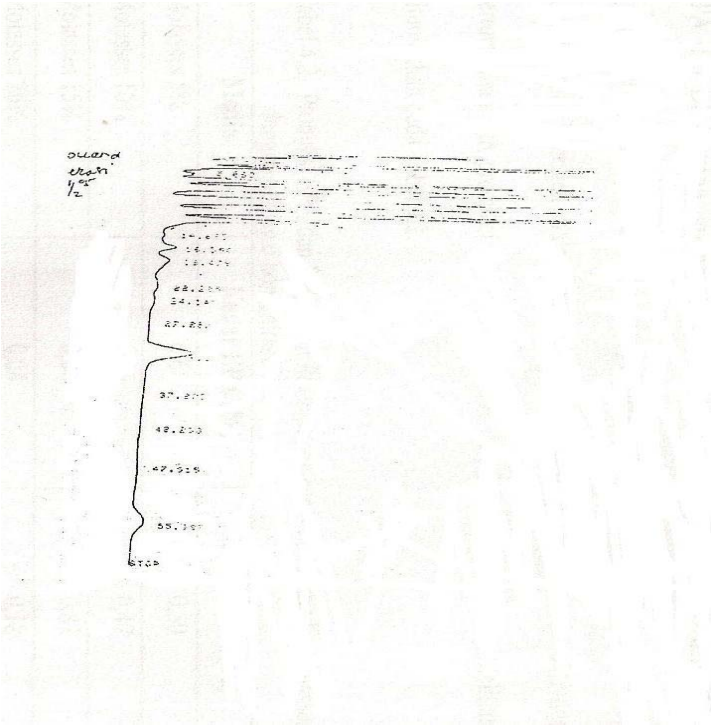


Figure 5. Chromatogram of PUFA of Sample Oil Extracted from *Rhizomucor miehei* on cane molasses.

LA = *Linoleic Acid*, LNA = *Linolenic Acid*, EPA = *Eicosa Pentaenoic Acid* and DHA = *Docosa Hexaenoic Acid*

Table 2 shows the EPA and DHA production with *Rhizomucor miehei* on cane molasses at different concentration. Higher concentration of molasses until 15% produced higher EPA and DHA, however more higher concentration showed decreasing production of those fatty acids. The optimum concentration of cane molasses was 15% with EPA and DHA of 0.86% and 0.82%, respectively.

Table 2. EPA and DHA production with *Rhizomucor miehei* on cane molasses at different concentration.

Medium	EPA (%)	DHA (%)
Molasses 5%	0.64	0.30
Molasses 10%	0.46	0.47
Molasses 15%	0.86	0.82
Molasses 20%	0.04	0.18
Molasses 25%	0.05	0.07

Conclusions

Rhizomucor miehei showed a greater potentiality than *Rhizopus microsporus* to produce polyunsaturated fatty acids by submerged fermentation. The results showed that cane molasses was the most effective as medium for PUFA production than wheat bran and pollard. 15% cane molasses was the optimum concentration for eicosapentaenoic acid and docosahexaenoic acid production. At this concentration, *Rhizomucor miehei* produced eicosapentaenoic acid and docosahexaenoic acid of 0.86% and 0.82%, respectively.

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