Influence of carbon source and culture condition on arachidonic acid production by *Candida krusei* in submerged culture

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***Abstract*—** The growing interest in the application of arachidonic acid (AA) in pharmaceutical and dietary requirements has obtained much attention on production of AA by microorganism. The influence of carbon sources and culture condition on arachidonic acid production was investigated in cultures of *Candida krusei* using shake flasks. The optimum culture condition for high yield of AA was determined by varying the pH (in range of 5 to 9) and agitation speed (in range of 50 to 250 rpm). The carbon sources that were used are sucrose, starch and the mixture of sucrose and starch. The highest dry biomass concentration (0.1473 g/ml) was obtained under culture condition at pH 7 and 250 rpm agitation rate by using sucrose as a carbon source. While, the highest yield of AA (5.0916 g/ml) was produced at pH 7 and 150 rpm agitation rate by using sucrose as carbon source.

***Keywords*—**arachidonic acid, *Candida krusei*, carbon source, lipids.

I. Introduction

The role of yeast in biotechnology had been discovered for a long time. Other than used in production of food and beverages, yeasts also play important role in pharmaceutical field. They are involved in production of medicines, dietary supplements and probiotics. As the importance of yeast in biotechnology is expanding, huge amounts of yeast are cultivated nowadays. Despite the fact that yeast can cause many diseases, researchers continue to investigate other angelic side of yeast that can be developed for humans' sake. One of a newly discovered application of yeast is found in production of fatty acid, such as linoleic acid, alpha-linolenic acid, and arachidonic acid [1]. Arachidonic acid (AA) is a polyunsaturated fatty acid (PUFA) which is a fundamental nutrient component for human beings. It has extensive application in pharmaceuticals, cosmetics and chemical materials. AA is an essential dietary component for human beings and a precursor of many important eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes [2] with important function in circulatory as well as central nervous systems [3]. Ahern et al. [4] stated that AA serves as a starting material for the biosynthesis of the prostaglandin PGE2. AA also helps information transfer of nerve systems and can aid development of infants [5], [6]. Thus, AA is very important in baby formula. Higashiyama et al. [6] also stressed on the various physiological functions of AA, for example protection of gastric mucosa, treatment of skin psoriasis, reduction of fatty liver, killing of tumor cells, and improvement of lipid metabolism of cirrhotic patients.

AA is a precursor of numerous eicosanoids and other compounds which are presently the subject of extensive medical research. Researchers are looking for the best producers of AA which can be consumed by everyone without any limitations. AA can be produced from fish and swine, however the process is more complicated as the removal of cholesterol, odours and tastes is quite difficult. Besides, marine resources are unstable due to unlimited fishing season and geographic locations [7]. Swine, the major producer of AA, are prohibited in certain religion and vegetarians. With the rising consumer demand for a 'Halal' integrated lifestyle, it is a need to find alternatives in producing products that can fit 'Halal' standards. Although AA is presently isolated from animal adrenal gland and liver and from sardines, the yield is only 0.2% (wt/wt) [8]. AA is also found in the cells of ciliated protozoa, amoebae, algae, and other microorganisms [7], [9]-12]. Thus, in this research, works were done to extract AA from yeast as an alternative of the promising producer of AA.

II. Materials and Methods

A. Microorganism

A plate of yeast *Candida krusei* was obtained from Kuliyyah of Science (Biotechnology), International Islamic University of Malaysia, Kuantan. The strain was maintained on potato dextrose agar (PDA) and stored at 4°C after incubated at 36°C for 5 day.

B. Growth medium

2 g of glucose and 1 g of yeast extract were added to 100 ml of distilled water and was autoclaved. Three loopful of yeast from the culture plate were transferred into the flasks containing growth media. The medium was then placed in incubator shaker for 24 hr at 36°C with 180 rpm agitation.

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C. Production medium

The condition for production media was varied so that the optimum condition can be studied. The composition of production media consists of (g/l): carbon source, 30; yeast extract, 5.0; KNO_3, 1.0; KH_2PO_4, 2.4; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_20$, 0.1; $FeCl_3.6H_2O$, 0.015; ZnSO4.7H2O, 0.0075; $CuSO4.5H_2O$, 0.0005 [12]. The carbon sources used were sucrose, starch and mixture of sucrose and starch. The pH was varied at 5, 7 and 9. They were set by using hydrochloric acid (HCl) and sodium hydroxide (NaOH). After being autoclaved, the media was inserted with 10% of grow media and incubated for 48 hr at 36°C. The agitation speed was set to 50, 150 and 250 rpm.

D. Extraction lipid

Lipid was extracted by the two-step Bligh and Dyer method [13]. First, ultrasonication bath was used to disrupt the cell. 1ml of cell suspension was taken and was put in a centrifuge tube containing 3.75 ml of a mixture of chloroform: methanol (1:2). The mixture was vortex for 5-10 min. 1.25 ml of chloroform was added with 1 min of mixing, and next 1ml of water was added with 1 min of mixing. The sample was centrifuged for 15 min at 8000 rpm. The lower phase was collected in another tube. 1.88 ml of chloroform was added to the non-lipid residue was vortex before centrifuged. The lower phase was mixed with the upper phase from the first centrifugation. The lipid extract was evaporated until it was about to dry, and dissolved in a small volume of chloroform: methanol (2:1). The samples were placed in vial for further analysis.

E. Sample analysis

Arachidonic acid (AA) extract was analyzed by gas chromatography which was equipped with flame ionization detector and a 50m by 0.32mm capillary column. The carrier gas was N_2, operated at pressure of 0.4 kg cm^{-2}. The temperature program of the column was: injector 250°C, initial 10 min at 180°C, heating 3°C min^{-1} to 240°C and holding for 20 min at 240°C [14]. AA was identified by comparing the retention time with the commercial standard.

III. RESULT AND DISCUSSION

A. Arachidonic acid and biomass yield

Arachidonic acid standard was diluted to three different concentrations. The area obtained at retention time around 38.73 min [15] from the result of gas chromatography was recorded and calculation by interpolation was conducted. From the area obtained, the concentration of every sample can be determined from the interpolation. From experimental design, there were 15 runs consist of varying parameter such as pH, carbon sources and agitation speed with biomass and AA as the response (Table 1). Based on the results, show that parameter condition at pH 7 and agitation speed was 150 rpm with sucrose as the sole carbon source, gave the highest response for both biomass and AA yields.

TABLE 1
Biomass and arachidonic acid (AA) yield obtained from experimental design

Run	pH	Carbon source	Speed (rpm)	Dry biomass (g/ml)	AA yield (g/ml)
1	9	starch	250	0.0561	0.1493
2	7	sucrose	150	0.1473	5.0916
3	9	starch+sucrose	250	0.0211	0.0800
4	5	starch+sucrose	50	0.0169	0.0000
5	5	starch	250	0.1010	1.5350
6	7	starch+sucrose	150	0.0977	0.0000
7	9	starch+sucrose	50	0.0194	0.0232
8	5	starch+sucrose	250	0.0808	1.3092
9	7	sucrose	250	0.1289	0.0014
10	5	starch	50	0.0204	0.8094
11	9	sucrose	150	0.1175	1.0709
12	5	sucrose	150	0.1234	0.2886
13	7	starch	150	0.0837	0.2055
14	7	sucrose	50	0.0831	0.1993
15	9	starch	50	0.0210	0.0448

B. Effect of pH

The highest dry biomass concentration was obtained at pH 7. Barath et al., [16] studied the effect of pH for growth of cell of *Candida krusei* and concluded that the optimum pH was 7. Suitable pH is necessary for growth of yeast as the amount of acid and base can affect the growing process. Different microorganisms species require different pH value. The highest AA content also was obtained at pH 7. Biomass produced was higher at pH 5 compared to pH 9. At high pH value the minerals in yeast become exhausted and it can be in emergency alkalosis [17]. Hence high pH value is not suitable for growth of yeast. However, at pH 5 the yield of AA was the lowest among other pH value. This result shows that lower pH value is not suitable for AA production when using sucrose at 150 rpm. Figure 3.1 shows the biomass obtained for each pH value.

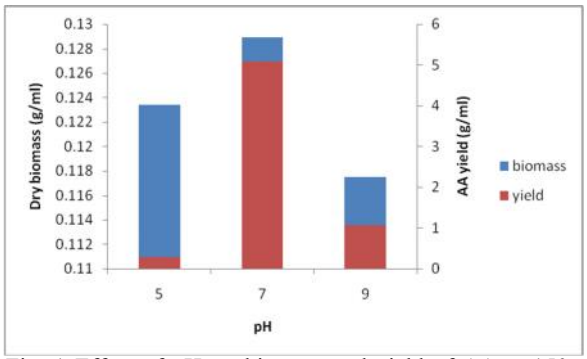

Fig. 1 Effect of pH on biomass and yield of AA at 150 rpm using sucrose

C. Effect of carbon source

Figure 3.2 shows the effect of carbon source on biomass and yield. From the graph we can see that the highest value of biomass (0.1289 g/ml) was obtained when sucrose was used as

carbon source. This result deflects from the findings by Yuan et al., [2] in which biomass of fungi *Mortierella alpina* was higher in starch compared to sucrose. The highest yield of AA was obtained when using sucrose (5.0916 g/ml). There was no AA yield when Yuan et al., [2] used sucrose and low yield produced when starch was used. Different carbon sources at 30 g/l were used. Most of the culture that used sucrose produced high biomass. According to Bajpai et al., [12] both sucrose and starch show poor fungal *Mortierella alpina* growth compared to other sources such as glucose, glycerol and maltose. The mixture of starch and sucrose was found to be not suitable production of AA as zero AA yields was obtained. The mixing of those two substrates may result in reaction that unable AA to be produced.

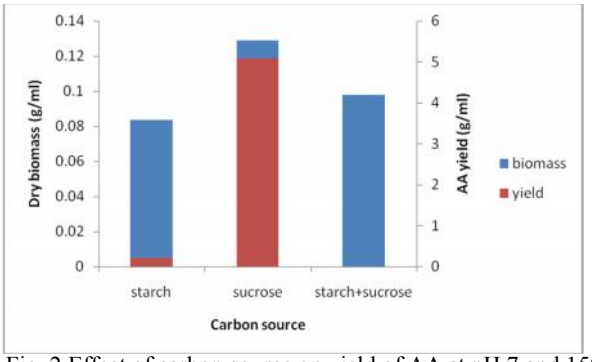

Fig. 2 Effect of carbon source on yield of AA at pH 7 and 150 rpm

D. Effect of agitation rate

Figure 4.7 shows the effect of agitation rate on biomass and yield production. The highest biomass concentration was obtained at 250 rpm. Agitation of culture allows more contact with air and thus increasing the cell produced. Liu et al., [17] used 200 rpm of agitation rate for culture of *Candida krusei* in their study. The best yield was obtained at 150 rpm. Most researchers used high agitation rate of orbital shaker in production of AA, showing that higher speed is preferable. The yeast growth was the highest at 250 rpm with pH 7 by using sucrose (0.1473 g/ml). However the AA yield was very low, which is 0.0014 g/ml. High speed of agitation may damage the cell and cause the enzyme that is needed in AA production to be disrupted. Hence the yield of AA became very low. As been reported by Palma et al., [18] excessive agitation resulted in low enzyme xylanase activity for *Penicillium janthinellum.*

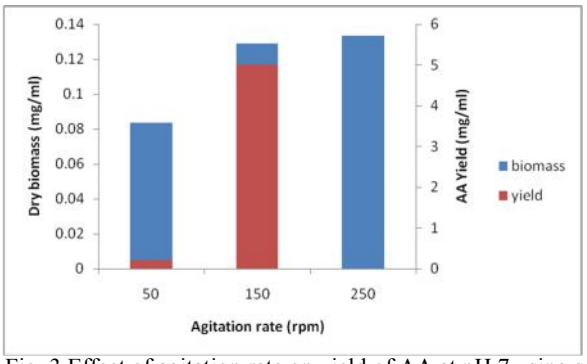

Fig. 3 Effect of agitation rate on yield of AA at pH 7 using sucrose

IV. Conclusion

The optimum culture condition for arachidonic acid (AA) production from *Candida krusei* was found to be pH 7 by using sucrose at 150 rpm because the dry biomass and AA yield were the highest. The dry biomass concentration is 0.1473 g/ml while AA yield is 5.0916 g/ml. whereby, pH 7 happens to be the most suitable pH for AA production. The best carbon source is sucrose compared to starch and mixture of sucrose and starch. Moderate agitation rate that is 150 rpm was favourable. *Candida krusei* had shown a potential to produce AA as an alternative producer rather than marine fish and swine. Thus, further studies on optimization of AA production should be directed at varying other significant parameters including nutrients requirements and method of extraction. The findings in this study will be helpful for other research about AA in the future.

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