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Research article

Biosynthesis and antioxidant activity of single cell carotenoids extracted from microbial cultures

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Abstract

Single cell carotenoids (SCC) as microbial metabolite was produced by a growing culture of yeasts (*Phaffia rhodozyma* and *Rhodotorula minuta*) and molds (*Neurospora sitophylla* and *Monascus purpureus*) in coconut water medium containing sucrose. The highest single cell carotenoid levels had been exhibited by culture of *P. Rhodozyma* after incubation for 168 h was approximately 46.12 µg/g, whereas it was shown by *M. purpureus*, *R. minuta* and *N. sitophila* were 25.30 µg/g, 14.72 µg/g and 12.50 µg/g, respectively. IC_{50} of 9.9896 µg/ml was shown by decreasing in absorption of DPPH for each sample of the tested samples that was measured at every 5 min for 30 min of observation. Although there was a decrease in absorbance, however, the antioxidant activity of crude SCC derived from culture of *N. sitophila* was 22 times lower compared to the antioxidant activity of vitamin C as a positive control, however, it was higher than the antioxidant activity of the crude SCC derived from culture of *P. rhodozyma*, *M. purpureus*, and *R. minuta*. This study was to provide important basic knowledge on physiological aspects of the biosynthesis of SCC derived from microbial cultures and to assay their antioxidant activities dealing with their potential properties as an alternative source for natural antioxidant.

Key words: Single cells carotenoid, Microbial culture, Biosynthesis, Antioxidant Activity.

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1. Introduction

Carotenoids are pro-vitamin A metabolite where its uses in the human body is needed both for cell growth as well as to prevent blindness, reproduction, maintenance of epithelial cells and enhance immunity against various diseases. They are pigments synthesized by photosynthetic and non-photosynthetic organisms. These substances are of commercial interest as coloring agents for food, pharmaceuticals, cosmetics, and animal feed. Because of their antioxidant

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and anticarcinogenic properties [4, 5], some of them have been proposed to act in the prevention of chronic diseases. Several carotenoids are currently produced for commercial purposes by chemical synthesis. However, there is a tendency to avoid the usage of synthetic materials for establishing safety and healthy food or feed supplements. Thus, there is a challenge to fulfill carotenoids requirements by applying microbial resources for mass production of single cell carotenoid, since microbes are easier to be handled and modified under controlled conditions than plants [6]. Carotenoids are pigments synthesized by photosynthetic and non-photosynthetic organisms. These substances are of commercial interest as coloring agents for food, pharmaceuticals, cosmetics, and animal feed. Because of their antioxidant and anticarcinogenic properties [4, 5], some of them have been proposed to act in the prevention of chronic diseases. Several carotenoids are currently produced for commercial purposes by chemical synthesis. However, there is a tendency to avoid the usage of synthetic materials for establishing safety and healthy food or feed supplements. For example, synthetic astaxanthin (3,3'-dihydroxy-β, β-carotene- 4,4'-dione) have been rejected on approval by the Food and Drug Administration (FDA), United of States for the addition those materials for improving fish quality [14]. Some types of microbes have the ability to produce a single cell carotenoids (SCC) qualified with a relatively high quantity. Through the utilization of bioprocess technology, can be produced and extracted products SCC as supplement products that was functioned also as natural antioxidant [1]. Antioxidants are compounds that can inactivate free radicals, which are unstable molecules, produced by various types of the body's normal chemical processes, solar radiation, cigarette smoke or other environmental influences. Today the addition of synthetic antioxidant compounds in a variety of cosmetic products, pharmaceutical and food is the most effective way to prevent fat oxidation (rancidity), in some products, but the use of synthetic antioxidants for use in the field of food has been on the wane. Several studies have shown the presence of toxic and carcinogenic effects of some synthetic antioxidants such products [8]. Antioxidants are generally negative effect is butylated hydroxylanisole (BHA), butylated hydroxyltoluene (BHT) and propyl gallates (PG). So has developed an attempt to find a natural antioxidant derived from plants, which are considered better than the synthetic antioxidant, especially in terms of health. Natural antioxidant contained in the plant section generally contains phenolic compounds and polyphenols [9]. Another source as a producer of natural antioxidants are cultured microbes producing carotenoid metabolites. Yeast and fungal cultures are potential as a producer of carotenoids including *Phaffia rhodozyma* and *Rhodotorula minuta*. In addition, *Neurospora sitophylla* and *Monascus purpureus* also potentially generate high levels of carotenoids, so the metabolites derived from the microbial culture, dubbed as a single cell carotenoids. Furthermore, this study is aimed to investigate the biosynthesis and profiles of single cells carotenoid derived from microbial strains and bioassay on their antioxidant activities.

2. Materials and Methods

Microbial Cultures

To produce single cell carotenoid *P. rhodozyma* was from BCCM (Belgian Coordinated Collections of Microorganism), *R. minuta*, *M. purpureus* and *N. sitophila* were from LIPI MC, Cibinong, Indonesia were cultivated on respective liquid media**.**

Production of Single Cell Carotenoids

A basal medium to produce SCC was coconut water containing 4% sucrose. The respective microbial cultures were grown on media with a particular pH and temperature of incubation. *P. rhodozyma* was grown at optimum temperature of 22°C and pH 5.5, and *N. sitophila* at 37°C and pH 5.5, whilst *R. minuta* has optimum temperature at 25-30°C and pH 7.0 and *M. purpureus* at 25-30°C and pH 5.5. The growth media were sterilized by autoclaving at 121°C for 15 min and pressure of 1.0 atm. Media were inoculated with 5% (v/v) starter culture at a density of 3.0-4.0 x10^7 cells/mL, i.e. after 24 h of incubation. Cell density was estimated using a hemocytometer. The cultures were shaken at 120 rpm at room temperature for 7 days [2]. Sampling was done at every 24 h. Parameters were observed, including biomass cells, according to Gravime Method [16], reducing sugar analysis through 3,5-dinitrosalicylic acid, DNS method, catotenoid extraction according to Sedmak method [11], and antioxidant activity test using 1,1-diphenyl-2-pikril hidrazil, DPPH method.

Production of carotenoids

Productions of single cell carotenoids of *P. rhodozyma* were carried out in three cultivating media: laboratorial glucose medium (MG medium: 3 yeast extract, 3 g malt extract, 5 g bactopeptone, and 10 g glucose in 1 litre distilled water) and two waste-based media, i.e. molasses medium (MM medium: 20 g molasses, 40 ml green bean sprout extract in 1 litre coconut water) and coconut water medium (MC medium: 40 g sucrose in 1 litre coconut water) *P. rhodozyma* cells were grown at 22 ºC in the flasks with 250 ml of broth media (MG, MC and MM) on a rotary shaker at 150 rpm for 168 h. Samplings were conducted every 24 h.

Biomass measurement

Cell growth was monitored by determining the dry cell weight according to the method of [3]. To collect the cell pellet, five milliliters of culture from the flask was transferred to a 15 ml corning tube and washed twice with distilled water by centrifugation at 12,000 x *g* for 5 min. The biomass was weighed after drying cells at 80ºC for 36 h. Growth kinetics of *P. rhodozyma* cells growing in the media were calculated as follows: $\mu = \ln (N_1\text{-}N_0) / \ln (T_1\text{-}T_0)$, where μ is the specific growth rate (h^{-1}), N_0 is the biomass at initial growth (g), N_1 is the biomass at final growth (g), T_0 is time at initial growth (h), and T_1 is time at final growth (h).

Extraction of single cell carotenoids

One milliliter of homogenous cell suspension was centrifuged at 10,000 x g, 4ºC for 10 min and washed twice with distilled water. Two milliliters of dimethyl sulfoxide (DMSO) (Merck, Schuchadt, Germany) was added to the dried pellet incorporation with one milliliter of 1 M phosphate buffer (pH 7.0) and in 15 ml corning tube with glass bead (5 mm in diameter). After centrifugation, cell pellet was added with 5 ml petroleum ether (Mallinckrodt Baker Inc., Phillipsburg, USA) in reaction tube and homogenized again for 10 min. After centrifugation at 10,000 x *g*, 4ºC for 10 min, carotenoid in

petroleum ether phase was transferred to clean reaction tubes and dried. Finally, an aliquot of 2 ml methanol (Merck KGaA, Darmstadt, Germany) was added for analysis of carotenoid concentration and profiles.

Analysis of total carotenoids

To calculate the total astaxanthin and β-carotene, extracted carotenoids were analyzed by high-performance liquid chromatography (HPLC, LC-20AB) (Shimadzu Co. Inc., Tokyo, Japan) on a Spherisorb ODS2 4.0- by 250 x 4.6 mm C18 column (Luna 5u Silica (2) 100 A) by using a 15-min gradient of ethyl acetate (0 to 100%) in Hexan-Aceton (86 : 14, v/v) at a flow rate of 2 ml min^{-1} at 20^oC. Absorption spectra for individual peaks were obtained with a photodiode array detector. Carotenoid peaks were identified by their absorption spectra and by their typical retention times at λ474 nm. Standards of 10 ppm astaxanthin (Sigma, Germany) and β-carotene (Sigma, Germany) were applied to identify the peak of those substances in sample.

Encapsulation of Single Cell Carotenoid (SCC)

For spray drying experiments, SCC, casein and soluble starch were added and dissolved into a solution of NaCl 0.85%. The solution was vigorously homogenized at 2500 rpm for 10 min. The homogenized mixture was microencapsulated in a spray drier. Processing conditions for spray drier was determined as 25000 rpm for spray drier disk rotation, 210-220°C and 100-110°C for entrance and exit air temperature, respectively. Feed flow was applied as 10-20%. The dried microcapsules were packed and stored at room temperature. Another encapsulation method that was applied is freeze dryer. For this reason, 0.2 g of SCC was added to 200 ml of NaCl 0.85%, 5% modified cassava flour, and then 5% casein was added as emulsifier agitated with mixer, and frozen at -20°C for 24 hours. After freeze drying, the pellet was broken into powder, washed with hexane and dehydrated under vacuum.

3. Results and Discussion

Cell Biomass

We observed that cells grow well in all tested media used in this study. Generally, the highest biomass was obtained after 96 h of cultivation. Measurement of the growth of mold and yeast culture is done by weighing cell biomass. Table 1 showed that the highest yields of biomass production cells for each treatment could be achieved at about the same time of incubation. The growth of molds and yeasts in the culture medium coconut water can be seen visually with changes in turbidity of their liquid media. A good growth was shown by the high cell biomass production in the media. The highest cell biomass production (84.2 g/L) was shown by fungal culture of *N. sitophila* after 48 h incubation, followed by *M. purpureus* (18.2 g/L) after 144 h incubation, however, the highest cell biomass production of yeast cultures (10.7 g/L) was shown by *R. minuta* after 120 h incubation and *P. rhodozyma* (14.6 g/L) after 96 h incubation. However, the cell biomass production did not affect the growth of mold and yeast cultures significantly. It indicated that the nutrients in the media containing coconut water could not increase the cell biomass significantly. The cell biomass production could be observed also regarding with a consumption pattern of sugar in the medium as a carbon source by measuring

some reducing sugars of the respective media.

Reducing Sugar Analysis

Analysis on reducing sugars indicated that the addition of sugar at approximately 1.0 g/L into media containing coconut water, however, it did not provide a significant impact on growth of cultures. Figure 1, showed the results on measurement of reducing sugar in production, media of mold and yeast cultures. The sugar content in medium containing coconut water at pH 5.5 was decreased along with the incubation period, indicating that the consumption of sugar has increased in line with the period of incubation. Each type of microbial cultures showed different optimum growth pH and growth temperature. Culture of *P. rhodozyma* has an optimum growth pH of 5.5 and growth temperature of 22°C while *R. minuta* has an optimum pH of 7.0 and a temperature of 25-30°C, and *N. sitophila* has optimum pH 5.5 and growth temperature of 37°C and *M. purpureus* has optimum pH and growth temperature of 5.5 and 25-30°C, respectively. Observing the curve it could be seen that reducing sugar content of each culture grown onto the same media exhibited different levels of reducing sugars. Regarding with culture of *N. sitopila* and *M. purpureus* reducing sugar content in the respective media showed the highest since on the day-1 period of incubation, while the media for culturing *R. minuta* and *P. rhodozyma* showed the highest reducing sugar content on the day-7 of incubation. This was due to differences in capability of each microbial culture to reduce sugar as a sole carbon source. The nutrients in coconut water, such as carbohydrate, protein, fat and mineral are also as essential nutrients dealing with the formation of carotenoid pigments.

Table 1. Average of cell biomass production of molds and yeasts during fermentation.

Incubation time (h)	Avarage of Cell Biomass (g/L)			
	P. rhodozyma	*R. minuta*	*M. purpureus*	*N. sitophila*
24	5.9	5.5	6.5	3.6
48	9.4	10.0	16.4	84.2
72	14.2	7.8	12.2	7.0
96	14.6	9.8	14.5	26.6
120	13.2	10.7	8.3	29.5
144	9.9	9.6	18.5	20.1
168	12.9	10.0	10.7	16.2

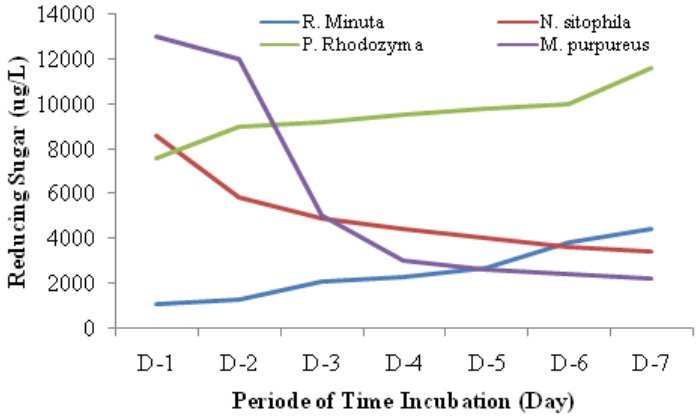


Figure 1. Measurement of reducing sugar in media of fungal and yeast cultures.

Carotenoid Pigments Extraction

Obtaining pellets from cultivating of molds and yeasts were extracted with some organic solvents such as dimethyl sulfoxide, ether petrol, phosphate buffer and methanol, and then centrifuged for 15 min at 4000 rpm and absorbance of the respective extracts were measured by using spectrophotometer at λ474 nm to estimate a total coefficient of extinction at 1%. Profile on formation of single cell carotenoid pigment derived from each microbial culture was shown in Figure 2. Single cell carotenoid pigment (SCC) is a secondary metabolite, which is closely related to primary metabolite that is strongly associated with growth of cells; however, it is not exactly as same as increasing in their cell biomass (Johnson and Schroeder, 1995). The average of the highest production of SCC of the respective cultures was achieved at different incubation period. The highest production of SCC was demonstrated by the culture of *P. rhodozyma* (46.12 µg/g) after incubation for 168 h, however the lowest production of SCC was indicated by the culture of *N. sitophila* (12.50 µg/g) after incubation for 72 h, whilst *M. purpureus* (25.30 µg/g) and *R. minuta* (14.72 µg/g) achieved the highest production of SCC after incubation for 120h, respectively. *P. rhodozyma* culture and *R.minuta* started producing SCC after 24h incubation. This was as indicated by the performance of the media of *P. rhodozyma,* which turned into reddish. The medium increasingly turned to reddish in line with incubation period, whereas the medium for growing of *R. minuta* turned into orange in line with the length of incubation. The occurrence on the formation of SCC was monitored along with 7-day of incubation. The fungal cultures of *M. purpureus* and *N. sitophila* started producing the pigment after 24h incubation. It could be observed from the performance of media of *M. purpureus,* which turned to red-brick colour in line with the length of incubation, while the media for growing of *N. sitophila* was turned into yellowish in line with the incubation time. The production of SCC was tended to increase in line with the length of incubation period, however, it then declined in line with the incubation time. The decrease in production of SCC of each cultures occurred at different time of incubation (Figure 3).

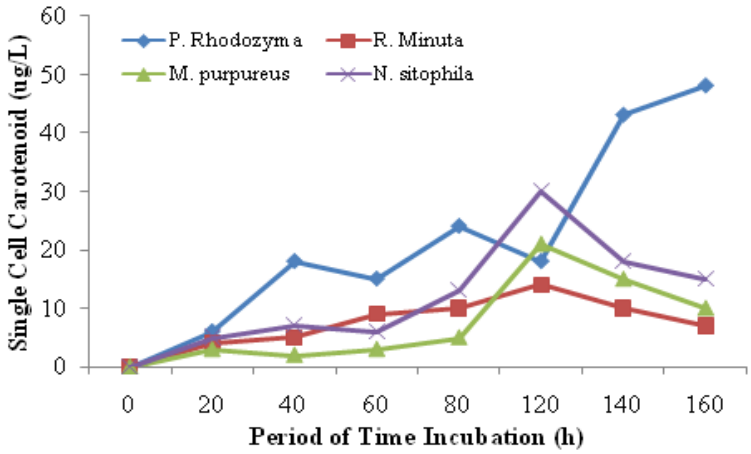


Figure 2. Curves on synthesis of SCC for short incubation period.

The decrease in production of SCC among of the cultures was due to depletion of carbon source in the medium as a primary source for the synthesis of this pigment constituent. In addition, there is an accumulation of toxins from lysis cells that occurred during cultivating that may inhibit carotegenesis [12]. The shortage of carbon source and the presence of toxic compound and decreasing in oxygen level may influence the carotegenesis process significantly.



Figure 3. Performance of SCC producing cultures after a week incubation.

Antioxidant Activity Test

Assay for antioxidant activity of SCC was performed by using DPPH as oxidator. The DPPH is a stable radical agent and can be measured its intensity at 515 nm. The measuring of the antioxidant activity of the extract of SCC derived from the respective cultures against DPPH was tested using vitamin C as a positive control that is known well as a commercial antioxidant. Figure 4 showed the antioxidative activity of SCC derived from each culture in comparison to vitamin C against oxidizing of DPPH. In this study, the concentration that was used for both extracts of SCC and vitamin C was 100 ppm. Results indicated that the extract of SCC derived from the culture of *N. sitophila* showed the highest antioxidant activity compared to the other culture extracts of SCC within IC_{50} value of 9.9896 µg/ml. This was evidenced by a decrease in absorbance of the sample of SCC against DPPH for 30 min. The result showed that the antioxidant activity of the crude SCC derived from *N. Sotiphila* showed highest antioxidant activity among of the crude SCC derived from

other cultures. The antioxidant activity of the crude extract of SCC derived from *N. sitophila* against DPPH was lower than that of vitamin C. However, the antioxidative activity of crude extract of SCC derived from *P. rhodozyma*, *M. purpureus*, and *R. minuta* showed lower antioxidative activity than the antioxidative activity of crude extract of SCC derived from *N. sitophila*. The decrease indicated that although the antioxidant activity of the crude SCC of *N. Sotiphila* was 22 times lower than the antioxidative activity of vitamin C against DPPH, however, IC_{50} is the concentration of the crude SCC that is capable in providing a percentage on scavenging free radicals at approximately 50% compared to vitamin C as control positive. Assay for antioxidant activity against oxidizing DPPH is one of method to test the antioxidant activity of the crude extract of SCC derived from culture of *N. sitophila*, *M. purpureus*, *P. rhodozyma* and *R. minuta* as a natural antioxidant compounds. Although a tested sample shows high antioxidant activity using DPPH as oxidator, however, the result does not always give just as good result as when tested using different methods, and therefore dealing with the assay for antioxidant activity of any biological compounds, it is recommended to test their antioxidant activities using different methods [15].

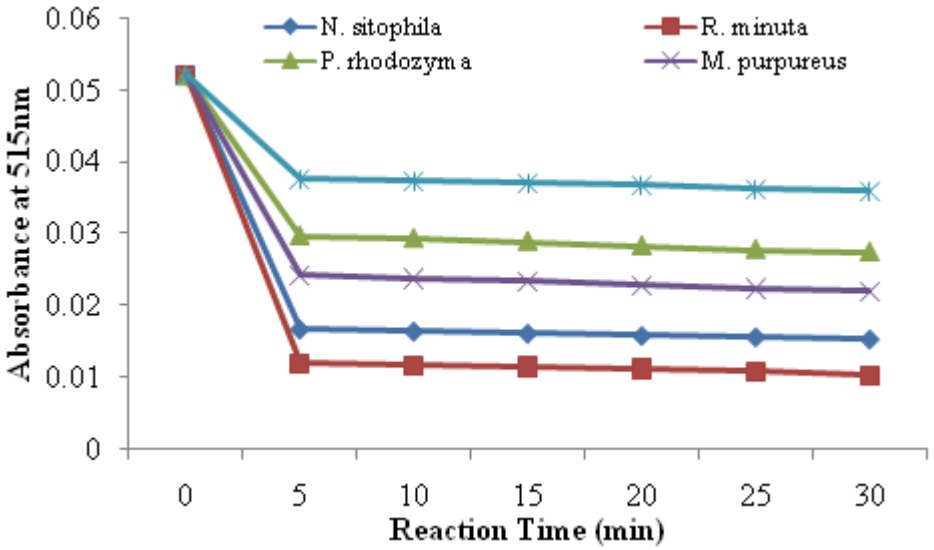


Figure 4. Assay on antioxidant activity of SCC.

Microencapsulation of SCC

There are some experiments for testing the stability of the wall materials used in the microencapsulation of carotenoids. It has been reported that hydrolyzed starches show the highest efficiencies in carotene retention. Acid modified tapioca starch, native tapioca starch, and maltodextrin were used as wall materials for carotenoids encapsulation. Microcapsules were prepared by spray drying. The ability of acid, modified tapioca starch, native tapioca starch, and maltodextrin were tested to serve as wall materials. The results obtained suggest that the modified tapioca starch can be considered as a potential wall material for encapsulation of β-carotene [7].

Nevertheless, Corralo Spada *et al*. [13] showed that microcapsules encapsulated by the freeze-drying method with 12 dextrose equivalent (DE) hydrolyzed starch exhibited the highest stability. In contrast, microcapsules prepared with native starch showed the lowest stability during storage. Modified starch could be used as a wall material in the microencapsulation process of carotenoids. Spray drying was used as an encapsulation method to evaluate the conditions of this process. The quantity of carotenoids was varied at 5, 10, and 15% in a solution containing 30% solids in order to obtain the microcapsules. According to the results, encapsulation efficiency, increased as the quantity of carotenoids decreased. Besides, retention of microencapsulated carotenoids was significantly greater when kept at 10°C compared to storage at 25°C [10]. Modified cassava flour (MCF) could be used in the microencapsulation of SCC by spray drying or freeze drying methods. In addition, they can be used for lycopene encapsulation. MCF provides more effective retention instead of in their native form. Moreover, MCF are encouraging biomaterial that can be applied for the encapsulation process. Numerous wall materials are available for food application such as whey protein, gelatin, sucrose, gum arabic, and soy protein. Additionally, according to the results, SCC retention in the microcapsules increased as the inlet air temperature increased. SEM views of spray dried encapsulated carotenes are shown in Figure 5.

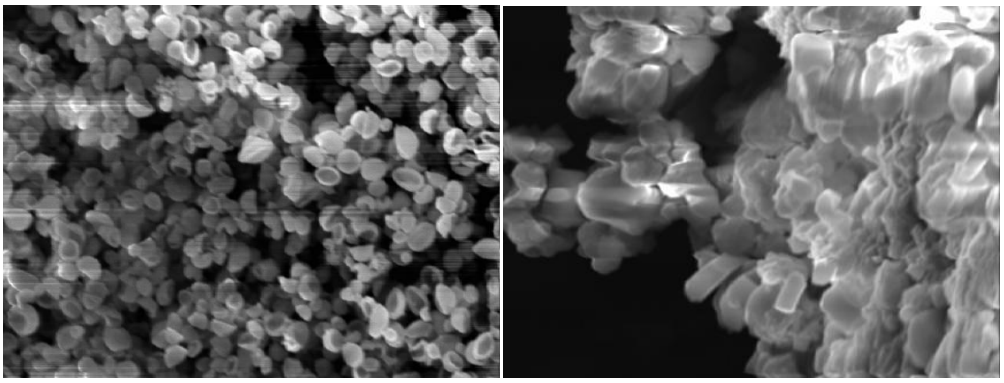


Figure 5. SEM image of SCC before (*left*) and after (*right*) microencapsulated.

Conclusion

From the results of this study, it could be concluded that the ability of fungal cultures tested dealing with sugar consumption had decreased in line with the length of incubation time, whereas the ability of yeast cultures tested had increased in line with the length of incubation time, as shown by their growth that was followed by changing in the performance of the growth media. The highest production of SCC was shown by culture of *P. rhodozyma* (46.12 µg/g), while the lowest production of SCC was indicated by the culture of *N. sitophila* (12.50 µg/g), however, the crude extract of SCC derived from culture of *N. sitophila* showed the highest antioxidant activity compared to the other crude extracts of SCC within IC_{50} value of 9.9896 µg/ml. The decrease indicated that although the antioxidant activity of the crude SCC of *N. Sotiphila* was 22 times lower than the

antioxidative activity of vitamin C against DPPH.

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