Malaysian Journal of Microbiology, Vol 14(4) Special Issue 2018, pp. 344-350
DOI: http://dx.doi.org/10.21161/mjm.144188

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Production of pigments by *Rhodotorula mucilaginosa*

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ABSTRACT

Aims: Pigments have a large and growing market in the world. Drawbacks in their production such as raw materials availability and low productivity prompt the search for fermentation routes for industrial production. A carotenoid-producing yeast identified as *Rhodotorula mucilaginosa* was isolated in our laboratory. The aim of this study was to investigate the growth and carotenoid production capacity of the yeast.
Methodology and results: A cost-effective substrate of sago starch hydrolysate (SSH) derived from sago fiber waste was used for the fermentation. The fermentation was carried out for 96 h at 27 °C in batch mode. The biomass produced during 5 days of fermentation was 9.6 g/L, which contained a carotenoid concentration of 8.1 mg/L and a specific yield of 845.9 μg/g.
Conclusion, significance and impact of study: The results demonstrated the capacity of *R. mucilaginosa* yeast to produce carotenoids and its potential for larger-scale production.

Keywords: Rhodotorula, carotene, torularhodin, torulene, sago starch hydrolysate

INTRODUCTION

The colourful splendour of nature is mainly due to the presence of compounds like carotenoids, anthocyanins, porphyrins, and chlorophylls. Carotenoids have high impact in nature since they are responsible for many of the brilliant red, orange, and yellow colours in plants and animals. The paramount interest in using microbial pigments is because of their natural character, safety, and medicinal properties, and their ability to provide controllable and predictable yields (Joshi *et al*., 2003). Carotenoids produced by the microbial route are very significant due to seasonal and geographic variability in the marketing and production of some pigments of plant origin (Frenova and Beshkova, 2009). Microbial processes are low-cost, with the use of natural carbohydrate sources as substrate bringing economic advantages. Carotenoids are synthesized by many microorganisms and plants in nature with acetyl-CoA being the key precursor during carotenoid biosynthesis (Olson, 1964; Moise *et al*., 2014). The production of carotenes such as Torularhodin and Torulene by the yeast *Rhodotorula mucilaginosa* (*R. mucilaginosa*) has been demonstrated previously (Aksu and Eren, 2005; Irazusta *et al.*, 2013; Cheng and Yang, 2016; Yen *et al*., 2016; Yoo *et al.*, 2016). While use of this yeast is currently limited due to the prohibitive cost of production, it remains of interest since carotenoids have high demand and price in the market. The market value of β-carotene, estimated at around $250 million in 2007, increased to nearly $261 million in 2010. This market is expected to grow to $334 million by 2018 at a compound annual growth rate of 3.1%. Lutein was around $233 million in 2010 and is expected to reach $309 million by 2018 with a compound annual growth rate of 3.6% (McWilliams, 2011). In the fermentation industry, the market is affected by the availability of the raw materials to produce the desired products. In the case of carotenoids, diverse raw materials have been tested to decrease the production cost and at the same time to maintain or even increase the productivity (Cheng and Yang, 2016). The consensus is that the production cost could be reduced when the yield of the product is increased, and a less expensive substrate is utilized. Fermentation parameters have a strong influence on productivity, and control of production facilitates the extraction of intracellular carotenoids from yeast (Yoo *et al.*, 2016). To reduce the production cost, efforts to use cost-effective substrates such as food waste

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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or agricultural wastes have been reported. The use of agricultural wastes such as glucose, molasses sucrose, and whey lactose sugars as the carbon sources also have been reported (Aksu and Eren, 2005; Cheng and Yang, 2016). On the other hand, since *R. mucilaginosa* can accumulate a large amount of lipids from the hydrolysate of cassava starch, Li *et al.* (2010) performed fermentation using seawater, with the aim to reduce the consumption of water (Li *et al.*, 2010). The direct cultivation of oleaginous *R. mucilaginosa* in a 5-L airlift bioreactor using seawater instead of pure water led to a slightly lower biomass being achieved, at 17.2 compared to 18.1 g/L, respectively (Yen *et al*., 2016). The capacity of *R. mucilaginosa* to biodegrade chemical wastes such as the toxic pesticide diazinon also has been demonstrated (Pacia *et al*., 2016). The strain improved the production of pigments in the presence of this toxicant. In a similar approach, the relationship among carotenoid production, copper bioremediation and oxidative stress in *R. mucilaginosa* RCL-11 has been demonstrated as well (Irazusta *et al.*, 2013). The characteristic of biodegradation of toxicants confers additional potential to the strain to be used in polluted soils. Regarding media composition in this study, we explore the use of sago fibres as a starch source from which to produce glucose as the substrate, since sago fibres are an industrial waste without price in the market. In our laboratory, we isolated a contaminating strain that grew in a very simple media. It was interesting because the media was the filtrate of a glucose syrup passed through a powdered activated charcoal filter. Not many nutrients were there; just the filtrate and some micronutrients. This is what the fermentation industry is looking for. Microorganisms without complex nutritional requirements are desirable. Upon cultivating the strain in agar plates and using the same simple media (filtrate syrup), the strain produced an orange pigment. It was identified as *Rhodotorula mucilaginosa* by molecular techniques (not the focus of this study).

Starch from sago fibres

Typical sago starch mill releases the wastewater containing residual starch and fibres in the nearby streams. Sago fibres are a starchy lignocellulosic by-product in the production of sago flour. It is generated from the pith of *Metroxylon sagu*. In Malaysia, Sarawak is the largest sago-growing area and the biggest exporter of sago starch. Sago fibres on a dry basis contain 58% starch, 23% cellulose, 9.2% hemicellulose, and 4% lignin (Chew and Shim, 1993; Linggang *et al.*, 2012; Lai *et al.*, 2016). The dried-powder form of sago fibres has shown the ability to be converted into fermentable sugar via enzymatic hydrolysis or acid treatment. A total of 0.62 g of glucose per 1 g of fibres (g/g) can be produced by using sulphuric acid hydrolysis (Kumoro *et al*., 2008). The presence of a suitable carbon source is crucial for the synthesis of carotenoids. *Rhodotorula* has potential as a natural source of carotenoids. One alternative to find a cost-effective substrate is the use of glucose derived from the starch remaining in the sago fibres during the production of sago flour. Thus, the aim of this study was to assess the growth and production of carotenoids from *R. mucilaginosa* using hydrolysed sago starch (HSS) derived from sago fibres as a low-cost carbohydrate source.

MATERIALS AND METHODS

Microorganism

The strain of *R. mucilaginosa* used was a contaminant yeast strain locally isolated in a Universiti Malaysia Sarawak (UNIMAS) laboratory. Potato dextrose agar (PDA) was used to maintain the yeast culture. The culture was stored in a refrigerator at 4 °C after 30 °C incubation for 24 to 72 h. The purity of the culture was regularly checked. The yeast strain was activated weekly (Yehia *et al.,* 2013).

Maintaining media

Potato Dextrose Agar (PDA) media (Difco, USA) was used for the isolation and cultivation of the yeast. All media used throughout this study were prepared according to manufacturer directions.

Preparation of sago starch hydrolysate (SSH)

Sago fibres were obtained from Herdsen Sago Mill in Sarawak, Malaysia. Prior to composition analysis, the sago fibre samples were oven dried at 90 °C for 24 h before being ground to pass through a 1 mm sieve. Dried sago fibres were analysed for moisture content.

A suspension of sago fibres, 10% (w/v) dry basis, was dissolved in distilled water. The pH of the suspension was adjusted to pH 6.5. Enzymatic hydrolysis was performed according to the directions of Novozymes A/C for starchy materials.

Cultivation of yeast for carotenoid production
Inoculum preparation

A loop full of an active (24 h) *R. mucilaginosa* cultivated on PDA was transferred into a universal bottle containing 20 mL of a media consisting of 40 g/L glucose and 5 g/L yeast extract. The strain was incubated for 24 h at 36 °C. The activated strain was then transferred into 200 mL growth medium containing 8 g of glucose and 1 g of yeast extract. The cells were incubated for 12 h at room temperature as the inoculum (Figure 1). The strain was prepared at an approximate concentration of 10^5 CFU/mL. Centrifugation of cells was performed at 4500 × g for 10 min to obtain a cell pellet. Sterilized distilled water was used to rinse the cell pellet before transfer into the bioreactor.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Fermentation process

Batch fermentation was performed using a 2-L bioreactor (B. Braun Biotech International, Germany). The fermentation media consisted of 3.7% HSS and 5 g/L of yeast extract. The operating conditions were: temperature 27 °C; agitation 150 rpm; initial pH fixed at pH 6.5, but not controlled. The cells were incubated for 5 days with samples withdrawn daily at 24 h intervals to determine cell growth (O.D), dry cell weight (DCW), cell viability, residual glucose, and carotenoid concentration (Yehia *et al.*, 2013).

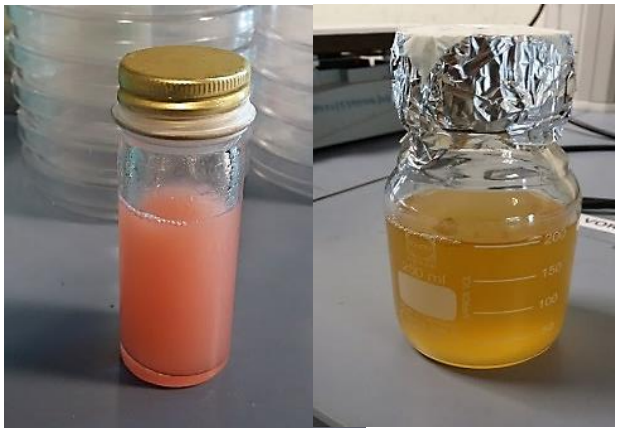

Figure 1: Inoculum preparation ready for performing fermentation.

Measurement of microbial growth

During batch fermentation, a total of 50 mL of broth sample was taken to determine optical density, cell viability, dry cell weight and residual glucose. Ten millilitres were taken from the 50 mL for pigment analysis. The sample was centrifuged for 15 min at 4500 × g. The supernatant was filtered using 0.45 µm filter membrane and stored at 4 °C for further analysis. The pellet was collected for DCW determination and analysis of pigment.

Optimal density (O.D)

The growth of yeast was recorded spectrophotometrically by measuring the optical density of yeast growth in the broth at 575 nm (UV mini-1240, Shimadzu Corporation, Japan).

Dry cell mass (g/L)

The dry cell weight of yeast was taken as a parameter for its growth. Ten millilitres yeast suspension was centrifuged for 15 min at 4500 × g, washed twice with distilled water, and finally dried at 105 °C till constant weight (2-3 h).

Cell viability

A haemocytometer was used to determine the percentage of living yeast cells using a solution of 0.1% (w/v) methylene blue dye. Equal volumes of yeast cell broth and methylene blue solution were mixed in an Eppendorf tube. The percentage of viable cells was counted under the microscope (Painting and Kirsop, 1990).

Reducing sugar

Dinitro salicylic acid (DNS) method was used to determine the concentration of reducing sugars present in the fermentation broth (Miller, 1959).

Test for carotenoids

A sample of DCW was mixed with 10 mL of chloroform in a falcon tube with vigorous shaking. The mixture was filtered using 0.45 µm filter membrane. The resulting filtrate was then treated with 85% H_2SO_4. A blue colour interphase indicated the presence of carotenoids in the sample (Ajayi *et al.,* 2011).

Carotenoid extraction and quantification

Carotenoid extraction was done according to Kanzy *et al*. (2015), with a sample of 10 mL taken from the bioreactor (Kanzy *et al.*, 2015). Cells were harvested using centrifugation at 4500 × g for 15 min.

The supernatant was discharged, while the cell pellet was washed three times with distilled water, then resuspended in 1 mL of dimethyl sulfoxide (DMSO) preheated to 55 °C. The mixture was vortexed for around 10 sec. After centrifugation, the pigment DMSO solution was pipetted off, and the DMSO extraction was repeated three times. The total carotenoid was determined, and optical density was measured using spectrophotometer (UV mini-1 240v, Shimadzu Corporation, Japan) at 501 nm. Total carotenoid content for *R. mucilaginosa* was calculated and expressed as cellular carotenoids (µg/g dry yeast) and also as volumetric carotenoids (mg/L) of culture. The extinction coefficient ($E_{1cm}^{1\%}$) 2040 was used according to the equation reported by Kanzy *et al.* (2015). All the operations were performed under subdued light to prevent degradation of pigment.

Data analysis

The production of pigments from *R. mucilaginosa* was analysed using mean (µ) of the analysis of the experiment done by duplicate.

RESULTS AND DISCUSSION

Characteristics of *R. mucilaginosa*

The colonies grew rapidly, smooth, mucoid and soft. They were orange or pink in colour and hyphae were absent. Figure 2 shows the pigment produced in PDA. Under

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microscopic examination, cell shape was oval with asexual reproduction, showing multi-lateral budding and the absence of ascospores and pseudohyphae.

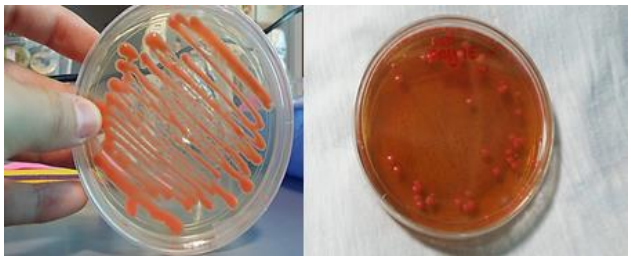

Figure 2: *Rhodotorula mucilaginosa* cultivated on PDA media at room temperature and used to prepare the inoculum.

Sago starch hydrolysate (SSH)

The presence of a suitable carbon source is fundamental for the production of carotenoids during the non-growth phase. It is the key for the production of secondary metabolites, various cell structures, and organic chemicals (Moise *et al.*, 2014). Agro-industrial raw materials in many cases result in serious environmental problems. Therefore, the use of sago fibres as a low-cost carbohydrate source could minimize the environmental problem (Aksu and Eren, 2005; Cheng and Yang, 2016). The medium was prepared with 10% (w/v) of sago fibres suspension, sufficient to keep the fermentation running properly. Sago fibres containing 55-60% of starch produced 37.8±4.4 g/L of glucose. Sago fibres served as an excellent raw material to produce glucose, and thus represent an effective alternative and readily manageable option.

Kinetics of cell growth

Table 1 summarizes the kinetics of the fermentation during the conditions tested. It was observed that fermentation started at DCW of 0.76 g/L, then grew exponentially during the first 48 h of the incubation period. The yeast gradually entered the stationary phase during the last three days of incubation. In the stationary phase, the highest OD from 72 h to 96 h was 1.014 (OD). Prior to fermentation, the cells were refreshed in universal bottles that contained fresh medium of 20 g/L of glucose and 5 g/L of yeast extract for 24 h. Then, the cells were transferred to the inoculum medium containing 8 g/L of glucose and 1 g/L of yeast extract for 12 h of inoculum. Transferring the cells ensured the cells avoided the long lag phase and accelerated growth during fermentation.

Cell growth

Dry cell weight (DCW) determination was used to monitor cell growth. Table 1 shows that the initial cell concentration was 0.76 g/L. DCW continued to increase as cultivation hours progressed. The highest DCW of 9.6 g/L was obtained at 96 h. A similar result was reported by Radulescu *et al.* (2009) where the result of DCW was 9.5 g/L at 84 h (Radulescu *et al.*, 2009). Therefore, the OD and DCW were directly correlated to each other. The overall yield of metabolites was concordant to the total biomass yield. Optimal cultivation conditions are crucial in order to maintain both high flow carbon efficiency to carotenoids and high fermentation growth rates to achieve maximum metabolite productivity (Marova *et al.*, 2011). The data for DCW was used to calculate the specific growth rate (μ). The μ of the yeast increased exponentially to 0.130 1/h in the first 12 h of incubation period (Table 1). The growth rate continued to increase after 12 h until it reached the maximum growth rate of 0.18 1/h at 48 h. After 48 h, the growth rate of yeasts decreased exponentially to 0.09 1/h at 72 h. At 96 h the cells did not register more growth. When specific growth factors or essential nutrients were exhausted, the cells entered stationary phase. From the data in Table 1, it is observed that cells experienced a faster decrease in overall growth rate when the cells entered stationary phase; therefore, cell proliferation was controlled at this stage.

Cell viability

Viability represents the survivability of *R. mucilaginosa* cells and was reported as CFU/mL. The percentage was obtained by dividing the number of living cells by the total number of cells. The haemocytometer technique alongside the use of methylene blue dye indicates the presence of an enzyme in a yeast cell, rather than the capability of the cell to divide. Although use of a hemocytometer is less accurate compared to slide count and plate cell count methods, it provides a very useful rapid indication of the number of viable yeast cells compared to traditional plate count methods which provide results only 3 or 4 days after inoculation (Painting and Kirsop, 1990). From Table 1, cell viability (reported as the Log_{10} of CFU/mL) increased as the period of incubation increased. Initial cell concentration started with 4.9 CFU/mL, then entered exponential growth phase where the viable cells increased to 5.39 CFU/mL in 48 h, and 5.43 CFU/mL at 96 h. Entering the plateau or stationary growth phase could be due to a combination of various adverse environmental conditions inhibiting the growth rate of cells. Viable yeast cells contain an enzyme that decolours methylene blue dye. Therefore, they appear as colour-less cells under the microscope. The blue colour was observed in nonviable yeast cells because they do not have the ability to reduce methylene blue dye (Painting and Kirsop, 1990). Table 1 shows the time profile of residual glucose. It is observed that the cells continuously metabolized glucose from the fermentation broth and not all the glucose was consumed, since 13.06 g/L of glucose remained when the fermentation was stopped. The percentage of glucose consumption was approximately 65% of the initial glucose concentration.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 14(4) Special Issue 2018, pp. 344-350
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Table 1: Kinetics for the fermentation of hydrolysed sago starch in batch mode.

					Residual	Productivity of total carotenoids	
Cultivation time (h)	OD	μ (1/h)	DCW (g/L)	CFU (log 10)	Glucose (g/L)	Volumetric (mg/L)	Specific (μg/g)
0	0.52	0	0.76	4.91	37.67	0.44	582.00
12	0.63	0.130	1.19	5.22	34.52	0.78	663.23
24	0.69	0.150	2.16	5.25	30.50	1.40	648.20
36	0.80	ND	3.96	5.26	24.02	2.64	664.61
48	1.013	0.18	8.47	5.39	20.72	6.27	739.47
72	1.014	0.09	9.01	5.42	17.02	6.87	761.80
96	1.014	0	9.59	5.43	13.06	8.11	845.89

Carotenoid assay

Production of pigment was confirmed by analysing the yeast cell pellets. Figure 3 shows a blue colour interphase appearing after the carotenoid assay was performed, indicating the presence of carotenoids in the sample. To establish the presence of carotenoids, concentrated sulphuric acid was used to produce the characteristic colouration (Karrer and Jucker, 1950). Polyene pigments are best identified by first destroying the surrounding substances, such as fats, before applying the colour tests. The pellet was first treated with concentrated aqueous alcoholic alkali that dissolved the fats and at the same time release the carotenoids within the cells. Chloroform was used as an extractant solvent for fats. Phytoxanthin esters were hydrolysed, and phytoxanthins were liberated. Hence, the presence of carotenoids was shown by a blue colour reaction. Polyene pigments are well known to liberate blue solution when treated with a range of strong acids such as concentrated sulphuric acid. This reaction occurs when a layer of concentrated sulphuric acid is formed under an ethereal solution of pigment. The sulphuric acid layer yielded an intense dark blue to blue-violet colour. Although the colour reactions were not specific, they could be used as qualitative tests (Karrer and Jucker, 1950). Carotenoids have a conjugated double bond system. This produce major problems associated with manipulation and work on carotenoids, especially their instability towards oxygen, heat and light. Acid and alkaline conditions are also detrimental to carotenoids. Degradation, transformation or even change in composition may happen when any of these factors are present in a sample (Oliver and Palou, 2000). Therefore, several precautions were taken.

Manipulations were performed under low light and temperature to avoid direct contact with sunlight. Carotenoid sample preparation and analysis hence required quick work. The pigments were supplemented with butylated hydroxyl toluene (BHT), an antioxidant to increase stability and prevent oxidation (Oliver and Palou, 2000). Finally, the samples were stored in the dark at about –20 °C for further analysis whenever necessary.

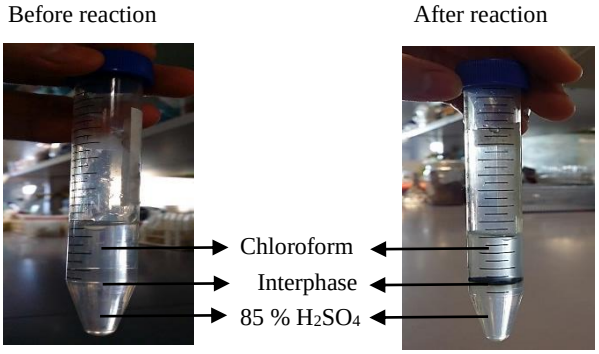

Figure 3: Colour change when the pellet was treated with chloroform and 85% sulfuric acid. A blue colour interphase indicates the presence of carotenoids.

Carotenoid extraction and quantification

Secondary metabolism refers to pathways of metabolism that are not essential for the survival of the organism. When nutrients are depleted, *Rhodotorula* starts to synthesize an array of secondary metabolites (pigments) in order to ensure the survival of cells (Marova *et al.*, 2011). From Figure 4, it is interesting to notice the greater carotenoid pigment accumulations in late logarithmic phase proceeding to stationary phase (starting at 48 h). Radulescu *et al.* (2009) reported total carotenoids of *R. rubra* ICCF 209 obtained using crude extract was 722.23 μg/g dry cell weight. Similarly, the data in Figure 4 shows that in the 96 h of cultivation, the highest carotenoid production in *R. mucilaginosa* was 845.89 μg/g dry cell weight. Figure 4 shows the lowest value of dry cell weight and cellular carotenoid, 0.763 g/L and 582 μg/g dry yeast respectively, at 0 h. The highest value of dry cell weight was 9.588 g/L and highest value of cellular carotenoid yield was 845.89 μg/g dry yeast at 96 h. This shows that the carotenoid yield was directly related to total biomass yield. Therefore, the data obtained suggests that *R. mucilaginosa* is a promising microorganism for commercial carotenoid production using SSH media. One constraint was the complicated liberation and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

bioavailability of carotenoids due to the presence of a thick cell wall (Marova *et al.,* 2013). Therefore, effective and well-characterized techniques of disruption were needed. There were some cellular properties that affect the ease of cellular disruption, requiring varied methods. For example, size or type of cell and the structure of the cell wall. Yeasts synthesize carotenoids when provided with a synthetic medium that contains various simple carbon sources; for example, glucose, sucrose, glycerol, cellobiose, xylose and sorbitol (Marova *et al.*, 2013). DMSO was employed to disrupt the cells in this study due to the previous study has reported that it is efficient to extract carotenoids (Sumanta *et al.*, 2014; Kumar and Keun, 2018).

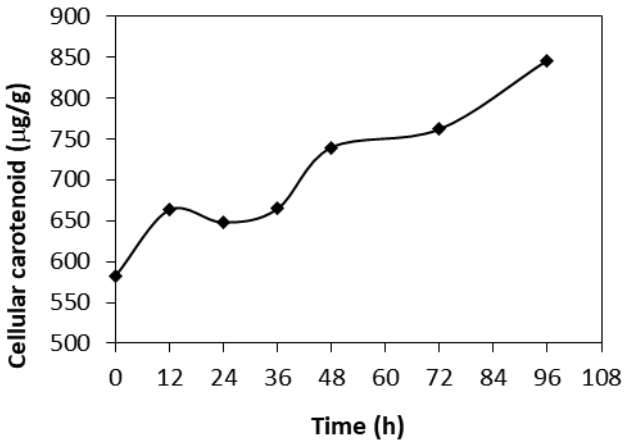

Figure 4: Kinetics of carotenoid production obtained by cells disruption using DMSO.

CONCLUSION

Rhodotorula mucilaginosa grown on SSH reached a highest dry cell weight of 9.6 g/L. The carotenoid produced was 8.11 mg/L and the specific yield was 845.89 μg/g at 96 h of cultivation. *R. mucilaginosa* grown at room temperature on substrate derived from sago fibres shows great potential to be an inexpensive and environmentally friendly method for carotenoid production. It could lower the overall cost of production. Bioconversion of sago fibres could be economically valuable for the production of fermentable sugar, while helping to minimise environmental problems associated with agro-residue.

ACKNOWLEDGEMENTS

The authors would also like to thank UNIMAS and biochemistry laboratory for continuously providing the materials and apparatus for the completion of the project. The project was funded by the grant F02/TOC/1514/2016.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538