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Carotenoid Production by *Rhodosporidium paludigenum* Using Orange Peel Extract as Substrate

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Abstract. Carotenoids are fat-soluble pigments that have various benefits in health and beauty. The demand and market of carotenoids are increasing; thus, the faster and cheaper biological production of these pigments using microorganisms is desired. In this study, the optimum medium pH and nitrogen concentration for carotenoid production by *Rhodosporidium* (*R.*) *paludigenum* using orange peel extract as substrate were determined. The soluble sugars from orange peels were extracted using distilled water at 100 °C. The yeast inoculum was cultivated in Yeast Peptone Dextrose (YPD) media before fermentation. The independent variables in the fermentation factorial design included initial medium pH (5, 6, and 7) and nitrogen concentration (0, 1, and 1.75 g/L urea in the medium). The decrease in reducing sugar concentration and the increase in biomass dry weight during fermentation demonstrated carbon source consumption by *R. paludigenum* for growth and carotenoid production. On the sixth day of fermentation (D6), the culture was harvested for the total carotenoid extraction and determination. The highest total carotenoid yield (107.63 µg/g) was achieved by the treatment group with an initial medium pH of 6 without nitrogen supplementation. This result indicated the orange peel extract potential as a substrate for carotenoid production using *R. paludigenum* as a workhorse. This was the first reported research in natural carotenoid generation using *R. paludigenum* (strain InaCC Y-236) locally isolated in Indonesia as the producer and orange peel extract as the substrate. Thus, this research would enrich the microbial carotenoid exploration and waste valorization to value-added products at the same time. The study can then be developed further and upscaled for industrial applications.

Key words: Carotenoids; Nitrogen; Orange Peel; pH; *Rhodosporidium paludigenum*

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INTRODUCTION

Carotenoids are a group of pigments that show a range of colors from red to yellow and consist of 40-carbon isoprenoid with more than 600 characterized structures. In general, these pigments are divided into two groups, i.e., carotenes (such as β-carotene) and xanthophylls, which are the oxidized form of carotenoid (such as astaxanthin). These pigments are naturally found in lipid droplets of fruits, vegetables, flowers, and microorganisms (Machado & Burkert, 2015; Mannazzu et al., 2015). Carotenoids are beneficial in various fields, including pharmaceuticals and food industries, due to their antioxidant activity and function as vitamin A precursor (Mata-Gómez et al., 2014).

Carotenoids can be chemically synthesized, however, the social demands of natural food and supplements are increasing gradually. Therefore, biotechnological methods to produce natural carotenoids via *de novo* microbial synthesis become attractive alternatives to be developed. Moreover, microbial carotenoid production is faster, does not depend on seasons, and requires relatively smaller facilities compared to the process using plants. Culture conditions of microbial carotenoid production can be controlled and adjusted to obtain natural pigment quality and quantity as expected (Machado & Burkert, 2015; Mannazzu et al., 2015).

The ability of Basidiomycetous yeasts in producing carotenoids has been known before. These yeasts grow in a red-pigmented colony and thus are usually classified as red yeasts. Red yeasts are highly potential to be developed as cell factory in the biotechnological production of carotenoids. The biosynthesis pathways of carotenoid generation in red yeasts have been investigated rigorously, and thus, the study of industrial-scale carotenoid production has gained more attention (Mannazzu et al., 2015). Various genera of red yeasts such as *Rhodotorula* (Tarangini & Mishra, 2014; Zhang et al., 2014), *Xanthophyllomyces* (Castelblanco- Matiz et al., 2015), *Rhodosporidium* (Dias et al., 2015; Singh et al., 2016), *Sporidiobolus* (Han *et* al., 2016), and *Sporobolomyces* (Cardoso et al., 2016) are known to produce carotenoids naturally. Red yeasts from genus *Rhodosporidium* are vastly studied for their potential carotenoid production. However, carotenoid production by species *Rhodosporidium* (*R.*) *paludigenum* is still poorly investigated. This species grows individually or in pairs, shows a smooth, shiny, salmon orange-colored colony (Kurtzman et al., 2011), and can be discovered in soil, tree extrudates, freshwater, sediments, and marine fishes (Yimyoo et al., 2011).

Selecting the right substrate is one of the essential factors for red yeast cultivation in carotenoid production. For that reason, a cheap and highly efficient substrate such as agricultural wastes will be more profitable for industrial-scale carotenoid production. Several types of lower-cost substrates have been investigated for carotenoid production by red yeasts, such as sugar cane juice (Bonadio et al., 2018), whey, molasses, corn steep liquor (Galal & Ahmed, 2020), and other agro-industrial waste (Mata-Gómez et al., 2014). Fruit waste is a potential substrate for microbial carotenoid production due to its high contents of carbons and minerals (Tarangini & Mishra, 2014). Orange is one of the most consumed fruits globally because it has many beneficial substances, such as alkaloids, flavonoids, lycopene, vitamin C, pectin, and tannins (Rafsanjani & Putri, 2014). Thus, orange peel belongs to the main contributor of food waste every year worldwide (FAO, 2016). Orange peel waste is biodegradable and highly potential to be valorized for value-added products. It has various valuable compounds such as limonene (Jha et al., 2019; Ozturk et al., 2019), carbohydrate polymers, and fermentable sugars (Ozturk et al., 2019). Moreover, orange peel also contains carotenoid precursors including geranylgeranyl pyrophosphate (GGPP) that involves in the carotenoid biosynthesis by the methyl-D-erythritol-4-phosphate (MEP) pathways (Loto et al., 2012). The phytoene synthase enzyme was also found in the orange peel, which converts GGPP to phytoene in the biosynthesis pathway (Lado et al., 2019). Phytoene was subsequently transformed through a series of biochemical reactions to generate carotenoids (Córdova et al., 2016; Lado et al., 2019; Rodrigo et al., 2013). Red yeasts such as *R. paludigenum* can be employed to enhance the production of carotenoid pigment from orange peel waste due to its ability to multiply fast and produce intracellular carotenoids. However, there is not much research investigating *R. paludigenum* as a carotenoid producer using agro-industrial waste as the substrate. Therefore, this study aimed to determine the optimum initial medium pH and nitrogen concentration for carotenoid production by *R. paludigenum* using orange peel extract as the substrate. Moreover, the strain of *R. paludigenum* used in this study, i.e., *R. paludigenum* InaCC Y-236, was locally isolated by Kanti et al. (2013) from *Piper* (*P*.) *nigrum* and *P. betle* plants in Bali and West Java, Indonesia (Kanti et al., 2013). This was the first reported research in carotenoid production by this specific *R. paludigenum* strain using orange peel extract as the substrate. The results of this study could complement the progression on the microbial carotenoid production and agro-industrial waste recovery. When implemented on a bigger scale, the biomass obtained could be developed as a nutritious feed, and the extracted carotenoid could be applied as natural food and cosmetics colorants (Mannazzu et al., 2015; Mata-Gómez et al., 2014).

METHODS

Substrate preparation

Malang *keprok* orange (*Citrus* sp.) peels were obtained from a fruit juice seller located in Intermoda Modern Market, Cisauk, Tangerang, Indonesia. The soluble sugars from orange peels were extracted by adding 2 liters of distilled water at 100 °C for 30 minutes. The extract was filtered using filter paper and stored at 4 °C for further use. The reducing sugar concentrations in the substrate were then adjusted to ± 20 mg/mL before experimentation.

Inoculum preparation

R. paludigenum (InaCC Y236) was purchased from Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia. The yeast was cultured on Potato Dextrose Agar (PDA) and incubated for seven days in 28 °C. Before being used for further fermentation, *R. paludigenum* was cultivated in Yeast Peptone Dextrose (YPD) media (20 g/L glucose, 10 g/L yeast extract, and 10 g/L peptone water) adjusted to pH 6.0 in 250 mL Erlenmeyer flask, and incubated in a water bath shaker (Model GFL 1086, Thermolab) at 120 rpm and 28 °C for 24 h.

Carotenoid production

The effect of initial medium pH (5, 6, and 7) and urea concentration (0, 1, and 1.75 g/L) as nitrogen source on carotenoid production by *R. paludigenum* was evaluated in 250 mL Erlenmeyer flask with working volume of 50 mL. The experiment was divided into nine treatment groups as showed in Table 1. The initial medium pH was adjusted using 1 M NaOH and 1 M HCl. Each flask was then inoculated with 1.53×10^6 cells/mL of *R. paludigenum* inoculum. Fermentation process for carotenoid production was performed in a water bath shaker (Model GFL 1086, Thermolab) at 120 rpm and 28 °C for 6 days. Each treatment group was performed in triplicate.

Table 1. Nine treatment groups for carotenoid production

Treatment group	I	II	III	IV	V	VI	VII	VIII	IX
Initial medium pH	5	5	5	6	6	6	7	7	7
Initial urea concentration (g/L)	0	1	1.75	0	1	1.75	0	1	1.75

Determination of biomass dry weight

Biomass dry weight was measured for six consecutive days by harvesting cell pellet after centrifugation of 1.5 mL culture at 2,400 x g for 10 minutes (Model Microcentrifuge, Sorvall Pico). Cell pellet was dried at 80 °C until constant weight was obtained (Tarangini & Mishra, 2014). The supernatant was kept for determination of reducing sugar concentrations.

Determination of reducing sugar concentration

Reducing sugar concentration was measured for six consecutive days using dinitrosalicylic acid (DNS) assay method. The supernatant was added with 0.5 mL distilled water and 1.5 mL DNS solution in a tube. The mixture was then heated for 10 minutes and cooled at room temperature. The absorbance of the mixture was measured at 540 nm using a vis spectrophotometer (Model Genesys 20, Thermo Scientific). Reducing sugar concentrations were then calculated based on the standard curve of absorbance versus glucose concentrations (0.15, 0.30, 0.45, 0.60, and 0.75 mg/mL).

Carotenoid extraction

After six days of cultivation, the culture was harvested and centrifuged at 8,570 x g for 10 minutes. The pellet was lyophilized (Model FD-551, Eyela, Japan) for 24 hours. The lyophilized cells were mashed, and carotenoids were extracted by suspending 0.2 g mashed cells in 3 mL acetone. The suspension was vigorously mixed with a vortex for one minute and ultrasonicated (Model 150 VT, Ultrasonic Homogenizer, BioLogics) in 40 kHz for 10 minutes. It was subsequently centrifuged at 16,200 x g for two minutes (Model Microcentrifuge, Sorvall Pico) to separate the remaining cell debris (Hernández-Almanza et al. 2017; Manasika & Widjanarko, 2014).

Determination of the total carotenoid

The total carotenoid concentration was determined by measuring the absorbance of the first layer of supernatant using spectrophotometry method. The absorbance of the total carotenoid was measured using a vis spectrophotometer (Model Genesys 20, Thermo Scientific) at 455 nm. The total carotenoid concentration was calculated based on the standard curve of absorbance versus standard β-carotene (Merck, Darmstadt) concentrations (0, 1.6, 3.2, 6.4, and 9.6 µg/mL) (Hernández-Almanza et al., 2017; Manasika & Widjanarko, 2014).

RESULTS AND DISCUSSION

Changes in reducing sugar concentration and biomass dry weight

Changes in the medium opacity and biomass density had been noticeable from the third day of fermentation (D3). On the inoculation day (D0), fermentation medium was considerably translucent, whereas on the D3, it was visibly opaque, which indicated the increase of yeast biomass density (Figure 1).

Table 2 shows the reducing sugar concentrations of the medium for each treatment group on the day 0, 3 and 6 (D0, D3, and D6) of fermentation. The decrease in reducing sugar concentrations indicated carbon source consumption by *R. paludigenum* for growth and carotenoid production.

Table 3 demonstrates biomass dry weight for each treatment on D0, D3, and D6. In most treatment groups, the biomass dry weight increased until D3 and then decreased on D6. In treatment group II and IV, however, the biomass dry weight increased until D6.

A **B** **C**

Figure 1. *Rhodosporidium paludigenum* culture on potato dextrose agar (A), as well as in orange peel extract medium on the inoculation day (B) and the third day of fermentation (C)

Table 2. Reducing sugar concentrations

Treatment group	Initial medium pH	Initial urea concentration in the medium [g/L]	Reducing sugar concentration in fermentation day 0-6 [mg/mL]		
			D0	D3	D6
I	5	0	22.02 ± 0.00	5.93 ± 1.01	0.83 ± 0.02
II	5	1	21.98 ± 0.00	7.01 ± 0.45	0.99 ± 0.06
III	5	1.75	19.54 ± 0.00	1.07 ± 0.03	0.94 ± 0.03
IV	6	0	22.02 ± 0.00	7.29 ± 0.75	0.83 ± 0.03
V	6	1	20.16 ± 0.00	3.49 ± 0.99	0.86 ± 0.08
VI	6	1.75	19.54 ± 0.00	1.12 ± 0.04	0.89 ± 0.03
VII	7	0	20.69 ± 0.00	2.95 ± 0.53	0.57 ± 0.03
VIII	7	1	20.69 ± 0.00	0.72 ± 0.03	0.76 ± 0.02
IX	7	1.75	20.69 ± 0.00	1.15 ± 0.03	0.79 ± 0.05

Note: Data were mean of triplicate ± standard error; D0: the inoculation day, D3: third day of fermentation, D6: sixth day of fermentation

Table 3. Biomass dry weight

Treatment group	Initial medium pH	Initial urea concentration in the medium [g/L]	Biomass dry weight in fermentation day 1-6 [mg/mL]		
			D0	D3	D6
I	5	0	4.07 ± 1.76	13.40 ± 1.80	11.97 ± 0.87
II	5	1	5.53 ±0.32	11.97 ± 0.87	12.83 ± 0.37
III	5	1.75	11.87 ± 3.37	13.90 ± 2.22	8.60 ± 2.10
IV	6	0	6.00 ±0.49	12.80 ± 1.64	17.90 ± 3.52
V	6	1	5.37 ± 1.45	16.10 ± 1.66	14.23 ± 1.62
VI	6	1.75	7.70 ± 0.40	11.60 ± 1.16	7.87 ± 2.27
VII	7	0	2.57 ± 0.52	7.93 ± 0.69	7.10 ± 0.40
VIII	7	1	7.03 ± 0.55	8.83 ± 0.49	7.37 ± 1.05
IX	7	1.75	7.90 ± 1.58	8.33 ± 0.59	6.77 ± 0.92

Note: Data were mean of triplicate ± standard error; D0: the inoculation day, D3: third day of fermentation, D6: sixth day of fermentation

Table 2 and 3 illustrate that reducing sugars in orange peel extract was utilized by *R. paludigenum* for its biomass growth. The reducing sugar concentration was depleted on D3 and D6 (Table 2). Orange peel contains soluble sugars, such as glucose, sucrose, fructose, and xylose (Javed et al., 2019). Insoluble polysaccharides such as pectin, cellulose, and hemicelluloses were also contained in orange peel (Torrado et al., 2011). However, until now, no study has shown that polysaccharides-converting enzymes can be produced by *R. paludigenum*. Therefore, in this study, soluble sugars extracted from orange peel might be the primary carbon source for biomass growth. The concentration of reducing sugars on D6 was less than 1 mg/mL, indicating the yeast's sugar consumption. Treatment group II and III with initial pH medium of 5 showed the highest level of the remaining reducing sugars, i.e., close to 1 mg/mL.

In this experiment, the yeast culture was exposed to oxygen since the system was not maintained anaerobically. Thus, the yeasts can use oxygen for their aerobic respiration, which eventually led to biomass growth. The yeasts consumed the sugar in the substrate, and it was broken down in a series of catabolic reactions to produce energy in the form of ATP. The complete breakdown of the sugar in aerobic respiration yielded more ATP than the process in anaerobic respiration. The ATP produced was then utilized for anabolic reactions in which the biomass was composed (Behera & Varma, 2017). Hence, it can be deduced that the cells underwent growth and replication by obtaining energy from glucose through glycolysis and the subsequent catabolic reactions. During yeast growth, there was a direct relationship between the amount of substrate consumed and the amount of biomass produced. Nevertheless, sugar as the carbon source was not the only substrate component that affects biomass growth. Temperature, pH, medium composition, as well as the nature of carbon and nitrogen source could influence biomass yield (Behera & Varma, 2017).

The logarithmic phase of the yeast growth was observed from D0 until D3. The lag phase was less than 24 hours (not shown), and the stationary phase was observed from D3 until D6 (Table 3). Tarangini and Mishra (2014) conducted a similar study to produce carotenoid by *Rhodotorula* sp. using fruit waste extract as the sole carbon source. The study showed

comparable results concerning the growth phase of the yeasts. The stationary phase was started on D3 and lasted until D6, which was exhibited by the constant biomass dry weight. In our study, the biomass dry weight decreased from D3 to D6 in all treatment groups, except group II and IV, which increased slightly. These results might indicate that the stationary phase of the yeast growth was not yet ended on D6 of fermentation in treatment group II and IV. Nasirian et al. (2018) demonstrated that the biomass concentration of yeast *R. diobovatum* reached its peak value at 144 hours of cultivation on initial glucose concentration of 40 g/L and pH 5.5. In that study, about 7 g/L glucose as substrate was also replenished into the culture at 120 hours of cultivation. In this experiment, the increase of biomass concentrations on D6 in treatment group II dan IV might be due to the relatively high levels of the remaining reducing sugars on D3, which were 7.01 ± 0.45 and 7.29 ± 0.75 mg/mL respectively. These concentrations were higher than those shown by other treatment groups on the same cultivation day. Thus, the yeasts in treatment group II dan IV still had more substrate to consume and stimulate their growth up to D6, after which the reducing sugar concentrations were negligible, and the cell mass decreased.

Total carotenoid concentration

In this study, the total carotenoid was extracted from the yeasts at the late stationary phase (on D6 of fermentation). The highest total carotenoid concentration was acquired in treatment group IV (107.63 µg/g) with an initial pH of 6 and without urea addition in the medium (Table 4).

Table 4. Total carotenoid concentration on the sixth day of fermentation

Treatment group	Initial medium pH	Initial urea concentration in the medium [g/L]	Total carotenoid concentration	
			[µg/g]	[µg/mL]
I	5	0	63.15	3.95
II	5	1	24.74	1.55
III	5	1.75	12.03	0.75
IV	6	0	107.63	6.73
V	6	1	50.27	3.14
VI	6	1.75	7.60	0.48
VII	7	0	42.18	2.64
VIII	7	1	14.91	0.93
IX	7	1.75	44.21	2.76

The availability of carbon sources, such as reducing sugars in this experiment, was essential for carotenoid biosynthesis at the late stationary phase of growth, close to the death phase when the biomass yield decreased. Lee et al. (2014) studied the carotenoid production at different growth phases by three strains of *R. toruloides* cultivated on glycerol. The maximum biomass concentration was reached during the late stationary phase, which was in line with the concentration of torularhodin, torulene, and β-carotene produced (Lee et al., 2014). Using the most eminent species of basidiomycetous carotenogenic yeast, *X. dendrorhous*, Loto et al. (2012) elucidated similar results. In that research, the highest carotenoid concentration was achieved at the late stationary phase, i.e., after 120 h of cultivation. Another study performed by Castelblanco- Matiz et al. (2015) showed that the total carotenoid content produced by the wild-type and mutant *X. dendrorhous* achieved its highest values (99.2 and 470.1 µg/g respectively) at the late stationary phase. By employing the same species of red yeast, Córdova et al. (2016) investigated carotenogenesis in wild-type and mutant strains. In both strains, the maximum carotenoid generation was also obtained at the late stationary phase (Córdova et al., 2016). It was generally discovered in most microorganisms that the secondary metabolites are expressed at the late stationary phase during nutrient starvation or limitation (Yang et al., 2014). The secondary metabolites are produced after the logarithmic phase because they are not essential for growth under normal circumstances. The metabolic profiling that was conducted by Lee et al. (2014) indicated the metabolic changes in *R. toruloides* during different growth phases, i.e., logarithmic or exponential phase (day 1 and 4), stationary phase (day 7), and late stationary phase (day 12). It was revealed that during the late stationary phase, the majority flow of the metabolic flux from acetyl-CoA was directed toward fatty acid and carotenoid biosynthesis, rather than tricarboxylic acid (TCA) cycle and amino acid metabolic pathway (Lee et al., 2014). The expression of carotenoids as secondary metabolites, however, might be regulated by different molecular mechanisms that may lead to possible diverse results under certain conditions (Thapa & Grove, 2019).

The carotenoid concentrations obtained in this experiment compared to those in other studies might be

different due to the difference in the species of carotenoid producers, carbon and nitrogen concentration, substrate and medium composition, mode of culture, as well as the method of extraction and carotenoid content analysis. Nevertheless, most findings showed that the optimum initial medium pH for carotenoid production was below 7. Yimyoo et al. (2011) used glycerol as the sole carbon source for carotenoid production by *R. paludigenum* strain DMKU3-LPK4. In that experiment, 400 µg/g of total carotenoid was extracted from 6.90 g/L dry cell mass on 132 hours of cultivation, with initial pH of 6, 0.559 g/L urea as nitrogen source, and 40 g/L glycerol as carbon source (Yimyoo et al., 2011). El-Banna et al. (2012) used 25 g/L glucose and 5 g/L urea to produce 279 µg/g total carotenoid from 6.67 g/L dry biomass of *Rhodotorula glutinis* var. *glutinis* with initial medium pH of 6.2. Tarangini and Mishra (2014) showed approximately 1.8 mg/L total carotenoid content on D6 of *Rhodotorula* sp. fermentation using fruit waste extract as sole carbon source, with initial medium pH of 7 and initial glucose concentration of 19.9 mg/mL. Nasirian et al. (2018) acquired 324 µg/g carotenoid from *R. diobovatum* at 144 hours (D6) of fermentation with initial medium pH of 5.5. These results indicated the effect of pH on carotenoid biosynthesis.

In plants, it was known that a predominant control of photosynthesis depends on the xanthophyll cycle. This cycle includes the conversion of carotenoid violaxanthin into zeaxanthin to provide photoprotection mechanisms. Violaxanthin deepoxidase is the enzyme that plays a role in zeaxanthin synthesis. At neutral pH, violaxanthin deepoxidase is soluble and inactive. Under intense light, energy input surpasses the photosynthetic capacity, which leads to the over-acidification of thylakoid lumen. This event subsequently activates violaxanthin deepoxidase (Hallin et al., 2016). At acidic pH, violaxanthin deepoxidase attaches to the thylakoid membrane and binds its violaxanthin substrate (Fufezan et al. 2012). A similar enzyme might involve in the carotenoid biosynthesis pathways of any carotenoid-producing microorganisms such as *R. paludigenum*. However, the exact mechanism has not been identified in any available studies, although it was suggested that carotenoid production might be regulated post-translationally (Pinheiro et al., 2020).

The role of nitrogen has been known for lipid (Insan et al., 2018) and carotenoid (Han et al., 2016) biosynthesis in oleaginous microorganisms. However, in this study, the highest carotenoid concentration was obtained from *R. paludigenum* culture that was cultivated without urea addition as the nitrogen source. These results might happen due to the pre-existing nitrogen content in the orange peel extract. Jariwala and Syed (2016) reported that citrate peel powder consisting of orange, sweet lime, and pomegranate peel powder contained 9.1 mg/g total nitrogen. Santos et al. (2015) described that dried orange peel contained 1.5% (w/w) nitrogen. Pathak et al. (2017) showed the proximate and ultimate analysis of citrus/orange peel. They stated that citrus/orange peel contained 0.64 - 1.15% nitrogen. This information confirmed that the orange peel extract in this study might have already carried nitrogen. Therefore, adding urea to the culture medium might increase the nitrogen content to the level that could negatively influence microbial carotenoid biosynthesis. The addition of urea as a supplemented nitrogen source decreased the C/N ratio in the medium. Studies have shown that the C/N ratio can affect lipid and carotenoid production in oleaginous yeasts, including *R. paludigenum* (Elfeky et al., 2019; Han et al., 2016; Lopes et al., 2020; Yimyoo et al., 2011).

Yimyoo et al. (2011) demonstrated that the carotenoid concentrations produced by *R. paludigenum* at the C/N ratio of 40 (2.61 mg/L), 60 (2.96 mg/L), and 80 (2.63 mg/L) were higher than those at the C/N ratio of 20 (0.64 mg/L). Nevertheless, the C/N ratio of 100 yielded a lower carotenoid concentration (1.80 mg/L) than those obtained at the C/N ratio of 40, 60, and 80 (Yimyoo et al., 2011). Lopes et al. (2020) presented the highest carotenoid concentration (353.7 µg/g) in *Rhodotorula toruloides* at the high C/N ratio (120) using xylose as a carbon source.

Han et al. (2016) showed that although the cell dry weight increased, the lipid and carotenoid content of oleaginous yeast *Sporidiobolus pararoseus* decreased gradually with the increase of nitrogen concentration in the medium. A biochemical mechanism might explain this phenomenon. After exhaustion of nitrogen in the medium, the lipid and carotenoid synthesis pathway was initiated, which led to a rapid reduction of the cellular AMP concentration. The metabolism of citrate in the TCA cycle at the mitochondria was then halted, resulting in a higher level of citric acid inside the mitochondria than the critical value (Han et al., 2016). Consequently, citric acid was secreted to the cytoplasm. In the cytoplasm, citric acid was converted by ATP-citrate lyase into acetyl-CoA and oxaloacetate (Han et al., 2016). ATP-citrate lyase is a vital enzyme found in the oleaginous microorganisms, and under nitrogen exhaustion, this enzyme was expressed in higher levels (Lopes et al., 2020; Pinheiro et al., 2020). The acetyl-CoA in the cytoplasm was then available for fatty acid biosynthesis (Tchakouteu et al., 2014).

Another mechanism could be explained by the possible content of xylose in the orange peel extract. Xylose was preferably metabolized through the phosphoketolase pathway, depending on the C/N ratio (Lopes et al. 2020). Under nitrogen limitation (high

C/N ratio), phosphoketolase activity was increased, which further generated acetyl-CoA as a precursor of fatty acid synthesis. This pathway can generate acetyl-CoA by phosphate transacetylase without losing a carbon compared to the common pathway originating from pyruvate (Pinheiro et al., 2020). Acetyl-CoA can subsequently enter the mevalonate (MEV) pathway through acetyl-CoA C-acetyltransferase enzyme to produce carotenoids (Pinheiro et al., 2020). These mechanisms might elucidate the correlation between nitrogen limitation in this study and the availability of acetyl-CoA for carotenoid synthesis. In oleaginous yeast such as *Yarrowia lipolytica*, lipid accumulation has been known to be related to an increased carotenoid level under nitrogen limitation (Gao et al., 2017; Matthäus et al., 2014). It was elucidated that carotenoids such as lycopene (Matthäus et al., 2014) and β-carotene (Gao et al., 2017) were stored in lipid bodies. Hence, the increase of lipid body formation would lead to an enhanced carotenoid storage capacity.

Elfeky et al. (2019) showed that in a high C/N ratio, cellular carotenoid accumulation increased significantly in *Rhodotorula glutinis*. At the high C/N ratio, nitrogen depletion occurred faster, which afterward induced the shifting of lipid production to the carotenoid synthesis pathway, to protect the cells from oxidative damage (Elfeky et al., 2019; Han et al., 2016). The antioxidant activity of pigments in photosynthetic microorganisms has been recognized (Dimarti et al., 2020). Pinheiro et al. (2020) demonstrated that under nitrogen limitation, proteins related to oxidative stress response, such as catalase, was upregulated. In oleaginous microorganisms, reactive oxygen species (ROS) becomes a critical signalling molecule in response to various cellular stresses (Shi et al., 2017). Several studies using oleaginous microorganisms have shown that ROS accumulation stimulated lipid biosynthesis in response to nitrogen exhaustion (Chokshi et al., 2017; Fan et al., 2014; Liu et al., 2012) and increased the activities of antioxidant enzymes including catalase. Thus, lipid and carotenoid accumulation under nitrogen starvation might be mediated by oxidative stress (Yilancioglu et al., 2014; Zhang et al., 2013). This suggestion might also answer why the highest level of carotenoids produced by *R. paludigenum* in this experiment was obtained without urea addition in the orange peel extract medium.

According to the author's knowledge, up until now, there have been no experimental studies concerning the effects of initial pH and nitrogen concentration on the natural carotenoid production by *R. paludigenum* InaCC Y-236 using orange peel extract as the substrate. Therefore, this research could provide new insight into the microbial carotenoid investigation and agro-industrial waste valorization simultaneously. This study's results can be optimized further and used as the preliminary information for the bigger scale research and, eventually, industrial applications.

CONCLUSION

The optimum cultivation conditions for carotenoid production by *R. paludigenum* using orange peel extract medium in this experiment were achieved at initial pH of 6 and without nitrogen addition. It confirmed that a high C/N ratio was preferable for carotenoid production by *R. paludigenum* in this study. The highest carotenoid yield was 107.63 µg/g, which was promising but required optimization for future research and industrial applications.

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