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Carotenoid Production by *Rhodosporidium paludigenum* DMKU3-LPK4 using Glycerol as the Carbon Source

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ABSTRACT

Rhodosporidium paludigenum DMKU3-LPK4, a red yeast isolated from soil in the northern part of Thailand, exhibits the capacity to produce carotenoids with the application of glycerol as the sole carbon source. The optimum conditions for carotenoid production of this strain in shaking flask cultivation were a temperature of 32°C and an initial pH value of 6.0 in a medium of 40 g/L glycerol, 0.56 g/L urea, 1 g/L KH_2PO_4, and 0.5 g/L $MgSO_4.7H_2O$. The carotenoid concentration reached 3.42 mg/L with a dry cell mass of 7.59 g/L at 132 h. This study demonstrated the potential of *Rhodosporidium paludigenum* as a new carotenoid producer and of glycerol as a sole carbon source for carotenoid production.

Keywords: *Rhodosporidium paludigenum*, carotenoids, glycerol, culture conditions

INTRODUCTION

Biodiesel, one alternative energy source, is derived from vegetable oil or animal fats and blended with petroleum-based diesel fuels for use in present-day diesel engines (Kahraman, 2005). In biodiesel production processes, crude glycerol is generated as a by-product, amounting to approximately 10% of the weight of the oil or fat used. Crude glycerol is of low value and refining it to produce pure glycerol is considered too costly. The impurities of the crude glycerol consist of methanol, alcohol, bases and various inorganic salts. As the exponential increase in the demand for biodiesel generates increased amounts of glycerol, research is needed to find potential applications of this by-product, instead of simply disposal (Thompson and He, 2006).

Carotenoids are natural pigments that have been used commercially as food colorants, animal feed supplements, nutraceuticals and other applications (Gordon and Bauernfeind, 1982; Johnson and Schroeder, 1995). Carotenoids having over 600 different chemical structures have been isolated from natural sources and characterized (Britton, 1995). However, only some carotenoids, such as β-carotene, canthaxanthin, citranoxanthin and astaxanthin, have been produced commercially either by chemical synthesis or fermentation, and only small amounts have been isolated from natural sources (Bramley *et al*., 1993; Johnson and Schroeder, 1995). Increased demand for carotenoids is due to their antioxidant function and is driven by increased health concerns and an aging population, which have initiated renewed efforts to find ways for the production of additional carotenoids in the quantities demanded (Lee and Schmidt-Dannert, 2002).

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Red yeasts in the genera *Rhodotorula, Rhodosporidium, Sporidiobolus, Sporobolomyces, Cystofilobasidium, Kockovaella,* and *Phaffia* are known as potential natural alternative sources of carotenoids (Čertík *et al*., 2009). They offer versatility in commercial carotenoid production in industrial-scale fermentation because of their unicellular nature, relatively high growth rate, and their amenability to easily applied processes that have been established already and can be expanded. Red yeasts can use diverse sources of carbon, including low cost raw materials, such as molasses (Bhosal and Gadre, 2001), whey (Frengova, 2004) and agricultural industrial by-products, such as crude glycerol (Kusdiyantini *et al*., 1998; Mantzouridou *et al*., 2008; Easterling *et al*., 2009). In the current study, red yeasts, were isolated from various natural habitats and screened for carotenoid production. The most potent strain, *Rhodosporidium paludigenum*, then underwent preliminary evaluation of its potential for carotenoid production, using glycerol as a substrate with various culture conditions.

MATERIALS AND METHODS

Microorganisms

A total of 135 red yeast strains, isolated from various habitats in Thailand, were used from the private culture collection at the Department of Microbiology, Kasetsart University, Thailand. The yeast strains were maintained on slants of yeast extract-malt extract (YM) agar (3 g/L yeast extract, 3 g/L malt extract, 5g/L peptone, 10g/L glucose and 15g/L agar) at 8°C. Additional red yeast strains were isolated from soils, tree exudates, tree bark, natural water, sediments and marine fish collected in Thailand by enrichment techniques. Small samples were added to 50 mL of YM broth, adjusted to pH 3.7 with 1 N HCl , supplemented with 200 mg/L chloramphenicol and 1 g/L sodium propionate in a 250-mL Erlenmeyer flask, and incubated in a rotary shaker at 130 rpm and 15°C for 14 d. The enriched culture was then spread on YM agar plates and incubated at 28-30°C for 1-7 d. Red yeast colonies were selected and purified by cross streaking on YM agar and the pure cultures were kept on YM agar slants at 8°C.

Screening of red yeast for high carotenoid production

Red yeast was grown in 50 mL of basal broth (4 g/L yeast extract, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$) supplemented with 20 g/L glucose with pH 5.5 in a 250-mL Erlenmeyer flask and incubated on a rotary shaker (Model Bio-Shaker BR-300FL, TAITEC, Saitama-ken, Japan) at 130 rpm and 28°C for 84 h. The red yeast strains with high carotenoid production were selected for further evaluation. Secondary screening was performed by cultivation in 150 mL of basal broth supplemented with 40 g/L glycerol and incubated as outlined above.

Identification of red yeast

The morphological, biochemical and physiological characteristics of the selected strain were determined in accordance with the standard methods as described by Yarrow (1998). Assimilation of nitrogen compounds was examined on solid media with starved inocula, consistent with the method of Nakase and Suzuki (1986). The sequence of the D1/D2 domain of the large subunit (LSU) rRNA gene of the selected strain was determined. The methods used for DNA isolation, amplification of the D1/D2 domain of the LSU rRNA gene by Polymerase chain reaction (PCR), and sequencing with the ABI BigDye Terminator Cycle Sequencing kit (Applied Biosystems, California, USA) with the adoption of an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, California, USA) were as described previously (Limtong *et al*., 2007). The sequences were compared pairwise by using the BLASTN homology search (Altschul *et al*., 1997).

Evaluation of culture condition for carotenoid production of the selected red yeast strain using glycerol as a carbon source

Inoculum was prepared by cultivation of the selected red yeast strains on YM slants at room temperature for 24-48 h, transferring one full loop into basal broth with 20 g/L glucose and incubating on a rotary shaker at 130 rpm and 28°C for 18-24 h.

Main culture cultivation was carried out in a 500-mL Erlenmeyer flask containing 200 mL of basal broth supplemented with 40 g/L glycerol as the sole carbon source. Each flask was inoculated with the inoculum to obtain an initial cell concentration as an optical cell density of 0.5 at 660 nm. Incubation was performed on a rotary shaker at 130 rpm and 28°C for 132 h. All experiments were performed in triplicate.

The effects of the media components and environmental conditions on carotenoid production were studied. The effects of glycerol concentration were determined at the concentrations of 20, 40, 60, 80 and 100 g/L. Ammonium sulfate, sodium nitrate, urea, monosodium glutamate, mung bean meal, soybean meal and corn steep liquor at 0.456 g nitrogen/L were used to find the most suitable nitrogen source. Carbon to nitrogen (C:N) ratios investigated were 20:1, 40:1, 60:1, 80:1 and 100:1 at the optimal glycerol concentration.

The initial pH of the medium was adjusted to 5, 6, 7 and 8 with either 1 M NaOH or 1 M HCl to study the effects on growth and carotenoids production.

The effects of cultivation temperature were determined in a range from 14°C to 38°C in a 40-mL L-shape test tube containing 10 mL of medium of optimal composition and at the optimal pH, as determined in the shaking flask experiments; the test tubes were incubated in a temperature gradient incubator (Model TN-3, Toyo Kagaku Sangyo Co. Ltd., Tokyo, Japan).

Analysis of fermentation parameters

Yeast growth was determined at an optical density of 660 nm with a spectrophotometer (Model UV-1700, Shimadzu, Kyoto, Japan) after washing the yeast cells twice with reverse osmosis water.

The carotenoids concentration was determined after freezing cell pellets, which were obtained from 5 mL of culture broth kept at -20°C overnight. Carotenoids were extracted from cells by suspending the cell pellets in 2 mL dimethyl sulfoxide (DMSO). After the addition of 3 g of 0.5 mm diameter glass beads, the mixture was vigorously mixed with a vortex mixer for 30 sec. The DMSO phase was obtained by centrifugation at 3,500 rpm for 10 min and transferred to a new centrifugation tube. The remaining cell debris was extracted twice with 2 mL of acetone containing 0.05% (w/v) of butyrate hydroxytoluene and vigorously mixed. The blend was centrifuged and the acetone phase was combined with the DMSO extract. The extract was centrifuged again to remove insoluble particles. The amount of total carotenoid concentration in the mixture solution was calculated using the absorbance value, which was measured after the extraction procedure with an absorbance at 450 nm by a spectrophotometer using the extinction co-efficiency of $A_{1cm}^{1\%} = 2{,}500$, as proposed by Davies (1976).

Identification of major carotenoid pigment composition produced by strain DMKU3-LPK4 was performed with a high performance liquid chromatography (HPLC) (Agilent 1100 HPLC system, Palo Alto, USA) fitted with a Cosmosil C18-AR-II column (Nacalai Tesque Inc., Kyoto, Japan). Acetone and water were used as a mobile phase in a linear gradient from 50 to 100% of acetone during 15 min, followed by 100% acetone for 10 min at a flow rate of 1 mL/min. Diode array detection was performed at 450 nm and the spectral properties of each individual peak between 400 to 600 nm were assessed.

Glycerol concentration was determined by HPLC (HP/Agilent 1100 HPLC system, Palo Alto, USA) equipped with a refractive index detector (HP/Agilent 1100 series, RID G1362A). An Ultron PS-80C column (Shinwa Chemical Industries Ltd., Kyoto, Japan) was used for the separation at 80°C.

RESULTS AND DISCUSSION

Isolation of red yeast

A total of 76 samples (soil, tree exudates, tree bark, natural water, sediments and marine fish) were subjected to isolation and only 15 strains of red yeast were obtained. It was reported that red yeasts, especially from the genus *Rhodotorula*, were common in marine and freshwater fish (Gatesoupe, 2007), and more frequently isolated from sediments and aquatic environments (Nagahama *et al*., 2001). *Rhodotorula cresolica*, a red yeast species, was reported to have been isolated from acidic sandy soils (Wouter and Frans, 1997) and recently, Limtong *et al*. (2008) reported the presence of red yeast from the genus *Rhodotorula* in water from a mangrove forest in Thailand.

Screening of red yeast for carotenoid production

The 15 newly isolated red yeast strains and 135 red yeast strains from the private culture collection at the Department of Microbiology, Kasetsart University, were subjected to a selection scheme to obtain potential carotenoid production strains. Carotenoid extract from four red yeast strains, DMKU3-LPK2, DMKU3-LPK4, DMKU3-R25, and SKK-BM3, with absorbances in excess of 0.5 at 450 nm were selected for further screening. The remaining strains produced carotenoids in relatively low concentrations. The four selected red yeast strains were subjected to secondary screening by cultivation in a basal medium supplemented with 40 g/L glycerol. The results indicated that the strain DMKU3-LPK4 produced the highest carotenoid concentration of 2.67 mg/L at 84 h, while strain DMKU3-LPK2 produced carotenoids at a comparable level of 2.65 mg/L at 84 h. The strains DMKU3-R25 and SKK-BM3 produced lower carotenoid concentrations of 2.27 mg/L at 84 h and 0.99 mg/L at 36 h, respectively (Figure 1). Only DMKU3-LPK4 was chosen for further studies.

Based on their individual UV spectral properties and chromatographic characteristics, three typical carotenoid pigments, namely torularhodin, torulene and β-carotene, were identified; they were most commonly found in red yeasts in various proportions. HPLC analysis proved that torularhodin and torulene were major carotenoids of the strain DMKU3-LPK4 (data not shown). These two carotenoids were also reported as major pigments of *Rhodosporidium babjevae* (Sperstad *et al*., 2006).

Optimization of carotenoid production with the application of glycerol as a carbon source

The effects of initial glycerol concentration on carotenoid production by the strain DMKU3-LPK4 were investigated with a basal broth supplemented with 20-100 g/L glycerol at pH 5.5. The results indicated that low glycerol concentrations of 20 and 40 g/L effected higher growths of 7.00 and 7.36 g dried cell mass/L after 120 h, respectively (Table 1). In contrast, higher glycerol concentrations of 60, 80 and 100 g/L, resulted in lower dried cell mass yields. Accumulations of carotenoids followed the same pattern as growth, that is, increasing glycerol concentration from 20 to 40 g/L resulted in an increase in carotenoid production, while at higher glycerol concentrations (60-100 g/L), carotenoid production decreased. These results indicated that carotenoid production of the strain DMKU3-LPK4 was consistent with its growth performance. The highest carotenoid concentration at 2.99 mg/L was achieved with a glycerol concentration of 40g/L,

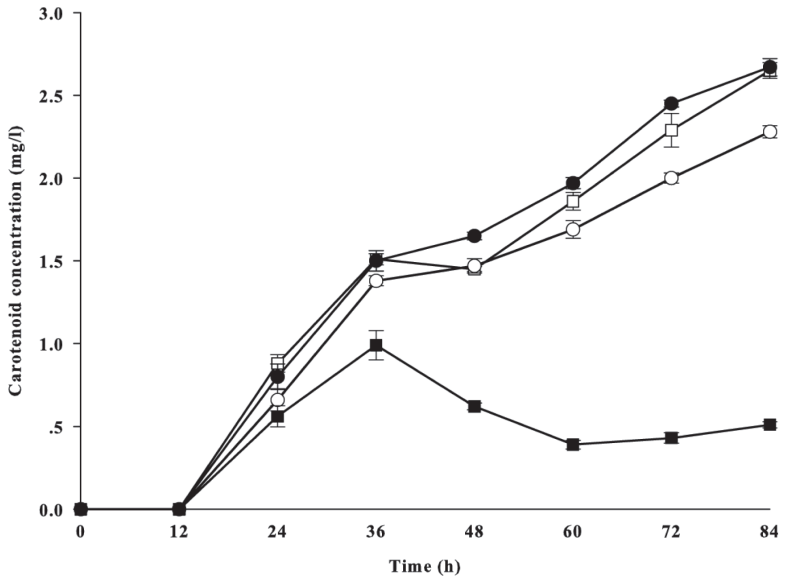

Figure 1 Time course of carotenoid production by the strains DMKU3-LPK4 (●), DMKU3 LPK2 (□), DMKU3-R25 (○) and SKK-BM3 (■), in basal broth supplemented with 40 g/L glycerol and incubated on a rotary shaker at 28°C and 130 rpm for 84 h.

which was applied for further assessment.

To study the effect of different nitrogen sources on carotenoid production, yeast extract was replaced by both inorganic nitrogen compounds (ammonium sulfate, monosodium glutamate, sodium nitrate and urea) and organic nitrogen sources (corn steep liquor, mung bean meal and soy bean) with equivalent nitrogen concentrations in the basal medium, supplemented with glycerol 40 g/L at pH 5.5. With the exception of yeast extracts, the highest carotenoid concentration of 1.92 mg/L and a high dried cell mass of 4.97 g/L were attained at 132 h with urea as the nitrogen source (Table 1). The other inorganic nitrogen compounds resulted in both lower dried cell mass and decreased carotenoid concentrations. The maximal dried cell mass (6.98 g/L) was produced when corn steep liquor was used, yet a lower carotenoid concentration (1.71 mg/L) was attained. Other organic sources of nitrogen had a relatively modest influence on growth and carotenoid production. These findings were contrary to those of An (2001), who found that ammonium sulfate supported carotenoid production by *Phaffia rhodozyma* slightly more favorably than urea. Moreover, An *et al.* (1989) reported that a reduced ability of *P. rhodozyma* to assimilate nitrogen increased its carotenoid content. Although the strain DMKU3-LPK4 produced a lower carotenoid level and had a lower growth rate when urea was used as the nitrogen source instead of yeast extract, the former was selected for further study due to its considerably lower price.

Numerous reports claimed that the C:N ratio has a major effect on cell growth and carotenoid accumulation by the yeasts, *Xanthophyllomyces dendrorhous* (anamorphic state: *P. rhodozyma*) (Liu and Wu, 2007) and *Rhodotorula glutinis* (Park *et al.*, 2005). The effect of the C:N ratio was studied with the basal broth supplemented with 40 g/L glycerol and replaced yeast extract with various concentrations of urea as the nitrogen source to adjust the C:N ratios of

the media to 20:1, 40:1, 60:1,80:1, and 100:1 at pH 5.5. The maximal carotenoid production of 2.96 mg/L and a dried cell mass of 5.5 g/L was obtained at 132 h with the C:N ratio of the medium at 60:1 (Table 1). When the C:N ratio of the medium was decreased from 60:1, lower dried cell mass and carotenoid production were obtained. The maximal dried cell mass of 5.73 g/L, was

Table 1 Carotenoid production and growth by the strain DMKU3-LPK4 in a basal broth supplemented with various glycerol concentrations as a sole carbon source, various nutrients and pH values by cultivation on a rotary shaker at 28°C and 130 rpm for 132 h.

Condition	Dry cell mass (g/L)	Carotenoid concentration (mg/g)	Carotenoid production (mg/L)
Glycerol [a]			
20 g/L	7.00 ± 0.19	0.34 ± 0.11	2.41 ± 0.25 (120h)
40 g/L	7.36 ± 0.24	0.40 ± 0.05	2.99 ± 0.10 (120h)
60 g/L	5.27 ± 0.24	0.49 ± 0.05	2.60 ± 0.03 (120h)
80 g/L	3.40 ± 0.16	0.55 ± 0.05	1.86 ± 0.03 (120h)
100 g/L	3.83 ± 0.33	0.53 ± 0.14	2.02 ± 0.21 (120h)
Nitrogen [b]			
Yeast extract	7.36 ± 0.24	0.40 ± 0.05	2.99 ± 0.10 (120h)
Urea	4.79 ± 0.08	0.40 ± 0.02	1.92 ± 0.03 (132h)
Sodium nitrate	3.79 ± 0.22	0.30 ± 0.07	1.15 ± 0.05 (132h)
Ammonium sulfate	1.91 ± 0.02	0.35 ± 0.06	0.66 ± 0.04 (132h)
Monosodium glutamate	1.51 ± 0.05	0.97 ± 0.04	1.46 ± 0.03 (132h)
Soybean mill	2.72 ± 0.17	0.28 ± 0.16	0.77 ± 0.11 (120h)
Mung bean mill	2.60 ± 0.08	0.40 ± 0.15	1.04 ± 0.15 (108h)
Corn steep liquor	6.98 ± 0.21	0.26 ± 0.06	1.79 ± 0.10 (132h)
C:N ratio [c]			
20:1	0.35 ± 0.02	1.83 ± 0.58	0.64 ± 0.37 (132h)
40:1	4.18 ± 0.06	0.62 ± 0.44	2.61 ± 1.51 (132h)
60:1	5.50 ± 0.05	0.54 ± 0.58	2.96 ± 1.71 (132h)
80:1	5.73 ± 0.32	0.46 ± 0.58	2.63 ± 1.52 (132h)
100:1	4.43 ± 0.03	0.04 ± 0.58	1.80 ± 1.04 (132h)
pH [d]			
5	5.29 ± 0.08	0.49 ± 0.06	2.59 ± 0.15 (132h)
6	6.90 ± 0.19	0.40 ± 0.05	2.74 ± 0.13 (132h)
7	5.53 ± 0.10	0.43 ± 0.05	2.38 ± 0.11 (132h)
8	5.20 ± 0.14	0.48 ± 0.03	2.48 ± 0.04 (132h)

Values shown are (mean ± standard error of the mean).

[a] Cultivation medium composed of 4 g/L yeast extract, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, with various glycerol concentrations and the initial pH 5.5.

[b] Cultivation medium composed of 40 g/L glycerol, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, with various nitrogen sources at a concentration of 0.456 g nitrogen/L and initial pH 5.5.

[c] Cultivation medium composed of 40 g/L glycerol, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$ with urea concentrations of 1.68, 0.84, 0.56, 0.42 and 0.34 g/L to adjust the C:N ratio of the medium to 20:1, 40:1, 60:1, 80:1 and 100:1, respectively, and pH 5.5.

[d] Cultivation medium composed of 40 g/L glycerol, 0.559 g/L of urea, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$ and the initial pH adjusted to 5, 6, 7 and 8.

achieved at a C:N ratio of 80:1; nevertheless the carotenoid production of 2.63 mg/L was inferior to the yield at a C:N ratio of 60:1. In a medium with a C:N ratio of 100:1, both dried cell mass and carotenoid production were reduced. Thus, a moderately higher C:N ratio had a positive influence on carotenoid production when effective carbon and nitrogen sources were applied. The influence of initial pH of the medium on the strain DMKU3-LPK4 was investigated and the results showed that the maximal carotenoid production (2.74 mg/L) and growth (6.90 g dried cell mass/L) were obtained at 132 h when cultivation was carried out at pH 6 (Table 1). Although the carotenoid production increased only slightly, growth was obviously superior in comparison to other pH levels.

The effect of temperature on growth and carotenoid production of the strain DMKU3-LPK4 was investigated with basal broth supplemented with 40 g/L of glycerol, 0.559 g/L of urea, pH 6 and an incubation range from 14°C to 38°C for 132 h. The results indicated temperatures in excess of 14°C caused improved growth and carotenoid production. The maximal growth, a dried cell mass of 9.74 g /L, was obtained with an incubation temperature of 30°C, while maximal carotenoid production of 2.45 mg/L was obtained at 32°C (Table 2).

The period of carotenoid production by the strain DMKU3-LPK4 was optimized in shaking flask cultivation at 32°C in a glycerol medium, composed of 40 g/L glycerol, 0.559 g/L of urea, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$ and pH 6.0. These optimal nutrient and pH conditions produced a carotenoid concentration of 3.42 mg/L at 132 h and a dried cell mass of 7.59 g/L. The pH level dropped slightly from 6.14 to 5.09 at the end of fermentation (Figure 2). Frengova and Beshkova (2009) reviewed the carotenoid production by some red yeast strains in the genus *Rhodotorula*, using various agricultural waste byproducts as carbon sources. It was shown that the strain DMKU3-LPK4 grown on glycerol accumulated higher carotenoid than other strains obtained, specifically by *Rhodotorula rubra* and

Table 2 Carotenoid production and growth of the strain DMKU3-LPK4 by cultivation in a 40-mL L-shaped test tube of 10 mL of a basal broth supplemented with 40 g/L of glycerol, 0.559 g/L of urea and pH 6 at various temperatures in a range from 14°C to 38°C for 132 h.

Temperature (°C)	Dry cell mass (g/L)	Carotenoid concentration (mg/g)	Carotenoid production (mg/L)
14	1.77 ± 0.50	0.18±0.04	0.32 ± 0.01
16	2.29 ± 0.99	0.16±0.43	0.37 ± 0.01
18	2.98 ± 0.00	0.12±0.03	0.37 ± 0.01
20	4.56 ± 0.03	0.18±0.01	0.83 ± 0.01
22	5.03 ± 0.41	0.29±0.08	1.44 ± 0.02
24	5.21 ± 0.65	0.29±0.13	1.49 ± 0.02
26	5.16 ± 0.45	0.33±0.09	1.72 ± 0.02
28	8.73 ± 0.98	0.24±0.11	2.10 ± 0.03
30	9.74 ± 0.00	0.23±0.00	2.24 ± 0.01
32	7.88 ± 1.08	0.31±0.14	2.45 ± 0.03
34	5.29 ± 0.67	0.05±0.27	0.29 ± 0.07
36	1.43 ± 0.27	0.34±0.19	0.48 ± 0.02
38	0.20 ± 0.06	2.10±0.33	0.21 ± 0.03

Values shown are (mean ± standard error of the mean).

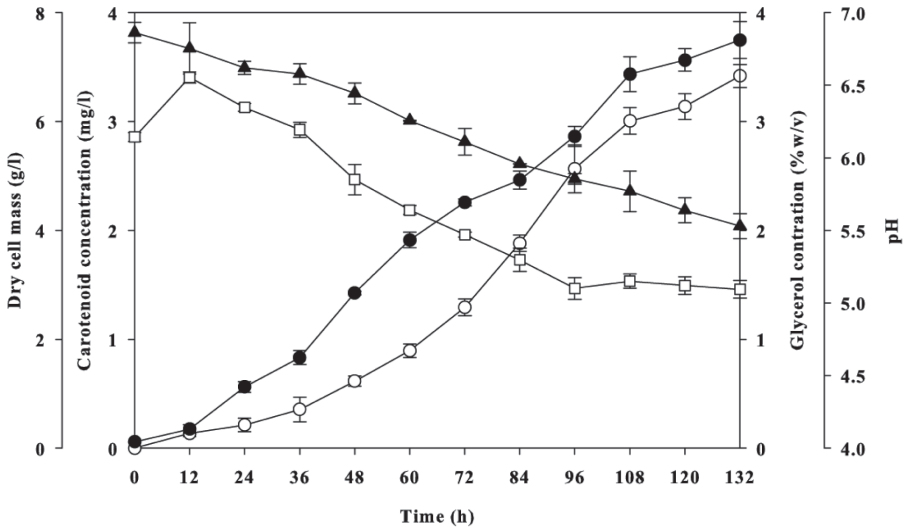

Figure 2 Time course of carotenoid production by the strain DMKU3-LPK4 in an optimum glycerol medium composed of 40 g/L glycerol, 0.559 g/L of urea, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$ and pH 6.0 by shaking flask cultivation on a rotary shaker at 32°C and 130 rpm for 132 h. Carotenoid concentration (○); Dry cell mass (●); Glycerol concentration (▲); and pH (□).

R. glutinis CCT 2186 in sugar cane juice and by *Rhodotorula mucilaginosa* CRUB 0195 when corn syrup was used. It appears that the slow growth of the strain DMKU3-LPK4 when glycerol was used as a carbon source was relatively low due to the glycerol consumption rate. This was probably due to an insufficiency of some nutrients and trace elements for the growth and carotenoid accumulation of the strain DMKU3-LPK4 in the present study. However, as inexpensive glycerol from biodiesel by-products was used as a substrate, possible improvement of carotenoid production by the strain DMKU3-LPK4 demands diverse investigation strategies. For instance, an appropriate stimulant could be appraised, such as white light or chemical treatment to induce carotenoid accumulation, as reported in another investigation (Čertík *et al*., 2009), or other cultivation parameters to improve growth rates could be appraised.

Identification of selected red yeast

Identification of the strain DMKU3-LPK4 was performed on the basis of a polyphasic approach consistent with a conventional taxonomic study by comparison of morphological, biochemical and physiological characteristics in accordance with the taxonomic key of Kurtzman and Fell (1998), as shown in Table 3, using a molecular taxonomic study by analysis of the similarity of nucleotide sequences of the D1/D2 domain of the LSU rRNA gene. The strain DMKU3-LPK4 possesses similar phenotypic characteristics to *Rhodosporidium paludigenum*. The D1/D2 domain of the LSU rRNA gene sequence of the strain was also identical to this species, thus confirming that they were the same species, in accordance with the report of Kurtzman and Robnett (1998). Therefore, the strain DMKU3-LPK4 was identified as *R. paludigenum*, as per Kurtzman and Robnett (1998). The nucleotide sequences of the D1/D2 domain of the LSU rRNA gene of the strain DMKU3-LPK4 were deposited at DNA Data Bank of Japan (DDBJ) with an accession number of AB519057.

Table 3 Biochemical and physiological characteristic of *R. paludigenum* DMKU3-LPK4.

Fermentation					
Glucose	-	Trehalose	-	Lactose	-
Galactose	-	Melibiose	-	Raffinose	-
Maltose	-	Sucrose	-		
Assimilation of carbon compounds					
2-Ketogluconic acid	w	Glucose	+	Methyl-D-glucoside	-
5-Ketogluconic acid	-	Glucuronate	-	N-Acetyl glucosamine	-
Cellobiose	+	Glycerol	+	Raffinose	+
Citrate	+	Inositol	-	Rhamnose	w
D-arabinose	w	Inulin	-	Ribitol	+
Erytritol	-	L-arabinose	w	Ribose	+
Ethanol	-	Lactate	w	Salicin	+
Galactitol	+	Lactose	-	Soluble starch	-
Galactose	-	Maltose	w	Sorbose	+
Galacturonic acid	-	Mannitol	+	Succinate	+
Glucitol	+	Melibiose	-	Sucrose	+
Gluconate	+	Melizitose	-	Trehalose	+
Glucono-lactone	+	Methanol	-	Xylose	+
Assimilation of nitrogen compounds					
Ammonium sulfate	+	Ethylamine-HCl	+	Potassium nitrate	+
Lysine-HCl	-	Sodium nitrate	+	Cadaverine	-
Other growth tests					
Vitamin-free medium	+	0.01% cyclohexaminde	-	0.1% cyclohexamide	-
50% Glucose	+	60% Glucose	+	10% NaCl	+
16% NaCl	+				
Other characteristics					
Hydrolysis of urea	+	Acid formation from glucose	-		

CONCLUSION

This was the first report of a newly isolated *R. paludigenum* strain (DMKU3-LPK4) and its capability to use glycerol as an alternative sole carbon source for carotenoid production in shaking flask cultivation. Optimum conditions for carotenoid production were a temperature of 32°C, pH 6.0 and a medium composed of 40 g/L glycerol, 0.559 g/L of urea to effect a C:N ratio of 60:1, 1 g/L KH_2PO_4, and 0.5 g/L $MgSO_4.7H_2O$. Under these conditions, the *R. paludigenum* DMKU3-LPK4 yielded 3.42 mg/L of carotenoids at 132 h and a maximal dried cell mass of 7.59 g/L.

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