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STUDIES ON EFFECT OF MEDIA COMPONENTS ON GROWTH AND â-CAROTENE PRODUCTION BY *RHODOTORULA GRAMINIS RC04*

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Abstract: Red pigmented yeast was isolated from a freshwater river flowing through mining area of Goa. The isolate was identified as Rhodotorula graminis using morphological, biochemical and physiological features. This isolate showed the presence of pigments β-carotene, torulene and torularhodin. Effect of different carbon and nitrogen sources was checked on biomass and β-carotene production. Among the inorganic salts, the isolate grew best with potassium nitrate. Although the yield of total β-carotene/gram of biomass was less than compared with other inorganic salts of nitrogen used in this study, the total β-carotene production was found to be maximum. Amino acids supported accumulation of β-carotene but gave poor yield. Complex nitrogen sources are the best substrates for growth and β-carotene production. Among these, yeast extract is the best nitrogen source for growth and β-carotene production. Glucose appears to be the best carbon source for biomass and β-carotene production followed by glycerol. Highest β-carotene production was observed at a 10:1 carbon to nitrogen ratio.

Key words: Rhodotorula graminis, β-carotene

INTRODUCTION

Carotenoids are a group of natural pigments produced by a wide range of microorganisms and plants. These are used commercially as food colorants and as a source in pigmentation of fish and shellfish in aquaculture. Due to the discovery of their anticancer and antioxidant properties, wider use of carotenoids as pharmaceuticals and nutraceuticals is expected. Recent research efforts have focused on the economic production of carotenoids in useful quantities. As a result, microbial production of carotenoids has attracted considerable interest [1,2]. Yeasts are more convenient than algae or molds for large-scale production in fermenters, due to their unicellular nature and high growth rate [3]. The synthesis of different natural commercially important carotenoids (β-carotene, torulene, torularhodin and astaxanthin) by several yeast species belonging to the genera *Rhodotorula* and *Xanthophyllomyces* (=*Phaffia*) has led to consider these red yeasts as potential pigment sources [4]. Carotenoids are produced with typical concentrations ranging from 15 to 200 µg/g dry weight and β-carotene values range from 1 to 40 µg/g dry weight in wild-type strains of *Rhodotorula* spp. [5]. There are several reports available on the development of suitable hyper-producing strains of *Xanthophyllomyces dendrorhous* (=*Phaffia rhodozyma*) and *Rhodotorula* spp for commercial carotenoids production [6,7].

A carotenogenic yeast culture performance is affected by numerous environmental and fermentation parameters especially concentrations of the medium components. The effects of different media components on biomass, carotenoids production and composition of carotenoids in *Rhodotorula glutinis* have been studied [8,9]. Although *Rhodotorula graminis* has been shown to accumulate high amount of carotenoids the potential of this yeast for β-carotene accumulation has not been investigated. Only one report is available on the effect of selected trace elements on carotenogenesis in *Rhodotorula*

graminis [10]. The present investigation therefore focused on studying the effect of carbon and nitrogen sources on growth and accumulation of β-carotene by *Rhodotorula graminis.*

MATERIALS AND METHODS

Isolation of pigmented yeast: The water sample was collected from freshwater river flowing through mining area of Goa in sterile screw capped bottles. One ml of sample was inoculated into 20mL of broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose, 1% glycerol, 0.05% chloramphenicol) pH 4.5 and incubated at room temperature for 48 hours [11]. The broth sample was diluted suitably and plated on agar medium of the same composition. The plate was incubated for 4 days at room temperature. Pigmented colonies were further purified on the same medium and stored at 4°C [12, 13].

Identification of pigmented yeast:

Morphological characterization: Identification was based on the comparative analysis of distinguishing morphological, physiological and metabolic characteristics of the isolate [14-16). Morphological characteristics of the isolate such as shape and type of cell division were observed by microscopic analysis of the culture grown in a liquid GPY medium (2.0% glucose, 0.5% yeast extract, 0.5% peptone, 0.2% monobasic sodium phosphate) at room temperature for 24 hours. Formation of pseudohyphae and mycelium was observed through the inoculation of the yeast on PGA (Potato glucose agar, Difco). The inoculated medium was incubated at room temperature and examined microscopically after 5 to 7 days of incubation.

Formation of ballistospores*:* The culture was inoculated on GPY-Sabouraud agar (Difco). On this plate, a PGA containing plate was placed inverted, sealed, incubated at room temperature and observed after three weeks to check for presence of ballistospores.

Physiological and biochemical characterization:

Fermentation of carbohydrate: The assay was carried out in test tube containing 2 ml of basal medium (0.45% yeast extract, 0.75% peptone and bromothymol blue solution), Durhams tubes and 1ml of glucose solution. The tube was inoculated with 0.1ml of culture grown in GPY-Sabouraud broth (Difco) and incubated at room temperature for 24 hours.

Utilization of carbon compounds: Assimilation of carbon compounds was verified with hexoses (glucose, galactose), pentoses (arabinose, xylose), alcohols (sorbitol, xylitol) and disaccharides (trehalose, maltose, sucrose, lactose). These carbon sources were used at a final concentration of 0.5%, in YNB agar (Difco). The inoculated media was incubated at room temperature to monitor the growth.

Growth on 50% (w/v) glucose-yeast extract agar: Actively growing culture was lightly inoculated as a streak on 50% (w/v) glucose-yeast extract agar in tube. The tube was inoculated at room temperature for four weeks and then examined for growth.

Growth at different temperatures: Test tubes containing GPY- sabouraud broth were inoculated and incubated in a water bath at 20, room temperature, 37 and 40ºC for 48 hours.

Amyloid composite synthesis: The growth obtained in the experiments for utilization of carbon compounds was also used to verify the synthesis of amyloid compounds. The growth was covered with Lugol's solution for a few minutes after 21 days of incubation. If the color of the growth turned blue, the test indicates synthesis of amyloid.

Cycloheximide test: 100 mg cycloheximide was dissolved in 2 mL of acetone and sterilized by filtration. This was added to 10-fold concentrated YNB medium (Difco) containing glucose (10.0%). The tube was incubated at room temperature and observed for three weeks for growth.

Urea hydrolysis: The culture was inoculated on Christensens-urea agar, incubated at room temperature and observed daily for five days for appearance of a deep pink color.

Production of β-carotene: Starter culture was prepared by inoculating one loopful of freshly grown culture in 50 mL of YM broth (Difco) in a 150 mL Erlenmeyer flask, incubating at room temperature in a shaker at 150 rpm for 24 hours in dark. Fermentation experiments were carried out in 500mL Erlenmeyer flasks containing 200 mL of fermentation media.

Each flask was inoculated with 5% v/v of starter culture and incubated at room temperature for 3 days at 150 rpm in the dark.

Effect of different nitrogen sources on biomass and β-carotene production: The isolate *Rhodotorula graminis* was cultivated in YM broth (Difco) containing (per litre) 3.5 g ammonium sulphate and 1.5 g asparagine as the source of nitrogen to serve as control for the experiment. For studying the effect of different nitrogen sources, ammonium sulphate and asparagine were replaced by other sources of nitrogen to supply equimolar amount of nitrogen. To compensate sulphate, 5.29 g of anhydrous sodium sulphate was added. The inorganic salts as sole source of nitrogen used for the study included ammonium nitrate, ammonium sulphate, sodium nitrate, sodium nitrite, potassium nitrate and calcium nitrate. The amino acids used as sole source of nitrogen included lysine, glycine, serine, threonine, histidine and tyrosine. The other organic nitrogen sources used were peptone, tryptone, meat extract, yeast extract, gelatin and urea.

Effect of different carbon sources on biomass and β-carotene production: The isolate was cultivated in YM broth (Difco) containing equimolar amounts of different carbohydrates. Glucose, glycerol, sorbitol, xylose, mannitol, arabinose and starch were used in this study. To check the effect of carbon: nitrogen ratio, all the above carbohydrates were used in desirable amounts to give carbon: nitrogen ratios of 4.4:1, 10:1 and 20:1.

Analytical methods

Dry weight estimation: Culture broth (100 ml) was centrifuged at 10,000x g for 20 minutes. Pellet was obtained and was washed twice with sterile distilled water. Pellet was transferred to preweighed aluminium cups and dried at 105°C till constant weight was obtained [17]. Dry cell weight reported is average of two samples.

Table 1: Colonial, morphological and biochemical characteristics of the yeast isolate RC04

Characteristic	Test result
Color of colony	Coral Red
Shape of colony	Circular
Shape of cells	Ovoidal, single or pair
Conjugation	-
Pseudohyphae	-
Mycelium	-
Ballistospore	-
Urease	+
Glucose 50%	-
20ºC	+
28ºC (Room Temperature)	+
35ºC	+
37ºC	-
Glucose Fermentation	-
Utilisation of	
D-glucose	+
D-galactose	+
D-xylose	+
Arabinose	+
Glycerol	+
Trehalose	-
Xylitol	-
Glucitol	+
Maltose	-
Lactose	-
Sucrose	+
Synthesis of starch	-
Cycloheximide Resistance	+

Extraction and Determination of β-carotene: Culture broth (100 ml) was centrifuged at 10,000x g for 20 minutes. Pellet obtained was washed twice with saline and centrifuged again. Pellet was finally suspended in 5 ml normal saline. The suspension was sonicated at 20 kHz for 10 minutes. Suspension was then mixed vigorously with DMSO, acetone and petroleum ether and kept standing in the dark for 18 hours at 4°C. The suspension was repeatedly mixed and kept standing in the dark for 18 hours at 4°C until there was no visible color in the aqueous layer. Organic layer was collected and absorbance was taken at 451 nm and amount of β-carotene was determined using the extinction coefficient 2680 for 1% solution of β-carotene (18). Value reported is average of two samples.

Visible absorption spectra for the extracts in

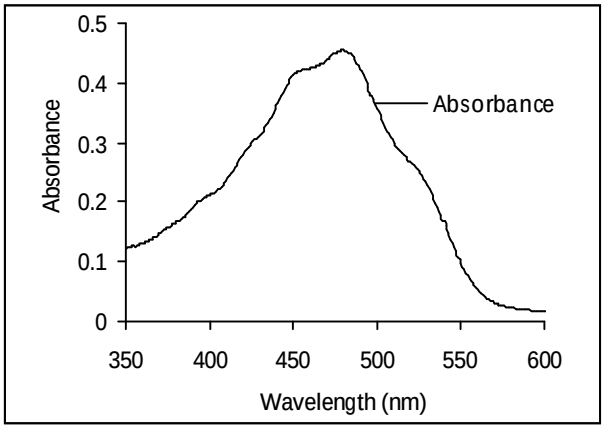
Table 2: Effect of different nitrogen sources on cellular content of β-carotene. *µg/g dry weight; # YM medium contains the Ammonium sulphate and asparagine as nitrogen source

In-organic Nitrogen source	β-carotene*	Amino acids	β-carotene*	Organic Nitrogen source	β-carotene*
YM medium# (Control)	29.10	Glycine	26.89	Peptone	32.19
Ammonium chloride	138.92	Lysine	110.60	Tryptone	26.69
Sodium nitrate	73.33	Serine	23.05	Meat extract	27.55
Ammonium nitrate	31.60	Tyrosine	59.55	Yeast extract	77.73
Potassium nitrate	138.20	Histidine	86.55	Gelatin	58.65
Ammonium sulphate	19.09	Threonine	48.39	Urea	43.42
Calcium nitrate	75.06				

Table 3: Effect of carbon sources and different C:N ratios on cellular content of β-carotene. µg/g dry weight

Carbon source	Cell bound β-carotene with different C:N ratio		
	4.4:1	10:1	20:1
Glucose	26.60	22.53	46.77
Glycerol	11.91	33.03	38.62
Sorbitol	33.12	40.82	50.18
Xylose	42.00	53.88	34.90
Mannitol	20.03	16.23	33.38
Starch	33.77	28.32	40.21
Arabinose	20.81	29.87	39.00
Sucrose	55.03	41.03	34.66

Fig. 1: Visible absorption spectra of pigment extracted from *Rhodotorula graminis* RC04 in petroleum ether.



petroleum ether were determined in the range of 350-600 nm using UV-Vis spectrophotometer (Shimazu Co., Japan) to find the other carotenoids produced by the isolate.

RESULTS

Colonial morphological and biochemical characteristics of the isolate are listed in Table 1.These characteristics match with the description of *Rhodotorula graminis*. Visible absorption spectra for the pigment extracts show peaks at 451, 479 and 520 nm (Fig.1) that correspond to λ_{max} of β-carotene, torulene and torularhodin, respectively.

Effect of different nitrogen sources on production of biomass and β-carotene by the isolate is shown in Fig 2. Cellular content of β-carotene with respective nitrogen sources is shown in table 2. This isolate grew best with potassium nitrate. Although the yield of β-carotene/gram of biomass was less than compared with other inorganic salts, the β-carotene production was found maximum. Cell bound β-carotene was found to be maximum with ammonium sulphate, sodium nitrate and calcium nitrate. However, with these salts growth of the yeast was poor. Among the organic nitrogen sources, peptone, tryptone, meat extract and yeast extract supported biomass accumulation to similar extents. Of these four, yeast extract gave both maximum biomass and cell bound β-carotene accumulation. Urea and gelatin supported both biomass and β-carotene accumulation poorly. In general the amino acids used in this study are poor nitrogen sources compared to their counterpart organic and inorganic nitrogen sources. Among the aminoacids, glycine, tyrosine, serine and threonine supported maximum biomass. Histidine, tyrosine, lysine and threonine supported maximum accumulation of β-carotene.

Rhodotorula graminis RC04 grew in all the carbon sources tested with accumulation of β-carotene (Figs 3, 4, 5). Interestingly, the utilization pattern and accumulation of β-carotene is changing with change in carbon to nitrogen ratio in medium. With low levels of carbon, glucose, glycerol and sorbitol were found to support biomass accumulation equally. However, maximum production of β-carotene was found in glucose, sorbitol, xylose, sucrose and starch. On increasing the carbon to nitrogen ratio to 10:1 both biomass and β-carotene accumulation improved with all the carbon sources tested. Glucose appears to be the best carbon source for biomass and β-carotene production followed by glycerol. Interestingly, β-carotene production was to similar extents in glucose, glycerol, sorbitol, xylose and sucrose. With an increase in the carbon to nitrogen ratio to 20:1, both biomass and β-carotene accumulation decreased considerably with all carbon sources tested. With carbon to nitrogen ratio of 20:1, glycerol was the best carbon source followed by sucrose. With a carbon to nitrogen ratio of 4.4:1, sucrose showed maximum cell bound β-carotene (Table 3). With an increase in the carbon to nitrogen ratio to 10:1, cell bound β-carotene increased with all carbon sources tested. With a further increase in the carbon to nitrogen ratio to 20:1, cell bound β-carotene decreased considerably with all carbohydrates tested.

DISCUSSION

β-carotene has been recognized as a potential anticancer agent. Plant sources of β-carotene usually are affected due to seasonal variations. Microbial production of β-carotene therefore has been visualized as major source of β-carotene to provide the needs of the food and pharmaceutical industries. Yeasts producing β-carotene have in the recent past become industrially important for production of β-

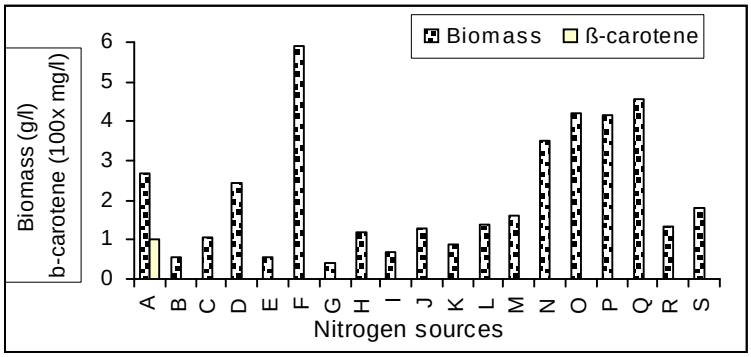


Fig. 2: Effect of different nitrogen substrates on growth and β-carotene accumulation in *Rhodotorula graminis* RC04. Empty bar – Biomass, Filled bar - β-carotene. A – Control (Ammonium sulphate and asparagine), B – Ammonium sulphate, C – Ammonium chloride, D – Ammonium nitrate, E – Sodium nitrate, F – Potassium nitrate, G – Calcium nitrate, H – Glycine, I – Lysine, J – Tyrosine, K – Histidine, L – Serine, M – Threonine, N – Peptone, O – Tryptone, P – Meat Extract, Q – Yeast extract, R – Gelatin, S – Urea

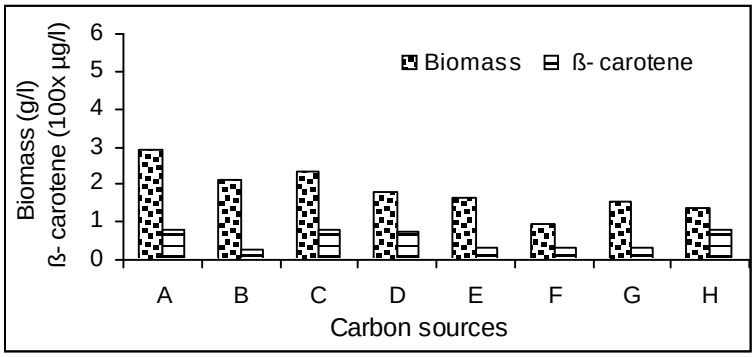


Fig. 3: Effect of different carbon substrates on growth and β-carotene accumulation in *Rhodotorula graminis* RC04 at C:N ratio of 4.4:1. Empty bar – Biomass, Filled bar - β-carotene. A – Glucose, B – Glycerol, C – Sorbitol, D – Mannitol, E – Starch, F – Arabinose, G - Sucrose**,** H - Xylose

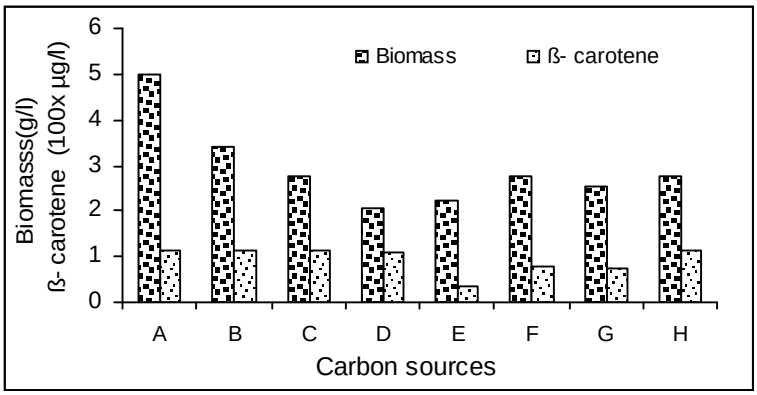


Fig. 4: Effect of different carbon substrates on growth and β-carotene accumulation in *Rhodotorula graminis* RC04 at C:N ratio of 10:1. Empty bar – Biomass, Filled bar - β-carotene. A – Glucose, B – Glycerol, C – Sorbitol, D – Mannitol, E – Starch, F – Arabinose, G - Sucrose**,** H - Xylose

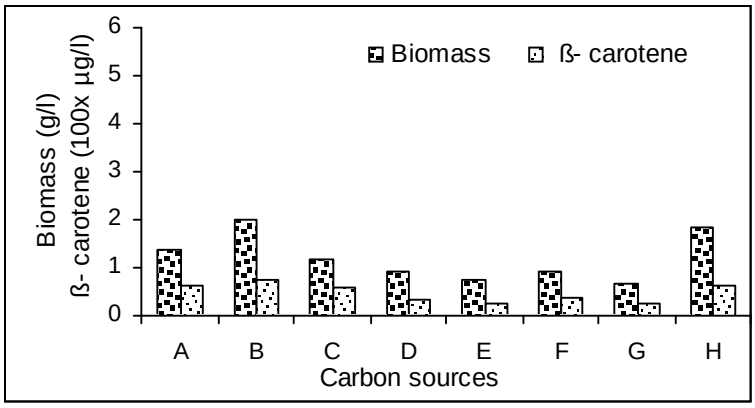


Fig. 5: Effect of different carbon substrates on growth and β-carotene accumulation in *Rhodotorula graminis* RC04 at C:N ratio of 20:1. Empty bar – Biomass, Filled bar - β-carotene. A – Glucose, B – Glycerol, C – Sorbitol, D – Mannitol, E – Starch, F – Arabinose, G - Sucrose**,** H - Xylose

carotene. The yeast species of *Rhodotorula* and *Xanthophyllomyces dendrorhous* have been identified as β-carotene accumulating yeasts. Mutant strains of *Xanthophyllomyces dendrorhous* have been investigated *for* production of β-carotene for commercial use (19, 20). Interestingly, *Rhodotorula graminis* has been reported as highest producer of total carotenoids among *Rhodotorula* spp. but no attempts have been made to investigate potential of this species for production of β-carotene. *Rhodotorula graminis* RC04 isolated from freshwater sources showed the accumulation of β-carotene. It was seen that β-carotene values range from 5-75 µg/g cell dry mass in species of *Rhodotorula* (21). This study has shown a differential production of β-carotene in the presence

of different carbon and nitrogen sources. β-carotene values range from 29-150µg/g cell dry mass. These are the highest reported values for β-carotene in *Rhodotorula graminis*.

This isolate accumulated the maximum content of β-carotene in the presence of yeast extract as sole source of nitrogen. Buzzini *et al* has shown the importance of trace elements in production of total carotenoids from *Rhodotorula graminis* (10). Yeast extract therefore possibly served not only as a source of nitrogen but fulfilled requirements of all microelements including vitamins for good growth and β-carotene by *Rhodotorula graminis* RC04. It is interesting to note that among the inorganic salts used as sole source of nitrogen, potassium nitrate served as source for good growth and β-carotene production by *Rhodotorula graminis* RC04. In contrast, growth and β-carotene production was relatively poor with sodium nitrate and calcium nitrate. This indicates there is possible role of K^+ in growth and β-carotene production by *Rhodotorula graminis* RC04. Further, organic nitrogen sources like peptone, tryptone, meat extract gave good growth but less β-carotene production than yeast extract. Aminoacids poorly supported β-carotene accumulation. This needs further investigation.

With the exception of mannitol, all the carbohydrates tested served as good substrates and glucose was best for growth. All the carbohydrates tested supported β-carotene accumulation to different extent. This isolate performed better for both growth and β-carotene production at carbon to nitrogen ratio of 10:1. Interestingly, a higher carbon to nitrogen ratio showed inhibitory effect on growth and β-carotene accumulation. This indicates that optimization of carbon and nitrogen is needed using empirical methods like Response Surface which will give exact relationship between carbon and nitrogen for improved production of β-carotene. This investigation highlighted that improvement in accumulation of β-carotene by *Rhodotorula graminis* RC04 is possible with manipulation of cultural conditions. Media components affect accumulation of β-carotene drastically both in terms of overall productivity and cell bound yield of β-carotene. Cheaper substrates capable of providing overall nutritional requirements of *Rhodotorula graminis* RC04 will need investigation for a viable commercial solution. Further, development of mutant will definitely open up the possibility of using this strain at commercial scale.

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