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Utilization of date syrup as a substrate for carotenoid production by *Rhodotorula glutinis*

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ABSTRACT-The potential use of date syrup, for the production of carotenoids by *Rhodotorula glutinis* in batch fermentation process, was investigated during 7 days. The results revealed that carbon (glucose or date syrup) and nitrogen sources [yeast extract, $(NH_4)_2SO_4$ and NH_4NO_3] had a significant influence on biomass and carotenoid production. Maximum yield of total carotenoid production (7.94 mg/L) with carotene content (2040 µg/g) and biomass (3.90 g/L) was obtained from *R. glutinis* after 7 days of fermentation in a substrate containing date syrup and yeast extract. The highest biomass (8.03 g/L) was obtained in the culture containing glucose and yeast extract, while the total carotenoid content of 6.72 mg/L with 836.86 µg/g carotene was produced in this medium. Significant differences were observed when comparing the average biomass and total carotenoid productions in different cultures and fermentation times. Our results demonstrated that date syrup (from low quality dates), as aby-product at a lower price, could be profitably used as a suitable carbon source for carotenoid production by *R. glutinis*.

INTRODUCTION

The agro-industrial by-products are economical sources of carbon and nitrogen for use in fermentation process. Date palm (*Phoenix dactylifera*) is one of the oldest cultivated plants in the Middle East and North Africa (Al-Farsiet al., 2005a). Date fruits contains, typically, total sugars (44-88%), fat (0.2-0.5%), protein (2.3-5.6%), dietary fiber (6.4-11.5%), minerals (0.1 to 0.916 mg/100 g date) and vitamins such as vitamin C, B_1, B_2, A and niacin (Al-Shahiband Marshall, 2003; Al-Farsiet al., 2005b; Biglariet al., 2008).

Date syrup is a product obtained from matured dates (tamar) and is used in the preparation of some traditional and industrial foods. The date fruits and date syrup are used for human consumption, in bakery and ice-cream products, and for the production of caramel color, ethanol, vinegar, and single cell protein (Mehaia and Cheryan, 1991). Because of the large amount of date production and also its high content of oligosaccharide and monosaccharide, this fruit, with its large amount of annual wastes, can be a good choice of microbial carotenoids production.

Carotenoids are natural bioactive compounds that play an important role in the function of all living organisms. They are used commercially as food colourants (yellow to red colour) and as a source in pigmentation of fish and shellfish in aquaculture, having crucial influence on the acceptability of many foods. Due to their anticancer and antioxidant properties and immune response stimulants, wider use of carotenoids as pharmaceuticals and nutraceuticals is expected (Edge et al., 1997; Hennekens, 1997). Recently, microbial production of carotenoids has attracted considerable interest. Yeasts in the genus *Rhodotorula* synthesize carotenoid pigments produce major pigments such as β-carotene, γ-carotene, torulene and torularhodin (Peterson et al., 1958; Simpson et al., 1971; Perrier et al., 1995).

Carotenoids production yield by *R. glutinis*is affected by numerous environmental and fermentation parameters such as aeration, agitation, temperature, pH, and the concentration of the medium components. For effective carotenogenesis, the use of inexpensive alternative carbohydrate sources, which typically are the by-products of various industries and contribute to contamination of the environment and the use of yeast strains that produce high amount of carotenoid are important (Frengova et al., 1994*)*. Recent researches have focused on the economical production of carotenoids (Edge et al., 1997; Bhosale and Gadre, 2001; Buzzini, 2001).

The aim of this study was to examine the potential of date syrup, an agro-industrial raw material, for the production of carotenoids by *Rhodotorula glutinis* at batch scale. In this study, date syrup was chosen as a substrate mainly because of its high sugar and other nutrient contents, low cost, ready availability and ease of storage. Date syrup is an important date fruit by-product which has not been investigated for β-carotene production. Furthermore, to the best of our knowledge, this is the first study investigating the carotenoids production by *Rhodotorula glutinis* using date syrup as an economical carbon source.

MATERIALS AND METHODS

Date syrup (DS) was obtained from the local shop (Kabkab). All media and chemicals were obtained from Sigma, Merck and Difco companies.

Microorganism and Growth Conditions

Rhodotorula glutinis Persian Type Culture Collection (PTCC) 5256 was purchased from Iranian Research Organization for Science and Technology (IROST). The yeast was maintained on a yeast and malt extract agar slant (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose and agar 20g/L at pH 6.2 ± 0.2) at 4ºC until used.

A yeast starter was prepared by adding one loop of a 24h old slant culture into 250 ml Erlenmeyer flask containing 50 ml of yeast and malt medium containing 15 g/L glucose, 2.5 g/L yeast extract, 2 g/L malt extract, 1 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4 and 0.25 g/L $MgSO_4.7H_2O$ (1) and incubating the flask at 30ºC for 72 h. Yeast cells were harvested by centrifugation (3500 rpm for 30 min), washed three times in sterile distilled water (A_{620}=0.5-0.75, corresponding to ca. 10^7cells/ml) in sterilized normal saline.

Fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml of sterilized medium. The basal medium used for carotenoid production had the following composition: 40 g/L glucose, 2 g/L yeast extract, 3 g/L K_2HPO_4, 3 g/L KH_2PO_4, 0.2 g/L $MgSO_4.7H_2O$ and 1% (v/v) Tween 80. For studies on the effect of other carbon source, glucose was replaced by the amount of date syrup (52.48 g/L). Similarly, yeast extract was also replaced by the $(NH_4)_2SO_4$ and NH_4NO_3, individually (Table 1). The initial pH of the medium was adjusted to pH 6 before autoclaving. Each flask was inoculated with the yeast starter of 2×10^7 cells and incubated at 30ºC for 7 days at 150 rpm, without illumination. All experiments were carried out in triplicate.

Table 1. Broth fermentation culture composition

Culture	Component				
	NH_4NO_3	$(NH_4)_2SO_4$	Yeast extract	Date syrup	Glucose
1[a]	*		*		
2	*			*	
3	*				*
4		*		*	
5		*			*
6		*	*		

* Presence in broth fermentation culture

[a] Basal medium

Analytical Methods

Total sugars and reducing sugars in date syrup were measured by the Munson and Walker method (Perrier et al., 1995).Total soluble solids were examined using refractometer by Brix measurement. Soluble proteins were measured by Biuret method (Córdova-Murueta1 et al., 2013); moisture and ash content were determined (AOAC, 2002). Final pH of each broth culture media was measured.

10 ml sample was taken from each flask every day. After cultivation, the cells were harvested by centrifugation at 3500 rpm for 30 min, washed twice with distilled water and centrifuged again. The dry mass was gravimetrically determined by drying centrifuged and washed cells at 45ºC to a constant weight. To extract carotenoids, cells were first harvested by centrifugation, washed twice with distilled water. Cells were resuspended in dimethyl sulfoxide (DMSO) and heated for 5 min at 55ºC to break the cells and intercellular carotenoid extracted to DMSO phase. The suspension was then centrifuged and the supernatant was collected. Acetone was added and mixed with pellet and centrifuged until it became colorless.Supernatants were collected and the absorbance was measured at 455 and 470 nm for detection of carotene and total carotenoids, respectively. Commercial pure β-carotene (from Merck Company) was used as a standard.

Statistical Analysis

Data were subjected to the analyses of variance(ANOVA) and multiple comparison tests at 95% of confidence level. All the analyses were carried out using the statistical software, SPSS13. Means were separated using Duncan's multiple range tests.

RESULTS AND DISCUUSION

The date syrup obtained from the local shop contained a high concentration of sugar. It consisted of 68.80% total sugar, 68.08% reducing sugar, 1.63% ash, 76.80 totals solid, 24.75% moisture and 0.50% soluble protein. The effects of carbon and nitrogen source on the kinetics of growth and carotenoids synthesis of yeast *R. glutinis* in 7 days were evaluated (Figs. 1 and 2).

In this research, the same weight of glucose and date syrup was used. Significant differences were observed when the average biomass and total carotenoid productions were compared in different cultures and at different times. Whereas sugar content of date syrup was lower, it was observed that the total carotenoids production was higher when culture media contained date syrup. Figs. 1 and 2 reveal that carotenoids were produced in association with yeast growth.

As shown in Table 2, the culture medium 6 containing date syrup and yeast extract appeared to be

the most suitable medium for carotenogenesis (higher than basal medium) in which carotenoid content reached 7.94 mg/L/day and average carotenoid production rate was 2.11 (mg/L/day).

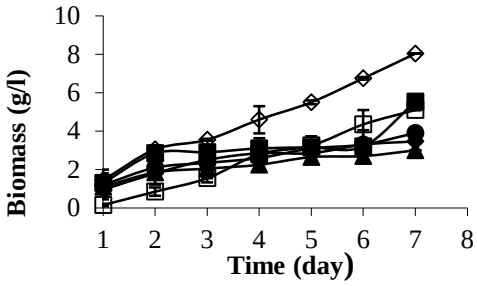

Fig. 1. The growth kinetic of *R. glutinis* in various culture compositions: (◇): Glucose and yeast extract; (□): Glucose and $(NH_4)_2SO_4$; (▲): Glucose and NH_4NO_3; (◆): Date syrup and $(NH_4)_2SO_4$; (■): Date syrup and NH_4NO_3; (●): Date syrup and yeast extract

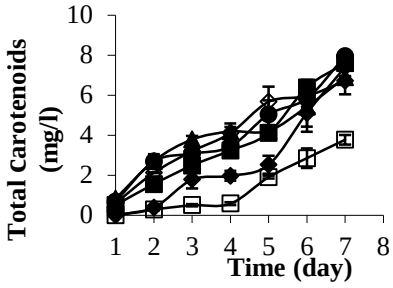

Fig. 2. Carotenoids production kinetic by *R. glutinis* in various cultures (◇): Glucose and yeast extract; (□): Glucose and $(NH_4)_2SO_4$; (▲): Glucose and NH_4NO_3; (◆): Date syrup and $(NH_4)_2SO_4$; (■): Date syrup and NH_4NO_3; (●): Date syrup and yeast extract

Table 2. Production of total carotenoids by *R. glutinis* grown in different fermentation culture, after 7days

Culture type[1]	Final pH[*]	Total carotenoids (mg/L culture fluid)	Biomass (g/L culture fluid)	Carotene content (µg/g dry cells)	Average carotenoid production rate (mg/L/ day)
1	5.60±1.06 [b]	6.72±1.47 [f]	8.03±0.97 [a]	836.86±53.77 [d]	0.77±0.05 [f]
2	1.82±0.74 [d]	3.77±1.02 [e]	5.10±1.06 [c]	739.22±2.78 [d]	0.92±0.12 [e]
3	5.90±1.21 [a]	6.89±2.41 [d]	3.00±0.88 [f]	2295.00±16.78 [a]	1.47±0.06 [c]
4	4.05±1.33 [c]	7.40±0.87 [c]	3.46±0.65 [e]	2136.94±11.31 [ab]	2.30±0.82 [a]
5	4.05±0.92 [c]	7.59±0.48 [bc]	5.53±0.23 [b]	1372.51±8.99 [c]	1.21±0.24 [d]
6	5.75±0.38 [ab]	7.94±1.34 [a]	3.90±0.56 [d]	2040.21±12.62 [b]	2.11±0.53 [b]

[*] Different superscripted letters indicate significant differences ($P<0.05$) among the observed values.

[1] Culture compositions have been reported in the text.

However, thebasal medium stimulated a maximum biomass production of 8.03 g/L which contained glucose and yeast extract (culture medium1). Enhancement of the β-carotene production would be performed by the addition of other nutrients such as yeast extract, glucose and urea. Ammonium sulphate as a nitrogen source was added to the growth media 2 and 4 (in which carbon sources was glucose and date syrup, respectively) that enhanced the carotenoid production rate to 3.77 and 7.40 mg/L/ day in culture 2 and 4, respectively. Thus, with the same nitrogen source, date syrup had a significant effect on carotenoid production. Minimum final pHs, total carotenoids, carotenoid content were obtained in culture 2 that contained ammonium sulphate instead of yeast extract and glucose as a carbon source. Furthermore, the addition of ammonium nitrate to culture 5 led to the enhancement of total carotenoid production and the organism growth was compared with ammonium sulphate when used as a nitrogen source in culture 4. Although the minimum biomass (3 g/L) was obtained in culture 3, the highest value of carotene content (2295 µg/g dry cells) was observed. Carotenoid is a second metabolite of yeast produced at the stationary phase. With a decrease in biomass, carotenoid production was increased. Time course of carotenoid production in culture 6 is shown in Table 3. About half of total dry cell weight (maximum dry cell weight production rate after 24 h) and about one third of total carotenoids were produced within 48 h.

As seen in Fig. 3, the growth rate of yeast was maximum in date syrup with NH_4NO_3 medium (2.33 g/L/ day) and decreased when other nitrogen sources were used in the media containing date syrup. On the other hand, in glucose media, maximum biomass production rate (1.28 g/L/day) was obtained in basal medium after 7 days. Also, the effect of carbon and nitrogen sources on carotenoid production rate was shown in Fig. 4. In media containing date syrup with $(NH_4)_2SO_4$ and glucose withNH_4NO_3 the highest carotenoid production rates were 13.94 and 4.88 mg/g/day after 7 days, respectively. Fig 3 indicated that the nitrogen sources associated with glucose and date syrup had significant effects on the growth and carotenoid production rate but there was not a parallel relationship between them.

Table 3. Fermentation parameters of carotenoid production by *R. glutinis* in the culture 6 containing date syrup

Parameter	Days						
	1	2	3	4	5	6	7
Total carotenoids							
Volumetric concentration (mg/L) *	0.69±0.01 g	2.69±0.86 f	3.08± 0.86 e	3.43±1.04 d	5.04±1.80 c	5.83±0.96 b	7.94±2.25 a
Produced carotenoids (%)	8.69±1.45 g	33.88±8.44 f	38.79±7.26 e	43.2±10.62 d	63.48±7.33 c	73.42±2.38 b	100±1.57 a
Volumetric rate (mg/L/ day)	0.69±0.04 d	2.00±0.81 a	0.39±0.01 e	0.35±0.03 e	1.61±0.13 b	0.79±0.15 c	2.11±0.61 a
Cellular rate (µg/g dry cell weight day)	575±11.89 g	2222±5.98 d	1560±12.36 e	1400±1.2 0 f	3220±118.50 c	3950±1.80 a	3517±15.72 b
Cell growth							
Cellular biomass (g/L)	1.20±0.92 f	2.10±0.07 e	2.35±0.44 d	2.60±0.48 c	3.10±0.83 b	3.30±0.12ab	3.90±2.25 a
Produced dry cell weight (%)	30.77±3.55 g	53.85±1.50 f	60.26±1.07 e	66.67±1.42 d	79.49±2.08 c	84.62±1.91 b	100±1.57 a
Dry cell weight production rate (g/L/ day)	1.20±0.04 a	0.90±0.12 b	0.25±0.01 e	0.25±0.04 e	0.50±0.10 d	0.20±0.03 e	0.60±0.61 c

* Different superscripted letters indicate significant differences (P<0.05) among the observed values.

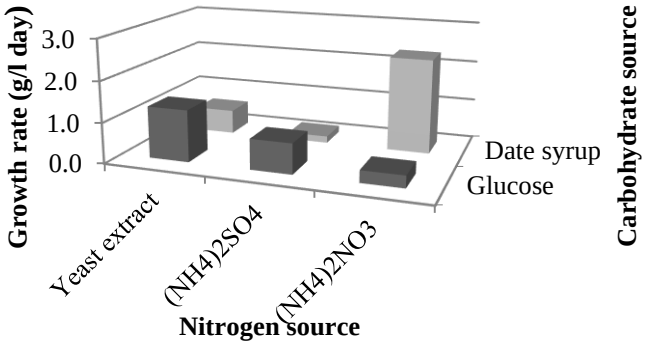

Fig. 3. Effect of carbohydrate and nitrogen sources on the growth rate of *R. glutinis*after 7 days in date syrup

The maximum total carotenoids (7.94 mg/L) and average carotenoid production rate (2.11 mg/L/day) were obtained from the culture containing date syrup and yeast extract as carbon and nitrogen sources that were higher than those at basal medium, respectively. *R. glutinis* is carotenoid producing yeast and synthesizes β-carotene as the major carotenoid (Perrier et al., 1995). The results (Table 3) suggest that date syrup had the potential to be employed as a sole substrate for *R. glutinis* for β-carotene production. Maximum carotenoids production, in line with some previous reports, occurred after the exponential phase (Buzzini and Martini, 1999; Buzzini, 2001). In addition, the maximum cell dry weight obtained from date syrup (8.03 g/L) was higher than the amount obtained from using glucose syrup (7.5 g/L), soybean flour extract (4.8 g/L) and maize flour extract (5.2 g/L) (Buzzini and Martini, 1999). Perhaps the difference is due to the differences of the yeast strain used, the nutrient compositions and chemical concentrations in the date syrup, the cultivation condition and the β-carotene extraction method.

The results revealed that the two factors, i.e., carbon and nitrogen sources, had a significant influence on biomass and carotenoid production. The inorganic nitrogen sources investigated produced only poor growth. In contrast to ammonium sulphate and ammonium chloride, ammonium nitrate resulted in distinctly higher β-carotene content, (Bhosale and Gadre, 2001). As seen in Table 2, the maximum total carotenoid was obtained from the date syrup medium containing yeast extract and the glucose medium containing NH_4NO_3. However, date syrup media containing NH_4NO_3 and $(NH_4)_2SO_4$ had the highest yeast growth and carotenoid production rate, respectively. During fermentation, the maximum carotenoid production seemed to occur after the exponential phase, in good agreement with some previous reports (Frengova et al., 1994) but in disagreement with some other findings (Shih and Hang, 1996).

CONCLUSIONS

The results demonstrated that date syrup as a by-product of low quality date can be used as a good and economical substrate for the production of yeast biomass and carotenoids by *R. glutinis* but its production was affected by nitrogen source. The best total carotenoids production rate could be obtained from culture contained date syrup and yeast extract as carbon and nitrogen sources, respectively.

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کاربرد شیره خرما به عنوان سوبسترا جهت تولید کاروتنوئید توسط رودوترولا گلوتینیس

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واژه های کلیدی:
محصول جانبی
شیره خرما
کاروتنوئید میکروبی
رودوترولا گلوتینیس

چکیده– امکان استفاده از شیره خرما جهت تولید کاروتنوئید توسط رودوترولا گلوتینیس، در فرایند تخمیر غیر مداوم طی ۷ روز بررسی شد. نتایج نشان داد که منبع کربن (گلوکز یا شیره خرما) و منبع نیتروژن (عصاره مخمر، سولفات آمونیوم و نیترات آمونیوم) تاثیر قابل توجهی بر تولید زیست توده و کاروتنوئید داشت. حداکثر بازدهی تولید کاروتنوئید کل (۷/۹۴mg/L) با محتوی کاروتن ۲۰۴۰µg/g و زیست توده ۳/۹۰ g/L توسط رودوترولا گلوتنیس در مدت زمان ۷ روزدر سوبسترای حاوی شیره خرما به عنوان تنها منبع کربوهیدرات و عصاره مخمر حاصل شد بیشترین میزان زیست توده ۸/۰۳g/L در محیط حاوی گلوکز و عصاره مخمر ایجاد شد، درحالی‌که میزان کاروتنوئید کل (۶/۷۲ mg/L) حاوی ۸۳۶/۸۶ µg/g کاروتن در این محیط تولید گردید. بین زمان‌های مختلف تخمیر و نیز محیط‌های کشت مختلف از نظر تولید زیست توده و تولید کاروتنوئید تفاوت معناداری مشاهده شد. نتایج حاصل از این پژوهش نشان داد که شیره خرما(از خرماهای با کیفیت پایین) به عنوان محصول جانبی با قیمت پایین می‌تواند به عنوان منبع کربن مناسب برای تولید کاروتنوئید توسط رودوترولا گلوتینیس مورد استفاده قرار گیرد.