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Improving carotenoid pigments production in *Rhodotorula mucilaginosa* using UV irradiation

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Abstract

Rhodotorula yeasts were isolated from various local sources in Damascus, Syria, during 2012 and 2013. Fifty isolates (23 from leaves trees, 13 from different soils, 6 from different meats, 6 from dairy products, 1 pickle and 1 traditional sweet) were identified using API 20C AUX system. Isolated yeasts were classified into three *Rhodotorula* species: *R. mucilaginosa, R. glutinis* and *R. minuta* representing 76%, 20% and 4% of total isolates tested respectively. All isolates were able to produce carotenoid pigments that varied in their quantities ranging from 13.61 µg/g dry biomass for isolate A28 to 658.23 µg/g for isolate A23. The wild strain A23 of *R. mucilaginosa* was subjected to mutagenesis using UV irradiation at a wavelength of 254 nm. Highest carotenoid production within isolated mutants reached a value of 734.58 µg/g dry biomass after an irradiation period of 4.5 minutes. Overall, UV irradiation

Introduction

Carotenoid pigments are widely distributed in nature, with over 600 molecule types already identified (Fraser and Bramley, 2004). These molecules are often found in algae, filamentous fungi, yeasts, bacteria, and plants. However, animals are not able to synthesize them. Their main biological functions are the anti-carcinogenic and provitamin A properties as well as the effects against reactive oxygen elements (Young and Lowe, 2011).

Because of their specific properties, there is a growing demand for more suitable medium to produce these natural pigments, especially for food coloring and nutritional supplementation (Downham and Collins, 2000). Carotenoid pigments can be used for the desired yellow, orange and red color of many foods, e.g. fruits, vegetables, egg yolk, some fish like salmon and trout, and crustaceans (Astorg, 1997) . Aside from being natural pigments, carotenoids also have important biological functions (Bendich, 1994). In more recent years, vitamin A-active and inactive carotenoids have been found to have other beneficial effects on human health: enhancement of the immune system and reduction of the risk for degenerative diseases such as cancer, cardiovascular diseases, macular degeneration and cataract (Krinsky, 2001).

Color is an important attribute that determines consumers' acceptance of foods and carotenoids constitute one of the most valuable classes of compounds for industrial applications, e.g. in the pharmaceutical, chemical, food and feed industries The addition of coloring agents in processed foods has been a common practice for many years (Baraka *et al*., 2014).

Due to the possible toxicity of artificial coloring agents, natural coloring alternatives have been increasingly sought. The huge international market for carotenoids has been met mainly by synthetic carotenoids with structures identical to those of natural carotenoids, but there is growing demand for natural sources (Johnson and Schroeder, 1996). Traditionally, carotenoids have been marketed as dried powder or extracts from plants, such as annatto, paprika and saffron. Natural colorants from plant sources, however, suffer from a diminishing or unstable supply of raw materials, subject to climatic conditions, as well as varying colorant level and quality of the final product (Maldonade *et al*., 2008).

Microbial carotenoids have attracted much attention in recent years (Nelis and De Leenheer, 1991). The main reason for the interest in using microorganisms to produce compounds that can otherwise be isolated from plants and animals is the ease of increasing production by environmental and genetic manipulation. The commercial utilization of microorganisms with biotechnological potential to

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produce carotenoids is presently limited by the high cost of production. However, the cost of carotenoid production by fermentation can be minimized by optimizing its process, using high pigment-producing microorganisms cultured in cheap industrial by-products as nutrient sources (Aksu and Eren, 2005) .The main microbial sources able to synthesize these molecules are the yeast genera Phaffia and *Rhodotorula*, both characterized by high growth potential, high production of carotenoids (Naghavi *et al.*, 2013), and their ability to grow in low-cost culture media (Squina *et al*., 2002). Ultraviolet light is one of the radiation sources that directly induce alterations in DNA molecules. Short-wavelength radiation between 200 and 300 nm is the most effective for mutagenesis (Vijayalakshmi *et al.*, 2001). Therefore the objectives of this study were to isolate and identify *Rhodotorula* yeast by API 20C AUX system from various local sources for Carotenoid pigments production and improving the productivity of the best wild isolate of *Rhodotorula* by mutagenesis using UV irradiation at 254nm.

Materials and Methods

Sample collection and screening

Samples were collected from various local sources (23 samples of different tree leaves, 13 samples of different soils, 6 samples of meat varieties, 6 samples of dairy products, 1 sample of pickle and 1 sample of traditional sweet). The collected samples were stored in aseptic packing until analysis. Screening was performed for those strains that produced the desired coloration; colonies ranging from yellow to red color yeast cultures inoculated in extract malt agar incubated at 30ºC. Out of 500 collected colonies, only fifty colonies (From A1 to A50) had a yellow to red color of high intensity. The stock cultures were maintained on malt extract-agar at 4ºC.

Isolation and selection of yeasts

The samples were placed in Erlenmeyer flasks containing 20 mL of YM, broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1.0% glucose), and incubated at 30ºC for 48 hours. The samples were then inoculated in Petri dishes containing YM agar and incubated at 30ºC for 120 hours (Yehia *et al.*, 2013). Yellow to red colonies were transferred to slant tubes with YM agar and incubated at 30ºC until heavy growth was obtained. These colored cultures were re-isolated in Petri dishes containing YM agar (incubated at 30ºC for 72 hours). After this stage, the purity of the strains was verified by microscopic examination. The selected cultures were transferred to agar slants containing the YM agar and after incubation were stored at 4ºC.

Biochemical characterization

Rhodotorula yeast cells were examined under the microscope (for their appearance such as size, cell shape, color and budding by staining the cell slides with Methyl Red. For biochemical classification of yeasts species, API 20C AUX was used according to Maldonade *et al.* (2006) which depends on the ability of yeast to ferment 19 types of sugar distributed in special copules on API strips after inoculating them with 100 µl of yeast suspension with a turbidity of 2 MacFarland. The API strips were incubated for 24 and 72 hours at temperature, 30ºC. Results were recorded and compared according to instruction the manual of API.

Screening of Rhodotorula *yeast for high carotenoid production.*

Rhodotorula yeast was grown in 50 mL of basal broth (BB) (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 in a rotary shaker (Model Bio-Shaker BR-300FL, TAITEC, Saitama-ken, Japan) at 130 rpm and temperature, 28°C for 48 h. The red colored yeast strains with high carotenoid production were selected for further evaluation (Yimyoo *et al.*, 2011).

Extraction and determination of carotenoids

Exposure of the extract to heat, light, and oxygen should be minimized throughout the extraction procedure. Therefore, wet cells were collected by centrifuging the growth medium at 9000 rpm for 10 min and washed twice with distilled water. The yeast cells were ruptured with dimethylsulphoxide (DMSO) and extracted into diethyl ether. The total carotenoid concentration in the diethyl ether extract was determined by measuring the optical density at 460 nm with the UV-vis spectrophotometer (Somashekar and Joseph, 2000).

Quantification of carotenoids

The amount of carotenoids was determined according to (Vijayalakshmi *et al.*, 2001) and (Maldonade *et al.*, 2006).The extracted carotenoid color was measured by the spectrophotometer at 460 nm. Carotenoid contents determined by the formula:

Production of carotenoids (µg/g) = 5.405 A460 X / Y

where A460: the value of the sample absorption at 460 nm wavelength, X (ml): volume of the sample, Y

(g): dry weight biomass.

Mutagensis improvement of R. mucilaginosa

To improve the production of carotenoids by *R. mucilaginosa*, the selected yeast was radiated by UV at 254 nm. Cell culture were spread on sterilized Petri dishes containing malt extract agar media and irradiated at a distance of 10 cm above Petri dishes after removing the lids with time intervals ranging from 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, to 5 minutes. After irradiation, the plates were incubated in dark at temperature, 30ºC (Bhosale and Gadre, 2001). The irradiated colonies were transferred of the incubation to the production medium (BB) for extraction and analysis of carotenoids.

Results and Discussion

Isolation and identification of Rhodotorula *yeast from local sources*

Rhodotorula yeast from local Syrian sources were collected and pure isolates of selected yeast were examined microscopically (Olympus 1000X). They appeared as large cells with ellipsoidal form and peripheral budding as shown in Figure 1. The isolated *Rhodotorula* yeast species were identified by biochemical characterization using API 20cAUX (Maldonade *et al*., 2006).

Results showed that *R. mucilaginosa* exhibited negative reaction to seven carbohydrates (Glycerol, Galactose, Inositol, Methyleglucoside, N-cytel glucose, cellobiose and Lactose) while *R. glutinis* revealed negative reaction to ten carbohydrates (Arabinose, Xylose, Adonitole, Xylitol, Galactose, Inositol, Methyleglucoside, N-acytel glucose, Lactose, and Trehalose). In addition to that, *R. minuta* showed negative reaction towards thirteen carbohydrates(2-keto glucose, Adonitol, Xylitol, Galactose, Inositol, Sorbitol, Methyleglucoside, N-cytel glucose, Lactose, Maltose, Trehalose, cellobiose and Rafinose) as illustrated in Table 1.

Classification of *Rhodotorula* yeast species from local sources for Carotenoid pigments production is shown in Table 2 revealing that the fifty isolates *Rhodotorula* yeast belong to three species : *R. mucilaginosa, R. glutinis and R. minuta* with 76%, 20% and 4% of total isolated samples respectively. It appeared that *R. mucilaginosa* was dominant in different tree leaves and to a less extend in different soils compared with other sources such as meat varieties, dairy products, pickle and traditional sweet. These results came in agreement with other researches which recommended isolation of *R. mucilaginosa* from tree leaves (Libkid and Brook, 2006).

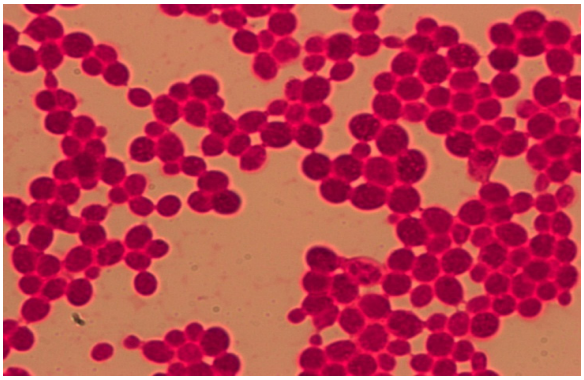

Figure 1. *Rhodotorula* yeast under microscopic examination

Table 1. Identification of Rhodotorula yeast species using API 20c AUX

No	Test	*R. mucilaginosa*	*R. glutinis*	*R. minuta*
1	GLU	+	+	+
2	GLY	-	+	+
3	2KG	+	+	-
4	ARA	+	-	+
5	XLY	+	-	+
6	ADO	+	-	-
7	XYL	+	-	-
8	GAL	-	-	-
9	INO	-	-	-
10	SOR	+	+	-
11	MDG	-	-	-
12	NAG	-	-	-
13	CEL	-	+	-
14	LAT	-	-	-
15	MAL	+	+	-
16	SAC	+	+	+
17	TRE	+	-	-
18	MLZ	+	+	+
19	RAF	+	+	-

Quantitative determination of carotenoids from Rhodotorula *isolates*

Carotenoid pigments from *Rhodotorula* isolates showed that the quantities vary in their production of carotenoids although they grow in low-cost culture media (Squina *et al*., 2002). Carotenoids production depends on the type of strains, and on the culture conditions which affect yeast growth and metabolite production. Table 3 shows that the minimum quantity of carotenoid pigments was recorded by isolate A28 (13.61µg/g) whereas the highest amount was produced by isolate A23 (658.23µg/g). However, the best five carotenoid producing isolates were A1, A2, A5, A23 and A24 which belong to *R. mucilaginosa* from different tree leaves except A5 which is a yogurt isolate and A24 isolated from meat, their production quantities of carotenoid pigments were 539.09, 323.45, 300.55, 658.23 and 428.53 µg/g, respectively. This result confirmed that microorganisms can exhibit genotype differences in spite of belonging them to the same type of strain according to the growth property and metabolism activity. The reason for that can be attributed to the response of the strain to environmental conditions (Elander and Chang, 1979). These results come in agreement with (Maldonade *et*

Table 2. Classification of *Rhodotorula* yeast species from local sources for carotenoid pigments production

Samples sources	No. of samples	*R. mucilaginosa*	*R. glutinis*	*R. minuta*
Tree leaves	23	18	5	-
Soils	13	10	1	2
Meat varieties	6	4	2	-
Dairy products	6	5	1	-
Pickle	1	1	-	-
Traditional sweet	1	-	1	-
Total of samples	50	38	10	2
Total species %		%76	%20	%4

Table 3. Total average quantities of Carotenoids from *Rhodotorula* isolates

Isolated No.	*Average of* Carotenoids pigment (µg/g dry weight biomass) ±SD
A1	***539.09±1.84***
A2	***323.45±2.17***
A3	*285.45±2.02*
A4	*111.65±1.4*
A5	***300.55±1.67***
A6	*109.64±0.71*
A7	97.54±1.51
A8	244.96±1.62
A9	91.99±0.86
A10	227.78±1.50
A11	65.94±0.71
A12	140.83±0.42
A13	95.23±2.07
A14	91.39±1.44
A15	111.78±1.67
A16	118.74±2.01
A17	214.61±1.95
A18	46.89±2.41
A19	66.6±1.33
A20	24.73±1.61
A21	43.14±1.45
A22	178.78±4.01
A23	**658.23±0.84**
A24	***428.53*±3.56**
A25	*42.63*±1.54
A26	36.34±1.87
A27	34.09±0.71
A28	13.61±0.76
A29	43.09±2.72
A30	31.71±1.31
A31	20.54±2.68
A32	19.42±1.16
A33	14.39±1.79
A34	154.58±1.59
A35	251.23±1.58
A36	230.58±1.97
A37	52.90±2.02
A38	148.23±2.22
A39	138.127±1.89
A40	217.28±1.63
A41	201.12±1.71
A42	39.53±2.08
A43	36.81±0.78
A44	23.480±1.16
A45	145.18±1.41
A46	195.41±1.12
A47	75.85±1.76
A48	94.88±1.30
A49	127.583±2.52

al. 2006) who isolated *R. mucilaginosa* from soil, it produced high quantities of carotenoid pigments reaching a value of 590 µg/g.

Moreover, Aksu and Eren (2005) reported that the quantity of carotenoid pigments reached a value of 355 µg/g for *R. mucilaginos*a isolated from soil. In addition, Maldonade *et al*. (2012) reported that the maximum carotenoid production by a strain of *Rhodotorula mucilaginosa*, isolated from the Brazilian ecosystem was 745 µg/ l.

Mutagenesis improvement of R. mucilaginosa *by UV irradiation at 254nm*

Table 4 illustrates the quantities of carotenoids produced by *R. mucilaginosa* after different intervals of irradiation where the results were compared with a control of isolate A23(658.23µg/g) which was not irradiated. It can be shown that increasing the radiation periods led to a decrease in colony numbers of *R. mucilaginosa* from 20 colonies at 0.5 minute period of irradiation reaching 5 colonies at the irradiation period up to 5 minutes and these result agree with other researchers such as Abd El-razek (2004) who concluded that long exposures to radiation with time led to a decrease in colony numbers, However, some colonies of *R. mucilaginosa* could survive the radiation and might improve the production of carotenoids such as the mutagen number 2 with 4.5 minute irradiation period that produced the highest amount of 734.58 µg/g followed by the mutagen number 10 at 3.5 minute irradiation period that produced 670.71 µg/g dry. Yehia *et al.* (2013) reported that 2 minutes irradiation were enough to produce high quantities of caroteniod pigments , he found that *R. glutinis* produced the highest amount of carotenoids under the same conditions.

Conclusion

It can be concluded that the best source for isolation *Rhodotorula mucilaginosa* is from tree leaves, it was show that these isolates produced the highest amounts of carotenoid pigments. It could also be concluded that by UV irradiation at 254 nm of those isolates, it is possible to obtain mutations with a higher production percentage reaching 89.6%.

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Table 4. Results of mutagenesis improvement of *R. mucilaginosa* by UV irradiation at 254nm

M.N	I.T	Quantities of carotenoids measured in µg/g dry weight biomass									
		0.5	1	1.5	2	2.5	3	3.5	4	**4.5**	5
1		191.17	221.34	225.55	99.71	146.13	171.97	137.82	185.44	238.39	125.47
2		178.78	293.41	271.42	172.96	214.78	151.13	294.57	258.87	**734.58**	148.63
3		189.17	254.97	233.76	231.47	114.62	203.27	248.37	185.57	157.82	160.92
4		203.22	177.16	211.28	172.44	99.41	205.9	109.06	189.17	266.09	119.68
5		223.57	170.74	256.09	239.36	186.17	116.1	121.17	296.04	142.84	314.47
6		131.43	213.49	269.02	254.03	203.91	260.42	121.02	190.07	257.95	-
7		224.79	192.55	205.14	152.49	298.62	146.87	239.17	191.17	226.33	-
8		190.35	122.11	144.58	192.9	227.01	217.48	162.15	618.05	-	-
9		259.44	139.12	131.26	109.1	193.17	160.86	106.23	-	-	-
10		162.15	217.16	194.76	177.59	178.06	108.1	**670.71**	-	-	-
11		158.28	121.61	124.87	99.38	149.86	198.52	-	-	-	-
12		139.82	237.08	163.32	227.95	153.14	112.6	-	-	-	-
13		189.17	168.9	170.79	224.79	-	-	-	-	-	-
14		173.41	198.63	149.17	183.16	-	-	-	-	-	-
15		186.82	177.42	191.17	117.11	-	-	-	-	-	-
16		145.42	164.01	-	-	-	-	-	-	-	--
17		229.71	163.11	-	-	-	-	-	-	-	-
18		227.25	185.31	-	-	-	-	-	-	-	-
19		158.46	-	-	-	-	-	-	-	-	-
20		169.2	-	-	-	-	-	-	-	-	-
T. Survival		**20**	**18**	**15**	**15**	**12**	**12**	**10**	**8**	7	**5**
Sig		**0**									
LSD(1%)		**0.012**									

M.T: Mutation Number I.T: Irradiation Time

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