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Using whey for Production of Carotenoids by *Rhodotorula glutinis*

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ABSTRACT

Optimization of fermentation conditions for maximum total carotenoids production by *Rhodotorula glutinis* NRRL YB-252 co-cultivated with *Lactobacillus casei* sub sp. *casei* NRRL B- 441 using whey as a tested medium was investigated. Maximum total carotenoids, torularhodin and dry cell weight were obtained from whey fortified with 3% glucose, 0.75% yeast extract and 0.5% magnesium sulphate as a carbon, nitrogen and mineral sources, respectively. At the optimal fermentation conditions, the total carotenoids, torularhodin and dry cell weight were 474.76 (µg/g), 101.56(µg/ml)and 7.10(g/L), respectively. Stability towards some technological factors of carotenoids produced under the optimal conditions was evaluated. The pigment was stable for most tested thermal treatments, sodium chloride and sucrose concentrations. High acidic conditions resulted in slightly low stable carotenoids.

Key words: *Rhodotorula glutinis, Lactobacillus casei* sub sp. *casei*, Carotenoids, Whey, carbon source, nitrogen source, mineral source, stability.

Introduction

The ever-increasing demand for food containing only natural ingredients is responsible for the market trend towards the use of natural rather than synthetic (Buzzini, 2001). Among pigments of natural origin, carotenoids seem to play a fundamental role, their presence in the human diet being considered positively because of their action as pro-vitamin, antioxidant or possible tumor-inhibiting agents. Despite the availability of a variety of natural and synthetic carotenoids, there is currently a renewed interest in microbial sources of pigments because of the problems of seasonal and geographical variability in plant origin (Latha and Jeevaratnam, 2010).

Carotenoids constitute a group of natural pigments that are ubiquitous throughout nature. They are present in photosynthetic organisms, as well as present in some bacteria, yeasts, and fungi. Over 600 different carotenoid species are found in bacteria, plants, fungi and yeasts. Carotenoids usually consists of 40 carbon atoms and their colour range from yellow to reddish with variations to brown and purple (yehia *et al*., 2013). Yeasts are more convenient than algae or fungi for large scale production of carotenoids in fermenters, due to their unicellular nature and high growth rate. *Rhodotorula glutinis* is known to produce characteristic carotenoids viz. torulene, torularhodin and β-carotene in various proportion. It is potentially useful for industries since it is able to grow in various cheap agricultural raw materials such as sugar cane juice, peat extract, whey, grape must, beet molasses, hydrolyzed mung bean waste flour, soybean and corn flour extracts and sugar cane molasses for carotenoid production (Aksu and Eren 2005; Bhosale and Gadre, 2001a; Buzzini and Martini,1999;Park *et al*., 2005;Simova *et al*.,2004 **a**nd Tinoi *et al*., 2005).

Raw material and by-products of agro-industrial origin have been proposed as low cost alternative carbohydrate sources for microbial metabolite production, with the view also of minimizing environmental and energetic problems related to their disposal (Demain *et al.*, 1998). A widespread natural substrate, a residue from cheese manufacture, is milk whey containing lactose as a carbon source. Carotenoid-synthesizing yeasts able to assimilate lactose are rarely found in natural conditions (Zalashko,1990). Carotenoid synthesis by lactose-negative yeasts of *Rhodotorula* genus in lactose substrates can be accomplished only by creating conditions in which the lactic acid bacteria transform lactose into carbon compounds (glucose, galactose, lactic acid)that are easily assimilated by the yeast in a process of co-cultivation of yeast and bacterial cultures (Simova *et al.*, 2003).

The objective of this work is to (A) Produce carotenoids by *Rhodotorula glutinis* NRRL YB-252 co-cultivated with *Lactobacillus casei* subsp. *casei* NRRL B- 441 using whey (tested medium) and yeast and malt extract broth (standard medium) at optimal fermentation conditions, (B) study the stability of carotenoids produced at optimal fermentation conditions.

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Materials and Methods

Microorganisms:

Rhodotorula glutinis NRRL YB-252 and *Lactobacillus casei* subsp. *Casei* NRRL B-441 were obtained from the Northern Regional Research Laboratory(NRRL), USA.

Rhodotorula glutinis NRRL YB-252 was maintained on a yeast and malt extract agar(YM agar) slant (10g/ldextrose, 3 g/l malt extract, 3 g/l yeast extract, 5g/l peptone and 20 g/l agar at pH 6.2±0.2) at 4°C until used. *Lactobacillus casei* sub sp. *casei* NRRL B-441 was maintained by mixing a pure culture that had been grown in MRS broth over night at 30°C with equal volume of 10% glycerol solution and storing at -20°C until used (Van Den Berg *et al*.,1995)**.**

Media for pigment production:

Pigment production was carried out in (a) yeast and malt extract broth(YM broth) as a Standard medium and (b) whey as a tested medium. Fresh sweet whey was obtained from Faculty of agriculture, Cairo University, Giza., Egypt. Whey was acidified by lactic acid to pH 4.6and heated for 10 min at 80°C to precipitate whey proteins. The precipitate was removed by cheesecloth and supernatant was filtered through two layers of whatman No.1 paper.The pH was then adjusted to pH 6 with 2 M – NaOH and autoclaved at 121°C for 20 min. The composition of the whey medium was as follows:4.9% lactose, 0.5% ash, 0.4% fat and 0.1% protein.

Effect of adding some components to the whey medium on carotenoids production:

Fermentation experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of sterilized YM broth or whey medium. The starch, sucrose or glucose as a carbon source (0, 1, 2, 3 or 5% w/v), ammonium sulphate, caso amino acid or yeast extract as a nitrogen source (0, 0.25, 0.50 or 0.75 % w/v) and potassium dihydrogen phosphate or magnesium sulphate as a mineral source (0, 0.25, 0.50 or 0.75 % w/v) were separately added with a mentioned concentrations to whey medium to examine the effect of activators on the growth and carotenoids productivity of the yeast. Each flask was inoculated with *Rhodotorula glutinis* NRRL YB-252 (5 % v/v) and *Lactobacillus casei* sub sp. *casei* NRRL B-441 (1% v/v) and incubated in shaker incubator at 220 rpm at 30°C.All shake flask experiments were carried out in triplicate. Samples were taken at zero time and every 48h throughout the tested incubation period. The samples were analyzed for, dry cell weight, residual lactose, pH, total carotenoids and torularhodin

Analytical Methods:

-Dry cell weight (g/l) of yeast was taken as a parameter for its growth. Aliquots (10 ml) of yeast suspension were centrifuged for 5 min. at 13.000 rpm washed twice with distilled water, and then dried at 105°C till constant weight (Simova *et al*., 2003). The pH was determined by using digital pH meter (Inolad model 720, Germany).The residual lactose of fermented whey samples was determined according to Nicckerson (1975).

Extraction and determination of total carotenoids & torularhodin:

R. glutinis cells were harvested by centrifugation at 10,000 g for 10 min and washed twice with distilled water. The cell pellets were centrifuged again and dried under vacuum (Latha *et al*., 2005). A known amount of dried yeast was hydrolyzed with 1N HCl in a water bath at 70°C for 90 min (Somashekar and Joseph, 2000). The acid free cells were soaked overnight in acetone: methanol (1:1) solution. The pigment was extracted with acetone and transferred to light-petroleum (40-60°C) in separating funnel and washed thrice thoroughly with distilled water (Frengova *et al*., 1994). The absorbance of the light – petroleum phase was read at 474 nm and the concentration of the carotenoids was determined using the absorption coefficient as $A^{1\%}_{1cm}$= 1600 (Andrewes *et al*., 1976). Total carotenoids content was calculated by using the formula (Baskar *et al*. 2010) given below and expressed as $\mu g \cdot g^{-1}$ of dry biomass and as $mg \cdot l^{-1}$ of culture. The carotenoids extract was concentrated by evaporation of solvent using a stream of nitrogen gas and kept at −8°C in dark.

Total carotenoids (µg/g) = A X V (ml) X 10^4/ $A^{1\%}_{1cm}$ X W (g)

Where: A= absorbance, V= total extract volume, W = sample weight, $A^{1\%}_{1cm}$ = absorption coefficient.

For the quantification of torularhodin (Pomeranz & Meloan, 1978), the absorbance of crude carotenoids extract was spectrophotometrically measured at 500 nm, which represent the maximum absorption of torularhodin. The crude carotenoids extract (5 ml) was treated with calcium hydroxide (0.25 g) to chelate the

carboxylic carotenoid torularhodin and the calcium hydroxide torularhodin chelate was removed by centrifugation at 3500 rpm (900× g) for 5 min. The absorbance of the torularhodin free carotenoids extract was remeasured at 500 nm and the torularhodin content ($\mu g \cdot ml^{-1}$) was obtained by subtraction of absorbance(A) at 500 nm before and after chelation and using the torularhodin absorption coefficient 2040 (Davies, 1965).

$C(\mu g \cdot ml^{-1}) = Ax\ 10^4 / A^{1\%}_{1cm}$

Where: C=torularhodin content ($\mu g \cdot ml^{-1}$),$A^{1\%}_{1cm}$ = absorption coefficient

Carotenoids production under the optimum conditions:

Carotenoids by *Rhodotorula glutinis* NRRL YB-252 co-cultivated with *Lactobacillus casei subsp. casei*NRRL B- 441 using whey asa tested medium were produced under the obtained optimal fermentation conditions at 30°C and 220rpm for 6 days.

The effect of some technological factors on stability of total carotenoids produced at the optimum fermentation conditions:

Stability of the caroteinoids produced by *Rhodotorula glutinis* at optimal fermentation conditions in petroleum ether was studied. Stability was investigated at different pH values (3.5, 4, 4.5, 5, 5.5 and 6), heat treatment (63 °C /30min, 72°C /15 s., 85 °C / 10 min, 85 °C / 30 min, 90°C/10min and 121°C / 20 min., and subsequently cooled to 25°C, NaCl (1, 2, 3, 5 and 7% (w/v)) and sucrose (5, 7, 9, 11 and 14% (w/v)). The optical density at 474nm of each sample was determined.

Statistical analysis:

The mean values and standard deviations were determined for all obtained data. Differences between samples were determined by T-test and were considered to be significant when $P \leq 0.05$ (Snedecor and Cochran (1989).

Results and Discussion

A- Effect of adding some components to the whey medium on carotenoids production:

1- Effect of different carbon sources and their concentrations on caroteinoids production:

Data presented in Table (1) show changes in the growth, pH, total carotenoids(µg/g) and torularhodin (µg/ml) production by *Rhodotorula glutinis* NRRL YB-252 co-cultivated with *Lactobacillus casei sub sp. casei* NRRL B- 441 using whey medium as a function of carbon sources and their concentrations. When tested carbon sources, starch, sucrose or glucose, were raised from 1 to 3 %, the total carotenoids, torularhodin and DCWincreased by progressing the incubation time to 6 days compared with control 1 (the whey without added carbon sources) or control 2 (YM medium). However, further supplementation of these carbon sources did not result in increase of the pigment production level. Therefore, the increase of cell mass is mostly responsible for the increase of the pigment production. Also, it could be noticed that the pH value and residual lactose decreased gradually with increasing tested carbon sources from 1 to 3 %. The highest total carotenoids (450 µg /g)and torularhodin (91 µg /ml) production was observed in the whey medium contained 3 % glucose after 6 days of incubation time on 30°C. When the incubation time was lengthened upon 6 days, a decrease in total caroteinoids, torularhodin and DCW was observed (data not shown). By comparison among the three sugars used, Table (1) clearly shows that glucose is the best carbon source at 3% followed by sucrose 3%. This result was in line with (Latha *et al*., 2005) who found that the maximum growth and pigmentation was observed in glucose and sucrose as carbon sources. Also, Aksu and Eren(2007) stated that the double reciprocal plots of specific growth and carotenoid production rates of the yeast cells as a function of initial glucose or sucrose concentration, both the rates increased with raising initial glucose or sucrose concentration up to 20 $g\ l^{-1}$.Sriyam *et al.* (2002) demonstrated that the 3% glucose resulted in the highest growth of *Rhodotorula glutinis* mutant (mB34) and total carotenoids.

2- Effect of different nitrogen sources and their concentrations on caroteinoids production:

The yeast extract, casoamino acid and ammonium sulphate at different concentrations, 0.25, 0.50 and 0.75% were individually used as a nitrogen source for production of carotenoids using *R. glutinis* co-cultivated with *Lactobacillus casei subsp. casei* NRRL B- 441(Table 2). The growth, total carotenoids and torularhodin production appeared to be stimulated at all nitrogen sources compared with whey medium (control 1) and yeast

and malt extract broth (YM, control 2). Also, the results indicated that the yeast extract at 0.75% concentration was the best nitrogen source for production of total carotenoids (381.15µg/g) and torularhodin (63.99µg/ml) using *R. glutinis* followed by casoamino acid and finally ammonium sulphate at the same concentration after 6 days of incubation at 30ºC. Asignificant decrease of pH and residual lactose were observed in case of yeast extract as a nitrogen source. Bhosale and Gadre (2001b) reported that the pigment production in the yeast was growth-associated. Also, the results are agreement with those obtained by Latha *et al.* (2005)who found that casein acid hydrolysate and yeast extract were among stimulant nitrogen sources for caroteinods production by *R. glutinis.* Aksu and Eren (2007) stated that the carotenoids formation and organism growth rate enhanced by adding ammonium sulphate as a nitrogen source.

Table 1: Effect of different carbon sources and their concentrations on carotenoids production.

Incubation time (day)	Responses	Control(1)	Control (2)	Carbon Source											
		Whey medium	YM medium	Starch				Sucrose				Glucose			
				Concentrations (%)											
				1	2	3	5	1	2	3	5	1	2	3	5
0	pH	6.00_{a}	6.02_{a}	6.01_{a}	6.03_{a}	6.02_{a}	6.05_{a}	6.01_{a}	6.04_{a}	6.02_{a}	6.01_{a}	6.03a	6.03a	6.02a	6.04a
	R.L.(%)	4.92_{a}	-	4.90_{a}	4.90_{a}	4.91_{a}	4.92_{a}	4.94_{a}	4.89_{a}	4.90_{a}	4.92_{a}	4.90_{a}	4.91_{a}	4.90_{a}	4.92_{a}
	DCW(g/L)	1.85_{a}	1.90_{a}	1.87_{a}	1.83_{a}	1.85_{a}	1.86_{a}	1.87_{a}	1.84_{a}	1.86_{a}	1.90_{a}	1.84_{a}	1.84_{a}	1.90_{a}	1.87_{a}
	T.C(µg/g)	23.53_{a}	23.57_{a}	23.50_{a}	23.53_{a}	23.50_{a}	23.42_{a}	23.46_{a}	23.55_{a}	23.42_{a}	23.45_{a}	23.46_{a}	23.48_{a}	23.51_{a}	23.49_{a}
	To.(µg/ml)	2.96_{a}	2.93_{a}	2.85_{a}	2.82_{a}	2.91_{a}	2.95_{a}	2.90_{a}	2.88_{a}	2.86_{a}	2.94_{a}	2.87_{a}	2.88_{a}	2.95_{a}	2.94_{a}
2	pH	5.61_{fe}	4.63_{n}	5.87_{a}	5.78_{b}	5.60_{gf}	5.70_{db}	5.75_{cb}	5.69_{ecd}	5.42_{kij}	5.50_{ih}	5.51_{h}	5.43_{jhi}	5.26_{m}	5.39_{ijk}
	R.L.(%)	4.47_{dc}	-	4.41_{fed}	4.38_{gf}	4.26_{ih}	4.53_{cb}	4.78_{a}	4.57_{b}	4.27_{h}	4.45_{ed}	3.85_{j}	3.27_{k}	3.08_{l}	4.53_{cb}
	DCW(g/L)	2.17_{k}	2.33_{ij}	2.20_{k}	2.35_{i}	2.67_{d}	2.10_{m}	2.42_{h}	2.62_{ed}	2.93_{b}	2.20_{k}	2.50_{g}	2.57_{fe}	3.00_{a}	2.75_{c}
	T.C(µg/g)	27.51_{n}	31.21_{lk}	38.78_{ij}	42.68_{jh}	47.78_{g}	27.78_{ml}	44.50_{hg}	61.15_{fde}	63.53_{ed}	32.75_{k}	63.71_{d}	85.53_{b}	89.75_{a}	81.56_{cb}
	To.(µg/ml)	3.75_{m}	4.23_{l}	5.66_{i}	6.65_{h}	11.13_{d}	3.63_{nm}	6.01_{i}	8.87_{g}	9.18_{fe}	4.44_{k}	9.24_{e}	12.81_{b}	13.15_{a}	12.09_{c}
4	pH	5.39_{fde}	4.50_{k}	5.77_{a}	5.58_{b}	5.17_{gf}	5.43_{c}	5.41_{dc}	5.39_{edc}	4.45_{lk}	5.22_{f}	5.03_{h}	4.76_{i}	4.13_{m}	4.66_{j}
	R.L.(%)	3.94_{b}	-	3.60_{d}	3.39_{gf}	3.37_{hgf}	3.98_{a}	3.57_{ed}	3.40_{f}	3.39_{gf}	3.76_{cb}	3.10_{j}	2.79_{k}	2.74_{lk}	3.09_{ij}
	DCW(g/L)	3.55_{m}	4.11_{ge}	3.55_{m}	4.00_{i}	4.13_{fe}	3.80_{k}	3.75_{lk}	4.10_{hg}	4.17_{ed}	3.93_{l}	4.22_{d}	4.35_{b}	4.73_{a}	4.30_{cb}
	T.C(µg/g)	57.3_{n}	99.30_{l}	95.32_{m}	175.12_{i}	183.82_{g}	165.60_{j}	186.14_{f}	189.98_{e}	197.62_{d}	110.92_{k}	177.74_{h}	257.7_{b}	265.2_{a}	235.12_{b}
	To.(µg/ml)	10.68_{n}	20.52_{ll}	21.18_{k}	32.45_{i}	32.67_{h}	22.17_{j}	33.24_{g}	35.41_{e}	36.50_{d}	18.91_{m}	33.82_{f}	47.94_{b}	50.00_{a}	46.10_{c}
6	pH	5.04_{a}	3.76_{h}	4.33_{b}	4.16_{d}	4.00_{g}	4.12_{ed}	4.23_{c}	4.10_{fed}	3.65_{i}	4.00_{g}	3.74_{ih}	3.55_{k}	3.46_{ml}	3.48_{l}
	R.L.(%)	3.33_{b}	-	3.00_{c}	2.06_{g}	1.37_{l}	3.74_{a}	2.64_{d}	2.57_{e}	1.43_{k}	1.59_{j}	2.23_{f}	1.77_{h}	1.00_{m}	1.57_{ij}
	DCW(g/L)	5.00_{j}	5.81_{dc}	5.10_{i}	5.64_{fe}	5.88_{ba}	5.10_{i}	5.61_{gf}	5.70_{e}	$5. 90_{a}$	5.83_{cb}	5.53_{h}	5.83_{cb}	5.95_{a}	5.90_{a}
	T.C(µg/g)	97.31_{m}	155.48_{l}	295.87_{i}	345.15_{h}	386.55_{d}	178.53_{k}	352.69_{g}	372.49_{e}	420.3_{c}	189.11_{j}	364.05_{f}	440.21_{b}	450.00_{a}	386.55_{d}
	To.(µg/ml)	19.41_{n}	31.13_{m}	63.04_{j}	71.94_{g}	81.74_{c}	33.48_{l}	66.39_{i}	68.30_{h}	76.84_{e}	46.10_{k}	76.23_{f}	81.15_{d}	91.00_{a}	88.77_{b}

R.L : Residual Lactose DCW: Dry Cell Weight T.C. : Total carotenoids To. : Torularhodin LSD of pH=0.0830 LSD of RL. = 0.0632 LSD of DCW=0.0671
LSD of T.C=0.1542 LSD of To.= 0.1363 Means with the same letter in the same raw are not significantly different

Table 2: Effect of different nitrogen sources and their concentrations on carotenoids production.

Incubation time(day)	Responses	Nitrogen Source											
		Control(1)	Control(2)	Ammonium sulphate			Casoamino acid			Yeast extract		
		Whey medium	YM medium	Concentrations(%)								
				0.25	0.50	0.75	0.25	0.50	0.75	0.25	0.50	0.75
0	pH	6.00_{a}	6.02_{a}	6.01_{a}	6.02_{a}	6.02_{a}	6.01_{a}	6.04_{a}	6.02_{a}	6.03_{a}	6.03_{a}	6.02_{a}
	R.L.(%)	4.92_{a}	-	4.90_{a}	4.90_{a}	4.91_{a}	4.93_{a}	4.89_{a}	4.90_{a}	4.90_{a}	4.91_{a}	4.90_{a}
	DCW(g/L)	1.85_{a}	1.90_{a}	1.88_{a}	1.87_{a}	1.87_{a}	1.86_{a}	1.89_{a}	1.86_{a}	1.85_{a}	1.85_{a}	1.85_{a}
	T.C(µg/g)	23.53_{a}	23.57_{a}	23.58_{a}	23.57_{a}	23.54_{a}	23.54_{a}	23.58_{a}	23.55_{a}	23.58_{a}	23.55_{a}	23.54_{a}
	To.(µg/ml)	2.96_{a}	2.93_{a}	2.94_{a}	2.97_{a}	2.96_{a}	2.95_{a}	2.94_{a}	2.95_{a}	2.93_{a}	2.98_{a}	2.96_{a}
2	pH	5.61_{a}	4.63_{k}	5.54_{b}	5.50_{c}	5.29_{gf}	5.42_{d}	5.33_{e}	5.30_{fe}	5.28_{hfg}	5.22_{i}	5.10_{j}
	R.L.(%)	4.47_{c}	-	4.43_{dc}	4.37_{e}	4.28_{f}	4.59_{b}	3.83_{g}	3.36_{h}	4.79_{a}	3.36_{h}	3.17_{i}
	DCW(g/L)	2.17_{j}	2.33_{h}	2.30_{ih}	2.39_{gf}	2.47_{e}	2.33_{h}	2.40_{f}	2.55_{c}	2.53_{dc}	2.64_{b}	2.80_{a}
	T.C(µg/g)	27.51_{k}	31.21_{j}	47.81_{i}	48.90_{f}	54.63_{ed}	48.08_{h}	48.38_{g}	54.64_{d}	63.15_{c}	64.91_{b}	77.26_{a}
	To.(µg/ml)	3.75_{k}	4.23_{j}	5.44_{i}	5.67_{h}	8.87_{c}	6.01_{g}	6.65_{f}	9.17_{b}	6.78_{e}	7.42_{d}	9.63_{a}
4	pH	5.39_{a}	4.50_{i}	5.38_{a}	5.27_{c}	5.03_{e}	5.31_{b}	5.22_{d}	4.92_{f}	4.76_{g}	4.65_{h}	4.63_{ih}
	R.L.(%)	3.94_{b}	-	3.70_{c}	3.36_{e}	3.35_{fe}	4.57_{a}	2.48_{g}	2.43_{h}	3.68_{d}	2.36_{i}	2.21_{j}
	DCW(g/L)	3.55_{k}	4.11_{j}	4.32_{h}	4.40_{g}	4.61_{fe}	4.27_{ih}	4.63_{e}	4.87_{dc}	4.93_{c}	5.00_{b}	5.13_{a}
	T.C(µg/g)	57.3_{k}	99.30_{i}	100.65_{h}	109.43_{f}	125.33_{e}	98.63_{j}	104.18_{g}	140.25_{b}	132.75_{d}	135.00_{c}	144.00_{a}
	To.(µg/ml)	10.68_{k}	20.52_{j}	31.61_{i}	32.25_{h}	47.69_{d}	33.58_{g}	36.96_{e}	51.03_{c}	35.32_{f}	51.74_{b}	54.78_{a}
6	pH	5.04_{a}	3.76_{i}	4.84_{b}	4.42_{e}	4.13_{h}	4.72_{c}	4.31_{f}	4.21_{g}	4.55_{d}	3.43_{j}	3.31_{k}
	R.L.(%)	3.33_{a}	-	2.97_{a}	2.88_{b}	2.71_{c}	2.44_{d}	1.67_{i}	1.64_{gf}	2.35_{e}	1.61_{hg}	1.48_{i}
	DCW(g/L)	5.00_{k}	5.81_{ed}	5.35_{i}	5.54_{g}	5.85_{d}	5.41_{h}	5.72_{f}	5.93_{cb}	5.15_{j}	5.95_{b}	6.15_{a}
	T.C(µg/g)	97.31_{k}	155.48_{j}	280.05_{g}	292.5_{f}	320.4_{d}	264.00_{i}	268.5_{h}	343.5_{b}	310.2_{e}	332.1_{c}	381.15_{a}
	To.(µg/ml)	19.41_{k}	31.13_{j}	36.07_{i}	37.08_{h}	54.78_{c}	41.89_{g}	43.31_{f}	52.33_{d}	44.36_{e}	56.66_{b}	63.99_{a}

R.L: Residual Lactose DCW: Dry Cell Weight T.C.: Total carotenoids To. : Torularhodin LSD of pH=0.0361
LSD of R.L. = 0.0490 LSD of DCW=0.0531 LSD of T.C =0.0591 LSD of To = 0.0713 Means with the same letter in the same raw are not significantly different

3-Effect of different mineral sources and their concentrations on carotenoids production:

The effect of different minerals on growth, total carotenoids and torularhodin production using *R. glutinis* co-cultivated with *Lactobacillus casei subsp. Casei* NRRL B- 441was examined and the results are given in Table(3).

The data revealed that, the growth, total carotenoids and torularhodin production has been affected with mineral sources and their concentrations where, the maximum reading of growth, total carotenoids and torularhodin were obtained with magnesium sulphate at a concentration of 0.5%. Generally, when the mineral source concentration was increased up to 0.5%, a decrease in aforementioned responses was observed. Also, it could be noticed that the yeast did not produce detectable pigment for control 1 or control 2 compared with treatments. The residual lactose percentage or pH value decreased with increasing of mineral concentration until

0.5% then, increased. These outcomes are in agreement with those obtained by Komemushi *et al*., 1994 who demonstrated that the magnesium stimulates caroteinoids production by *R. glutinis.* Buzzini *et al.* (2005) reported that certain trace elements have shown a selective influence on the carotenoid production in *R. graminis.*

Table 3: Effect of different mineral sources with different concentrations on carotenoids production.

Incubation time (day)	Responses	**M**ineral Source							
		Control(1)	Control (2)	Potasium di**h**ydrogen phosphate			Magnesiumsulphate		
		Whey medium	YM medium	Concentrations (%)					
				0.25	0.50	0.75	0.25	0.50	0.75
0	pH	6.00_{a}	6.02_{a}	6.00_{a}	6.03_{a}	6.04_{a}	6.02_{a}	6.01_{a}	6.01_{a}
	R.L.(%)	4.92_{a}	-	4.90_{a}	4.90_{a}	4.91_{a}	4.92_{a}	4.90_{a}	4.90_{a}
	DCW (g/L)	1.85_{a}	1.90_{a}	1.87_{a}	1.86_{a}	1.85_{a}	1.89_{a}	1.87_{a}	1.89_{a}
	T.C(µg/g)	23.53_{a}	23.57_{a}	23.59_{a}	$23..54_{a}$	23.55_{a}	23.53_{a}	23.55_{a}	23.54_{a}
	To.(µg/ml)	2.96_{a}	2.93_{a}	2.93_{a}	2.96_{a}	$2. 94_{a}$	2.96_{a}	2.92_{a}	2.94_{a}
2	pH	5.61_{a}	4.63_{e}	$5.50._{b}$	5.35_{d}	5.42_{gef}	4.54_{f}	4.43_{h}	4.49_{g}
	R.L.(%)	4.47_{a}	-	4.36_{b}	4.27_{d}	4.33_{c}	3.59_{e}	3.33_{g}	3.36_{f}
	DCW(g/L)	2.17_{j}	2.33_{h}	2.60_{e}	2.63_{dc}	2.51_{f}	2.75_{b}	2.80_{a}	2.65_{c}
	T.C(µg/g)	27.51_{h}	31.21_{g}	41.85_{f}	46.87_{b}	42.04_{d}	43.87_{c}	50.93_{a}	41.93_{e}
	To.(µg/ml)	3.75_{h}	4.23_{g}	5.26_{f}	5.96_{b}	5.88_{c}	5.67_{e}	6.03_{a}	5.84_{dc}
4	pH	5.39_{a}	4.50_{j}	4.12_{e}	3.85_{g}	4.01_{f}	4.44_{c}	3.79_{h}	4.30_{d}
	R.L.(%)	3.94_{a}	-	3.70_{b}	3.25_{d}	3.34_{c}	2.65_{e}	2.46_{g}	2.55_{f}
	DCW(g/L)	3.55_{h}	4.11_{g}	4.79_{dc}	4.81_{c}	4.63_{e}	4.85_{b}	4.93_{a}	4.46_{f}
	T.C(µg/g)	57.30_{h}	99.30_{g}	134.99_{f}	181.13_{c}	141.76_{d}	139.85_{e}	225.46_{a}	189.80_{b}
	To.(µg/ml)	10.68_{h}	20.52_{g}	25.29_{e}	28.04_{b}	27.12_{c}	26.66_{d}	33.13_{a}	22.64_{f}
6	pH	5.04_{a}	3.76_{d}	$3 .91_{b}$	3.73_{ed}	3.83_{c}	$3. 67_{f}$	$3. 60_{hg}$	$3. 61_{g}$
	R.L.(%)	3.33_{a}	-	2.98_{a}	2.65_{c}	2.89_{b}	1.74_{d}	1.45_{f}	1.66_{e}
	DCW(g/L)	5.00_{h}	5.81_{e}	5.51_{g}	5.93_{d}	5.77_{f}	6.00_{c}	6.22_{a}	6.10_{b}
	T.C(µg/g)	97.31_{h}	155.48_{g}	163.13_{f}	205.43_{c}	175.83_{e}	188.10_{d}	263.13_{a}	221.51_{b}
	To.(µg/ml)	19.41_{h}	31.13_{g}	34.06_{e}	35.04_{c}	31.37_{f}	34.90_{d}	44.68_{a}	39.21_{b}

R.L: Residual Lactose DCW: Dry Cell Weight T.C.: Total carotenoids To. : Torularhodin
LSD of pH=0.0177 LSD of R.L. = 0.0236 LSD of DCW=0.0258 LSD of T.C. =0.0587
LSD of To= 0.0433 Means with the same letter in the same raw are not significantly different

B- Carotenoids production under the optimum conditions:

Caroteinoids production from *Rhodotorula glutinis* NRRL YB-252 co-cultivated with *Lactobacillus casei subsp. casei* NRRL B- 441 using whey asa medium under the optimal fermentation conditions of 3% glucose, 0.75% yeast extract and 0.5% magnesium sulphate for 6 days at 30°C and 220 rpm are shown in Table (4). It could be observed that the maximum total caroteinoids (474.76 µg/g), torularhodin (101.56 µg/ml) and dry cell weight (7.10 g/L) were obtained at the optimum conditions. The obtained total caroteinoids by *R. glutinis* at the optimum conditions approximately in the same range was obtained by park *et al*., 2005 who achieved455 µg/g and Squina *et al*.(2002) recived 497.2 µg/g. Also, Frengova *et al*., 1994 found that the total caroteinoids from *R. glutinis* co- cultivated with *Lactobacillus helveticus* in whey ultrafiltrate was 268µg/g.

Table 4: Caroteinoids production from *R.glutinis*NRRL YB-252 co-cultivated with*Lactobacillus casei*subsp.*casei*NRRL B- 441 under the optimum conditions at220 rpm at 30°C for 6 days.

Responses	value
pH	3.45
R. L. (%)	1.31
DCW (g/L)	7.10
T.C(µg/g)	474.76
To.(µg/ml)	101.56

R.L: Residual Lactose DCW: Dry Cell Weight
T.C. : Total carotenoids To. : Torularhodin

C- Effect of some technological factors on stability of total carotenoids:

Data obtained for the stability of caroteinids produced under the optimum conditions at different technological factors are presented in Table (5). It is obvious that, the stability of caroteinoids has been affected with reducing pH values where, slightly low stability for caroteinoids was noticed at low pH values. The highest stability was recorded at the studied maximum pH (6). Similar result was reported by Kaur *et al*. (2009) who stated that at neutral conditions the intracellular caroteinoids produced by *Rhodotorula rubra* was shown to have more stability than acidic conditions.

Also, high concentrations of sodium chloride and sucrose result in low carotenoids stability. No significant differences for carotenoids stability was recorded at low concentrations of sodium chloride and sucrose. These

results confirm data presented by Rosso (2007) who reported that the addition of sugars and salts with high concentrations had a negative effect on the pigment stability.

Table 5: Effect of some technological factors on the stability of total carotenoids.

Factor		OD_{474nm}
pH	3.5	0.845_{ec}
	4.0	0.853_{dc}
	4.5	0.864_{c}
	5.0	0.898_{b}
	6.0	0.929_{a}
NaCl(%)	zero	0.975_{a}
	1	0.973_{a}
	2	0.966_{a}
	3	0.953_{ba}
	5	0.871_{c}
	7	0.695_{d}
Sucrose(%)	zero	0.975_{a}
	5	0.975_{a}
	7	0.961_{a}
	9	0.956_{a}
	11	0.941_{ba}
	14	0.928_{cb}
Thermal treatment		
	Without thermal treatment	0.975_{a}
	63ºC /30 min	0.961_{a}
	72 ºC/15 s	0.964_{a}
	85 ºC/10 min	0.968_{a}
	85 ºC/30 min	0.965_{a}
	90 ºC/10 min	0.895_{b}
	121ºC/20 min	0.866_{c}

LSD of pH=0.0123 LSD of NaCl = 0.0211 LSD of Sucrose=0.0251 LSD of thermal treatment=0.0140

Also, it could be observed that the stability of carotenoids affected with autoclaving process (121ºC/20 min) and heating at 90 ºC/10 min , but the other studied thermal treatments had no effect on carotenoids stability. The low stability at high thermal treatment has been explained by Bhosale *et al*. (2003) who found that *Rhodotorula caroteinoids* get chemically denatured on exposure to the high heat treatment

Conclusion:

The optimal fermentation conditions for the carotenoid production by *Rhodotorula glutinis* NRRL YB-252 co-cultivated with *Lactobacillus casei* sub sp. *casei* NRRL B- 441 using whey as a tested medium were3% glucose as carbon source, 0.75% yeast extract as nitrogen source and 0.5% magnesium sulphate as mineral source. These conditions supported high carotenoid production at 474.76(µg/g) of yeast. High concentrations of sodium chloride (7%) and sucrose(14%), low pH(3.5:4.5)and high thermal treatments (autoclaving process) showed slightly low stable carotenoids.

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