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ENVIRONMENTAL AND NUTRITIONAL PARAMETERS CONTROLLING THE PRODUCTION OF CAROTENOIDS BY *Rhodotorula* sp. AND USES AS FOOD ADDITIVES
1-USING SHAKE FLASKS AS A BATCH AND TWO STAGE BATCH CULTURE TECHNIQUES

BY

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ABSTRACT

Carotenoids production by *Rhodotorula glutinis* 32 were studied under batch and two stage batch culture conditions using shake flasks (150 rpm) incubated at 30°C for 3 days. The highest carotenoids yield as well as biomass was obtained on modified medium contains 40 gl^{-1} glycerol as a source of carbon, 0.111 gl^{-1} ammonium nitrate and 1.033 gl^{-1} yeast extract as a nitrogen sources, 6.25 gl^{-1} KH_2PO_4, 0.2 gl^{-1} $MgSO_4.7H_2O$, 0.75 gl^{-1} NaCl and 0.005 gl^{-1} $FeSO_4.7H_2O$ as mineral salts, pH 5.0. The amount of carotenoids (mgl^{-1}) and yield coefficient increased by 4.34 and 3.8 fold comparing to the production on basal medium. Four industrial wastes materials were used as a carbon source after carbon and nitrogen analysis. Also, under two stage batch culture, the highest carotenoid production was obtained on productive medium during 24 and 72 hrs of first and second steps of cultivation, respectively. Carotenoid concentration, content and yield increase 1.22; 1.23 and 1.26 fold, respectively than that obtained on one stage batch culture.

Key words: Carotenoids, *Rhodotorula* sp. and batch & two stage batch techniques.

INTRODUCTION

Carotenoids are composed of a polyene skeleton which usually consists of 40 carbon atoms it's a cyclic or terminated by one or two cyclic end groups. The collective terms xanthophylls refers to substituted derivatives containing hydroxy, keto, methoxy, eoixy- or carboxyl groups. Unsubstantiated derivatives are commonly called carotenes (Goodwin, 1980). Carotenoids of yellow orange and red color are among the most widespread and important natural pigment. They are found in higher plants, algae, fungi and bacteria, both in non-photosynthetic tissues and in photosynthetic tissue, accompanying the chlorophylls (Vandamme, 1989). Several bacteria, fungi and yeast are effective carotenoids producers. These carotenoids are natural pigments used in food and feedstuff, but only beta-carotene is produced commercially, on a limited scale,

and at high cost, from just one microbial source (Nelis and De Leenheer, 1991). β-carotene has also been the subject of several studies in *R. glutinis* (Martelli *et al.*, 1990 and Martelli & da Silva, 1993). The presence or absence of the other pigments, especially pigment torularhodin, pigments plectaniaxanthin-like and pigments torulene-like depended on the strain. Perrier *et al.* (1995) reported that the total carotenoid content varied widely in *Rhodotorula* strains studied from 10 μg (g dry weight)$^{-1}$ in *R. bogoriensis* to 100 μg (g dry weight)$^{-1}$ in *R. armeniae, R. mucilaginosa* and *R. aurantiaca*. β-carotene represented average 70 % of total carotenoids.

The carotenoids pigments are useful as food additives (Tsubokura *et al.*, 1999), active ingredients of antioxidants and vitamins which possess antitumor activity and protect humans from heart and age related diseases (Lient, 2000 and Steck *et al.*, 2004). They can also be used as color agents in food and drugs, and in agriculture to enhance flower, fruit or plant color (Johnson and Schroeder, 1996). Martelli and da Silva (1993) reported that synthesis of β-carotene in *Rhodotorula* is not associated with culture growth increases after all sucrose is consumed and attains a maximum at 42 hr of cultivation. They also added xylose, glycerol and acetate as a carbon source to the basic medium for β-carotene producing by *R. glutinis, R. lactosa* and *R. rubra*. Parajo *et al.* (1998) noticed that the concentrations of yeast extract, malt extract and peptone in xylose-containing media were considered as operational variables for assessing the effects of carotenogensis. They also stated that the presence of inorganic N-sources improved carotenoid production.

Medium containing molasses as a carbon source was used by Martelli *et al.* (1990), Mahattanatavee & Kulprecha (1991) and Fontana *et al.* (1995) for carotenoids production by different yeast strains. Martin *et al.* (1993) used acid extracts of peat as the main substrate source for high carotenoids production by *R. rubra*. Whereas the culture media contained apple, maize and grape ground oil cakes were tested by Podoprigora *et al.* (1996) for astaxanthin and β-carotene production.

The aim of the present investigation is to maximize carotenoids production by *Rhodotorula* strains using shake flasks as a batch and two stage batch culture. Also, some local industrial wastes were used to reduce the production cost.

MATERIALS AND METHODS

Yeast strains used:-

Four strains belonged to genus *Rohodotorula* were provided from Microbiology Dept. Fac. of Agric. Ain Shams Univ. Cairo, Egypt.

Media used:-

Saccharomyces rouxii medium (CAIM, 1987) were used for yeast preservation and propagation. Med (1) Martelli & da Silva (1993), Med (2) Frengova *et al.*, (1994) and Med (3) Shabati & Mukmenev (1995) were used for carotenoids production

Standard inoculum:-

Standard inoculum was prepared by inoculation of conical flasks (250 ml in volume) containing 100 ml of *Saccharomyces rouxii* medium (containing 10 gl^{-1} glucose) with a loop of tested culture. The inoculated flasks were incubated on rotary, shaker (150 rpm) for 24 hours at 30°C. The content of these flasks were used as a standard inoculum (1.25 gl^{-1} dry cell) for shake flasks experiments.

Industrial raw materials used:-

Four raw materials namely black strap cane molasses, sweet whey, potato starchy waste and glucose syrup were used. They were obtained from Sugar refinery factory (El-Hawamdia), Egypt dairy products Co. (El-Ameria), Farmfrits factory (10th Ramadan City) and Glucose & Starch Co. (Torrah), respectively.

Fermentation and culture condition:-

Fermentation was carried out in 250 ml cotton plugged Erlenmeyer flasks, each containing 100 ml of productive medium. The inoculated flasks were inocubated at 30°C for 3 days using rotary shaker (150 rpm). At the end of fermentation period, the cell were harvested by centrifugation at 3000 rpm for 5 min to determined the dry weight as well as carotenoids content.

Selection of suitable medium for carotenoids production:-

Media No.1, 2 & 3 were used in this experiments in order to select the most suitable medium for securing high carotenoids production by all tested yeast strains during fermentation period.

Factors affecting the production of carotenoids:-

Using the appropriate medium the effect of each of the following factors were studied.

1-Carbon sources:-

Eight carbon sources namely mannose, fructose, sucrose, lactose, glycerol, mannitol, ethanol and acetic acid were used. The amount of carbon compound added to the productive medium to replace the original carbon source in it, were calculated to give equal final concentration, to eliminate the errors which might occur as a result of differences in carbon in each source.

2-Organic and inorganic nitrogen source:-

The fermentation medium which selected for carotenoids production was devoid of any other nitrogen source. The media were then supplemented with different organic and inorganic nitrogenous compound with an equivalent amounts of N_2 to that present in the original medium. Therefore twenty trials were employed in order to select the best medium content.

3- Effect of mineral salts:-

Four mineral salts which constituent in medium 3 were taken and focusing to tested their effect on carotenoids as well as biomass production. These

salts were KH_2PO_4, $MgSO_4.7H_2O$, NaCl and $FeSO_4.7H_2O$. These experiments were constructed by adding four concentrations of each salt i.e KH_2PO_4 (0.0, 2.5, 6.25 &7.5 gl^{-1}), $MgSO_4.7H_2O$ (0.0, 0.15, 0.2 &0.25 gl^{-1}), NaCl (0.0, 0.5, 0.75 &1.5 gl^{-1}) and $FeSO_4.7H_2O$ (0.0, 0.005, 0.015 & 0.020 gl^{-1}) to fermented media. The previous procedures of propagation and chemical analysis were adopted.

4-Some vitamins:-

The effect of vitamins supplementation on carotenoids production by the tested strain was examined in these experiments using modified med.3. The mixture of vitamins were applied containing biotin (0.01 mgl^{-1}), riboflavin (1.0 mgl^{-1}), folic acid (0.01 mgl^{-1}) and thiamin hydrochloride (1.0 mgl^{-1}). The experiment was carried out by adding the mixture or omission one vitamin from it to get five treatments in addition to control (without vitamins).

5-Inoculum size:-

The inoculum size was adjusted at different volumes of standard inoculum ranged from 1 to 5 ml to inoculate 100 ml productive medium. Then followed by the previous procedures of propagation and chemical analysis.

6-pH of the medium:-

The pH of the productive medium was adjusted at different values ranging from 3.5-7.0 by using 6 N HCl or 6 N NaOH. Incubation was performed at 30°C using rotary shaker for 3 days.

Two-stage batch culture for carotenoids production:-

The production of carotenoid pigments was carried out by using two step flask culture. The selected yeast was first grown in 250 ml Erlenmeyer flasks containing 100 ml productive medium after inoculation with standard inoculum. The flasks were then incubated at 30°C using rotary shaker (150 rpm) for 24, 48 or 72 hrs. At the end of incubation period, samples (10 ml) were taken from the grown cultures to estimate the cell dry weight and carotenoids content. The remaining content of these flasks (90 ml) was centrifuged at 3000 rpm for 15 min then the harvested cells were washed twice with sterilized water and used to inoculate the second step flasks. The flasks of second step were incubated at 30°C for 72 hrs in order to determine the cell dry weight and carotenoids content.

Chemical analysis

1. **Total sugars** were determined according to Flood & Preistly (1973).
2. **Total nitrogen** were determined according to Jackson (1973).
3. **Carotenoid pigments** were extracted from productive yeast cells and determined according to method described by **Frengova *et al.* (1994).**

Statistical analysis

Regression analysis was carried out by Microsoft Excel (Microsoft Corporation, 1995).

Growth & carotenoids parameters

The specific growth rate (μ) and doubling time (t_d) were calculated from the exponential phase according to Painter & Marr (1963).

Number of generations and multiplication rate were calculated according to Stanier *et al.* (1970).

Carotenoid yield (g%) = gram carotenoids X 100/gram original sugar, according to Ramadan *et al.* (1985).

Carotenoids yield coefficient ($Y_{c/x}$) = gram carotenoids /gram biomass dry weight, according to Grothe *et al.* (1999).

Carotenoids content (%) according to Lee & Chol (1998)

Carotenoids productivity (p) according to Lee (1996)

RESULTS AND DISCUSSION

I-Carotenoids production by *Rhodotorula* strains using shake flasks as a batch culture.

A-Selection of suitable medium for carotenoids production:

Recently some investigators (Buzzini & Martini, 1999; Buzzini, 2000 and Buzzini, 2001) developed an interesting process for carotenoids production by yeast belonging to genes *Rhodotorula* which used as a natural coloring substance. Therefore, some local strains of *Rhodotorula* were used in this experiment to study the growth behavior and carotenoids production in different tested media in order to select the suitable productive medium at proper incubation period.

Results of carotenoids production indicated that the carotenoids production by *Rhodotorula* strains was increased gradually during the first 72 hrs to reach the maximum value then decreased till 120 hrs on all the tested media (as shown in Table 1). The highest carotenoids concentration (mgl^{-1}) and yield coefficient ($Y_{c/x}$) were observed on med. 3 by *R. glutinis* 32 (0.846 mgl^{-1} & 0.108 mgg^{-1} dry cells) and *R. glutinis* 34 (0.819 mgl^{-1} & 0.104 mgg^{-1} dry cell), whereas the highest cell dry weight of these strains being 9.2 and 10.9 gl^{-1}, respectively were obtained on med. 2 after 120 hrs fermentation period. The corresponding figures for specific production rate of carotenoids (μ_p), productivity (P) and content (%) were 0.013 h^{-1}, 0.012 $mgl^{-1}h^{-1}$ & 0.011% for *R. glutinis* 32 and 0.036 h^{-1}, 0.011 $mgl^{-1}h^{-1}$ & 0.01% for *R. glutinis* 34, respectively (Table 2). Generally, it could be noticed that the highest specific production rate of most tested strains on all different media was recorded during the first 48 hrs of fermentation and carotenoids production was continued to give the highest value after 72 hrs of fermentation period. These results are confirmed with that obtained by Frengova *et al.* (1994). They stated that carotenoid content of *R. glutinis* increased during the exponential phase of growth, with the highest value which recorded in the early stationary phase. Also, Xu *et al.* (1997) stated that carotenoids production occurred mainly in the late exponential phase of red yeast growth as determined from a growth curve.

Table(1):Cell dry weight and carotenoids production of different *Rhodotorula* strains grown on different media during 120 hours incubation period at 30°C using shake flasks as a batch culture.

Medium NO.	Time (hours)	*R.glutinis* 32			*R.glutinis* 34			*R.glutinis* 36			*R.mucilaginosa* 38		
		DW (gl^{-1})	Carotenoid		DW (gl^{-1})	Carotenoid		DW (gl^{-1})	Carotenoid		DW (gl^{-1})	Carotenoid	
			Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1})		Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1})		Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1})		Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1})
1	24	1.20	0.048	0.040	3.03	0.048	0.016	1.75	0.064	0.037	2.90	0.015	0.005
	48	5.00	0.3	0.060	5.85	0.117	0.02	2.90	0.070	0.024	6.97	0.127	0.018
	72	7.50	0.560	0.075	8.12	0.248	0.031	6.05	0.182	0.030	8.25	0.155	0.019
	96	7.70	0.209	0.027	9.78	0.155	0.016	6.00	0.095	0.016	8.50	0.101	0.012
	120	8.70	0.122	0.014	10.7	0.110	0.010	6.00	0.080	0.013	8.50	0.096	0.011
2	24	2.80	0.126	0.045	2.98	0.104	0.035	2.70	0.084	0.031	3.15	0.035	0.011
	48	5.50	0.525	0.095	5.90	0.325	0.055	5.77	0.254	0.044	7.30	0.139	0.019
	72	6.05	0.618	0.102	6.95	0.509	0.073	8.55	0.295	0.035	7.60	0.163	0.021
	96	8.45	0.337	0.040	9.90	0.418	0.042	8.15	0.336	0.041	8.60	0.116	0.013
	120	9.20	0.219	0.024	10.9	0.446	0.041	8.15	0.311	0.038	8.50	0.115	0.014
3	24	2.00	0.15	0.075	2.70	0.203	0.075	2.09	0.04	0.019	1.80	0.016	0.009
	48	5.85	0.617	0.105	6.75	0.349	0.052	5.23	0.157	0.030	5.60	0.095	0.017
	72	7.83	0.846	0.108	7.85	0.819	0.104	5.7	0.332	0.058	8.05	0.165	0.020
	96	8.15	0.591	0.073	8.55	0.737	0.086	7.5	0.205	0.027	8.80	0.119	0.014
	120	8.80	0.151	0.017	8.95	0.355	0.040	7.6	0.182	0.024	9.20	0.118	0.013

$Y_{c/x}$ = Carotenoid yield coefficient (mgg^{-1} dry cell)

Table(2):Specific production rate (μ_p), productivity (P) and carotenoid content (%) of different *Rhodotorula* strains grown on different media during 5 days incubation period at 30°C using shake flasks as a batch culture.

Medium NO.	Time (hours)	*R.glutinis* 32			*R.glutinis* 34			*R.glutinis* 36			*R.mucilaginosa* 38		
		μ_p	P	%	μ_p	P	%	μ_p	P	%	μ_p	P	%
	24	-	0.002	0.004	-	0.002	0.002	-	0.003	0.004	-	0.001	0.0005
	48	0.076	0.006	0.006	0.037	0.002	0.002	0.004	0.001	0.002	0.089	0.003	0.002
1	72	0.026	0.008	0.008	0.031	0.003	0.003	0.040	0.003	0.003	0.008	0.002	0.002
	96	-	0.002	0.003	-	0.002	0.002	-	0.001	0.002	-	0.001	0.001
	120	-	0.001	0.001	-	0.0009	0.001	-	0.0007	0.001	-	0.001	0.001
	24	-	0.005	0.005	-	0.004	0.004	-	0.004	0.003	-	0.001	0.001
	48	0.059	0.011	0.010	0.047	0.007	0.006	0.046	0.005	0.004	0.057	0.003	0.002
2	72	0.007	0.009	0.010	0.019	0.007	0.007	0.006	0.004	0.004	0.007	0.002	0.002
	96	-	0.004	0.004	-	0.004	0.004	-	0.004	0.004	-	0.001	0.001
	120	-	0.002	0.002	-	0.004	0.004	-	0.003	0.004	-	0.001	0.001
	24	-	0.006	0.008	-	0.008	0.008	-	0.002	0.002	-	0.001	0.0009
	48	0.059	0.013	0.011	0.023	0.007	0.005	0.057	0.003	0.003	0.074	0.002	0.002
3	72	0.013	0.012	0.011	0.036	0.011	0.010	0.031	0.010	0.006	0.023	0.002	0.002
	96	-	0.006	0.007	-	0.008	0.009	-	0.002	0.003	-	0.001	0.001
	120	-	0.001	0.002	-	0.003	0.004	-	0.002	0.002	-	0.001	0.001

μ_p = Specific production rate (h^{-1})
P = Carotenoids productivity ($mg l^{-1} h^{-1}$)
% = Content (%)

From the foregoing results, it could be concluded that all the tested *Rhodotorula* strains complete their growth phase in 48 hrs, whereas maximum carotenoids production obtained after 72 hrs on all the tested medium. *R. glutinis* 32 was selected as a good carotenoid-producing yeast for maximum production on med. 3 after 72 hrs and will be chosen for further studied.

B-Factors affecting the production of carotenoids

Nutritional requirements

Carbon sources

Data present in Table (3) clearly show that the highest cell dry weight as well as all carotenoid parameters were detected on medium 3 containing either sucrose or glycerol as a sole carbon source. The presence of lactose as sole carbon source on med.3 gave a drastic effect on biomass production and so carotenoid production. The results may be due to the disability of *R. glutinis* 32 to utilize lactose. Generally it could be stated that replacing glucose on med.3 by sucrose or glycerol increased the concentration of carotenoid 1.9 &1.86 fold. Therefore, an experiment was constructed to study the effect of sucrose and glycerol concentration on the production of carotenoid by *R. glutinis* 32.

Table (3): Effect of different carbon sources on carotenoids production by *Rhodotorula glutinis* 32 after 3 days incubation period at 30°C using shake flasks as a batch culture.

Carbon sources	Cell dry weight (gl^{-1})	Carotenoid produced			
		Concentration (mgl^{-1})	$Y_{c/x}$ (mgg^{-1} dry cell)	Content (%)	Productivity ($mgl^{-1}h^{-1}$)
Glucose (control)	7.500	0.800	0.107	0.011	0.011
Mannose	7.250	0.615	0.085	0.008	0.009
Fructose	5.250	0.652	0.124	0.012	0.009
Sucrose	8.750	1.517	0.173	0.017	0.021
Lactose	0.650	0.017	0.026	0.003	0.0002
Glycerol	8.500	1.490	0.175	0.018	0.021
Mannitol	4.250	0.569	0.134	0.013	0.008
Ethanol	5.000	0.543	0.109	0.011	0.008
Acetic acid	5.250	0.526	0.100	0.010	0.007

$Y_{c/x}$ = Carotenoid yield coefficient (mgg^{-1} dry cell)

Sucrose and glycerol concentrations

Data in Table (4) show that there was a gradual increase in carotenoids production by *R. glutinis* 32 with the increase of sucrose or glycerol concentration reaching a maximum at 30 or 40 gl^{-1}, respectively. With 40 gl^{-1} glycerol, the values of carotenoids concentration, yield coefficient ($Y_{c/x}$), yield, content and productivity were higher than attained in 30 gl^{-1} sucrose. These values were 1.941 mgl^{-1}, 0.268 mgg^{-1} dry cells, 0.005 %, 0.027 % and 0.027 $mgl^{-1}h^{-1}$, respectively. These results are in line with those obtained by Martelli and da Silva (1993) who found that the amount of ß-carotene produced by *R. glutinis* increased when sucrose replaced by glycerol as a sole carbon source in productive medium.Generally, it could be concluded that 40 gl^{-1} glycerol was the most favorable carbon source for carotenoids production by *R. glutinis* 32. Regression

analysis of carotenoids production as a function of sucrose or glycerol concentrations led to the following equations:-

$Y = 36.30 + (-6.73) X$ for sucrose concentrations

$Y = 20.06 + 9.99 X$ for glycerol concentrations

Table (4): Effect of sucrose and glycerol concentrations on carotenoids production by *Rhodotorula glutinis* 32 after 3 days incubation period at 30°C using shake flasks as a batch culture.

Carbon sources	Sucrose					
		Carotenoids production				
Concentration (gl^{-1})	DW (gl^{-1})	Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1} dry cell)	Yield (%)	Content (%)	P ($mgl^{-1}h^{-1}$)
10	4.75	0.574	0.121	0.006	0.012	0.008
15	6.52	0.822	0.126	0.005	0.013	0.011
20	7.25	1.020	0.141	0.005	0.014	0.014
25	7.75	1.330	0.172	0.005	0.017	0.018
30	7.95	1.380	0.174	0.005	0.017	0.019
35	6.75	1.253	0.186	0.004	0.019	0.017
40	6.00	1.125	0.188	0.003	0.019	0.016
45	5.55	0.548	0.099	0.001	0.010	0.008
50	5.25	0.374	0.071	0.001	0.007	0.005
	Glycerol					
10	5.50	0.427	0.078	0.004	0.008	0.006
15	6.25	0.553	0.088	0.004	0.009	0.008
20	7.50	0.714	0.095	0.004	0.010	0.010
25	7.75	1.110	0.143	0.004	0.014	0.015
30	8.00	1.402	0.175	0.005	0.018	0.019
35	7.25	1.672	0.231	0.005	0.023	0.023
40	7.25	1.941	0.268	0.005	0.027	0.027
45	7.00	0.952	0.136	0.002	0.014	0.013
50	6.50	0.485	0.075	0.001	0.007	0.007

P= Productivity ($mgl^{-1}h^{-1}$) $Y_{c/x}$ = Carotenoids yield coefficient (mgg^{-1} dry cell)

It is clear that the slop of the reaction between carotenoids concentrations and glycerol concentrations is considered as a good index for high production.

Nitrogen sources

Data presented in Table (5) show the production of *R. glutinis* 32 carotenoids as influenced by different nitrogen sources.

Generally, it could be stated that addition of yeast extract to inorganic nitrogen sources gave higher growth (gl^{-1}) and carotenoids concentrations (mgl^{-1}) than each organic or inorganic source alone. The cell dry weight ranged from 2.5

to 6.25, from 4.0 to 6.0 and from 6.0 to 7.25 gl^{-1}, and carotenoids concentrations ranged from 0.062 to 0.487, from 0.47 to 0.65 and from 0.607 to 2.093 mgl^{-1} were obtained when organic, inorganic and inorganic plus yeast extract were used as a nitrogen sources. The highest values of cell dry weight and carotenoids concentrations being 7.25 gl^{-1} and 2.093 mgl^{-1} were attained in different media containing yeast extract and supplemented with ammonium sulfate and ammonium nitrate, respectively. Also, the highest figures of carotenoids parameters being 0.31 mgg^{-1} dry cells, 0.0052 %, 0.031% and 0.029 $mgl^{-1}h^{-1}$ for carotenoids yield coefficient ($Y_{c/x}$), yield %, content (%) and productivity, respecivily, were observed by *R. glutinis* 32 on glycerol medium containing ammonium nitrate and yeast extract as a nitrogen source. The results are in agreement with those obtained by Parajo *et al.* (1998) who noticed that the concentrations of yeast extract in xylose-containing media supplemented with inorganic nitrogen sources were effected on carotenoids. Also, An *et al.* (1991) used yeast extract, malt extract and peptone as nitrogen source for carotenoids production by *Phaffia rhodozyma*.

Table (5): Effect of different nitrogen sources on carotenoids production by *Rhodotorula glutinis* 32 after 3 days incubation period at 30°C using shake flasks as a batch culture.

Nitrogen sources	Cell dry weight (gl^{-1})	Carotenoids produced				
		Conc. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1} dry cell)	P ($gl^{-1}h^{-1}$)	Yield (%)	Content (%)
Ammonium chloride	6.00	0.470	0.078	0.007	0.0012	0.008
Ammonium nitrate	4.75	0.550	0.116	0.008	0.0014	0.012
Ammonium phosphate	4.50	0.644	0.143	0.009	0.0016	0.014
Ammonium sulfate	4.25	0.650	0.153	0.009	0.0016	0.015
$NH_4Cl+(NH_4)_2SO_4$	4.00	0.649	0.162	0.009	0.0016	0.016
$NH_4NO_3+(NH_4)_2SO_4$	4.50	0.580	0.129	0.008	0.0015	0.013
$(NH_4)_2PO_3+(NH_4)_2SO_4$	4.25	0.520	0.122	0.007	0.0013	0.012
$(NH_4)_2SO_4$+ Yeast extract*.	7.25	1.850	0.255	0.026	0.0046	0.026
NH_4NO_3+ Yeast extract	6.75	2.093	0.310	0.029	0.0052	0.031
NH_4CL+ Yeast extract	6.00	0.607	0.101	0.008	0.0015	0.010
Ammonium phosphate + Yeast extract	6.25	0.680	0.109	0.009	0.0017	0.011
Yeast extract	5.95	0.468	0.079	0.007	0.0012	0.008
Urea	4.25	0.062	0.015	0.001	0.0002	0.001
Peptone	4.25	0.487	0.115	0.007	0.0012	0.011
Malt extract	2.50	0.365	0.146	0.005	0.0009	0.015
Yeast extract +Urea	6.25	0.483	0.077	0.007	0.0012	0.008
Yeast extract +Malt extract	4.25	0.470	0.111	0.007	0.0012	0.011
Yeast extract +Peptone	5.25	0.410	0.078	0.006	0.0010	0.008
Malt extract +Urea	4.50	0.350	0.078	0.001	0.0009	0.008
Malt extract +Peptone	3.75	0.342	0.091	0.005	0.0009	0.009

*= control treatment $Y_{c/x}$ = Carotenoids yield coefficient (mgg^{-1} dry cell)

Ammonium nitrate: yeast extract ratio (A/Y)

Different ratios of ammonium nitrate nitrogen to yeast extract nitrogen ranged from 0.09 to 11 were prepared by mixing different concentrations of both nitrogen source to give the same final nitrogen content being 0.182%. Results recorded in Table (6) clearly show that increasing yeast extract concentrations in the mixture nitrogen source gave high cells yield as well as carotenoids concentration than the ammonium nitrate. The maximum carotenoids concentration being 2.401 mgl^{-1} was obtained at A:Y ratio of 1:5. The corresponding figures for carotenoids yield coefficient ($Y_{c/x}$), content %, yield % and productivity were 0.356 mgg^{-1} dry cells, 0.036 %, 0.006 % and 0.033 $mgl^{-1}h^{-1}$, respectively. At A:Y ratio of 1.5, the carotenoids content, yield and productivity were increased about 1.29, 1.25 and 1.22 fold, respectively, as compared with that obtained at control treatment (A:Y of 1:1). This may be due to the role of yeast extract as growth promoters for biomass and carotenoids production.

Therefore, the productive med.3 will be used for further studies after replacing glucose and $(NH_4)_2SO_4$ by glycerol (40 gl^{-1}) and mixture of ammonium nitrate (0.111 gl^{-1}) and yeast extract (1.033 gl^{-1}), respectively and named modified medium No.3.

Mineral salts concentrations:-

The tabulated data in Table (7) revealed the stimulatory effect of each salts in the productive medium, since the omission of KH_2PO_4, $MgSO_4.7H_2O$, NaCl or $FeSO_4.7H_2O$ resulted in losing the productivity ($mgl^{-1}h^{-1}$) of carotenoids by 70.27, 94.59, 87.18 and 58.97, respectively comparing to the highest productivity of each salt.

Regarding the carotenoid yield (%), data clearly show that these is a positive relationship between the concentration of each salt and carotenoid yield till reached the maximum value by adding 6.25 gl^{-1} KH_2PO_4, 0.2 gl^{-1} $MgSO_4.7H_2O$, 0.75 gl^{-1} NaCl and 0.005 gl^{-1} $FeSO_4.7H_2O$.

The maximum biomass production which express as dry weight were coincide with previous proper salt concentration as well as carotenoids parameters.

The range of KH_2PO_4 selected for investigation were constructed according to Martelli & da Silva, 1993; Frengova *et al.*, 1994; Bhosale & Gadre, 2001 and Buzzini, 2001 who recorded that the maximum carotenoids production was obtained when KH_2PO_4 concentration was varied from 2 to 8 mgl^{-1} depended on the presence of other source of phosphate in productive medium, *Rhodotorula* strain and fermentation conditions.

The drastic effect of $MgSO_4.7H_2O$ omission in the productive medium which led to loss 94.6 % of carotenoids productivity are agreement with those obtained by Martelli & de Silva (1993), Buzzini (2001) and Bhosale & Gadre (2001) who revealed the importance of using $MgSO_4.7H_2O$ as a source of magnesium salt for the growth and carotenoids production by *R. glutinis*.

Table(6): Effect of nitrogen ammonium nitrate: nitrogen yeast extract ratio (A/Y) on carotenoids production by *Rhodotorula glutinis* 32 after 3 days incubation period at 30°C using shake flasks as a batch culture.

Ratio	Nitrogen ratio of Amm.nitrate :Y.extract (A:Y)	Amm.nitrate (gl^{-1})	Y.extract (gl^{-1})	Cell dry weight (gl^{-1})	Carotenoids production		Content (%)	Productivity ($mgl^{-1}h^{-1}$)	Yield (%)
					Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1} dry cell)			
0.09	1:11	0.055	1.137	5.9	1.79	0.303	0.030	0.025	0.0045
0.2	1:5	0.111	1.033	6.75	2.401	0.356	0.036	0.033	0.0060
0.33	1:3	0.166	0.93	7.1	2.228	0.314	0.031	0.031	0.0056
0.5	1:2	0.221	0.827	7.3	2.119	0.290	0.029	0.029	0.0053
0.71	1:1.4	0.277	0.723	6.95	2.085	0.300	0.030	0.029	0.0052
1.0	1:1 (control)	0.093	0.093	6.8	1.916	0.282	0.028	0.027	0.0048
11	11:1	6.609	0.103	6.25	1.782	0.285	0.029	0.025	0.0045
5	5:1	0.554	0.202	5.95	1.353	0.227	0.023	0.019	0.0034
3	3:1	0.498	0.31	5.00	1.248	0.250	0.025	0.017	0.0031
2	2:1	0.443	0.41	5.05	1.248	0.247	0.025	0.017	0.0031
1.4	1.4:1	0.388	0.517	4.90	1.157	0.236	0.024	0.016	0.0029

$Y_{c/x}$ = Carotenoids yield coefficient (mgg^{-1} dry cell)

Table (7): Effect of different concentrations of KH_2PO_4, $MgSO_4.7H_2O$, NaCl and $FeSO_4.7H_2O$ as ingredient salts in m.m.3 on carotenoids production by *R glutinis* 32 after 3 days at 30°C using shake flasks as a batch culture.

Salt (gl^{-1})	Cell dry weight (gl^{-1})	Carotenoids production		Content (%)	Productivity ($mgl^{-1}h^{-1}$)	Yield (%)
		Concentration (mgl^{-1})	$Y_{c/x}$ (mgg^{-1} dry cell)			
KH_2PO_4						
0.0	7.00	0.798	0.114	0.011	0.011	0.0020
2.5	7.10	1.547	0.218	0.022	0.021	0.0039
6.25	7.40	2.633	0.356	0.036	0.037	0.0066
7.5	6.90	1.203	0.174	0.017	0.017	0.0030
$MgSO_4.7H_2O$						
0.0	3.15	0.155	0.049	0.005	0.002	0.0004
0.15	7.95	2.598	0.327	0.033	0.036	0.0065
0.2	7.5	2.694	0.359	0.036	0.037	0.0067
0.25	7.4	0.155	0.021	0.002	0.002	0.0004
NaCl						
0.0	6.45	0.393	0.061	0.006	0.005	0.0010
0.5	7.30	1.892	0.259	0.026	0.026	0.0047
0.75	7.80	2.779	0.356	0.036	0.039	0.0069
1.5	5.40	1.145	0.212	0.021	0.016	0.0029
$FeSO_4.7H_2O$						
0.000	7.20	1.119	0.155	0.016	0.016	0.0028
0.005	7.65	2.786	0.364	0.036	0.039	0.0070
0.015	7.30	2.042	0.280	0.028	0.028	0.0051
0.020	7.25	0.659	0.091	0.009	0.009	0.0016
Control	6.75	2.401	0.356	0.036	0.033	0.0060

$Y_{c/x}$ = Carotenoid yield coefficient relative to biomass
m.m.3 = Modified med.3 containing glycerol ($40gl^{-1}$),ammonium nitrite and yeast extract

As regards to, the effect of NaCl and $FeSO_4.7H_2O$, the aforementioned result are in agreement with those obtained by Bhosale & Gardre (2001) who found that the total carotenoids content of *R. glutinis* mutant 32 increased with 2 fold using medium prepared in artificial seawater containing NaCl and other minerals. Also, Harker *et al.* (1996) added 40 mM NaCl to stimulate carotenoid synthesis in *Haematococcus plavialis.* Mahattanatavee and Kulprecha (1991)

added 0.2 mg Fe^{2+} ion per liter to productive carotene medium for tested some producing yeast strain.

Some vitamins:-

This experiment was carried out as a trial to increase the yield of carotenoids. So, five treatments were prepared as presented in Table (8). Data clearly show that there is no obvious difference could be detected between all the treatments comparing to the control. These data may be due to the content of these vitamins in yeast extract. Brock *et al.* (1994) stated that yeast extract contains some vitamins mgg^{-1} as thiamine 10, riboflavin 20, nicotinic acid 400, pantothenic acid 50, pyridoxine 25, biotin 1.0, inositol 1500 and choline 1500.

Table (8): Effect of some vitamins on carotenoid production by *R glutinis* 32 after 3 days at 30°C on m.m.3 using shake flasks as a batch culture.

Treatments	Biotin	Riboflavin	Folic acid	Thiamin hydrochloride	Cell dry weight (gl^{-1})	Carotenoids production	
						Concentration (mgl^{-1})	$Y_{c/x}$ (mgg^{-1} dry cell)
1 (control)	-	-	-	-	7.59	2.77	0.365
2	+	+	+	+	7.70	2.890	0.375
3	-	+	+	+	7.65	2.760	0.361
4	+	-	+	+	7.80	2.763	0.354
5	+	+	-	+	7.35	2.681	0.365
6	+	+	+	-	6.65	2.439	0.367

$Y_{c/x}$ = Carotenoid yield coefficient (mgg^{-1} dry cell)

Environmental factors

Initial pH values

In this experiments, all nutritional requirement were adjusted to the best findings in previous experiments, except that the only variable factor was the pH. Results of carotenoids production as influenced by initial pH values were illustrated by Fig. (1). These results indicate that pH 5.0 was the most favourable one for carotenoids production (3.215 mgl^{-1}) by *R. glutinis* 32. The corresponding figures for yield coefficient ($Y_{c/x}$), content, yield and productivity were 0.432 mgg^{-1} dry cells, 0.043%, 0.008% and 0.045 $mgl^{-1}h^{-1}$, respectively. Whereas highest cell dry weight (7.8 gl^{-1}) was noticed at pH 5.50. All other tested pH values, either lower or higher than pH 5.0 decreased carotenoids production. These results are in agreement with those obtained by Ho *et al.* (1999) who stated that the highest carotenoids production by *Phaffia. rhodozyma* was found to be an initial pH value of 5.0.

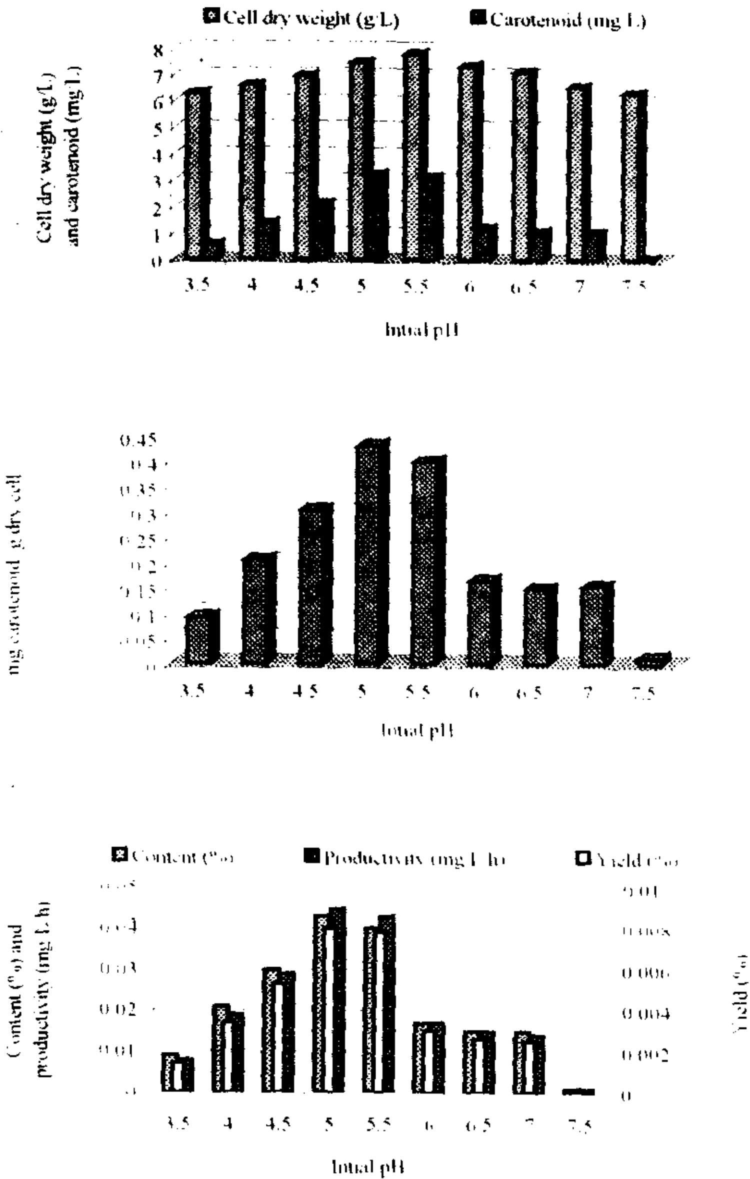


Fig.(1): Effect of initial pH on carotenoids production by *Rhodotorula glutinis* 32 after 3 days incubation period at 30°C using shake flasks as a batch culture.

Inoculum size

Data in Fig. (2) show that inoculation with 3 % of standard inoculum gave the highest amount of carotenoid pigments being 3.469 mgl^{-1} whereas highest cell dry weight (8.8 gl^{-1}) was obtained in modified med. 3 inoculated with 4 % and incubated at 30°C for 3 days on rotary shaker (150 rpm). Also, the figures of carotenoids yield coefficient (0.434 mgg^{-1} dry cells), content (0.043 %), yield (0.0087 %) and productivity (0.048 $mgl^{-1}h^{-1}$) were recorded by using 3 % of standard inoculum.

Therefore, it is recommended to use 3 % of standard inoculum of *R. glutinis* 32 to inoculate modified medium 3 for carotenoids production in further studies, instead of 2 % of basal medium.

The inoculum size used by Frengova *et al.* (1994) for all fermentations was 6% (v/v) and its cell concentration was about 1.2 g dry cell/L. Whereas 5 % inoculum size (v/v) was applied by Bhosale and Gadre (2001) for 18 hrs in shake flasks.

C-Use of some industrial raw materials

Some available industrial wastes such as sweet whey, potato starchy waste, glucose syrup and molasses were used for carotenoids production by *R. glutinis* 32 using shake flasks as a batch culture. The chemical analysis of total carbon in black strap molasses, sweet whey, potato starchy waste and glucose syrup were 47.4, 4.6, 3.9 and 41 %, respectively. The corresponding figures of total nitrogen were 0.11, 0.84, 0.81 and 0.27 %, respectively. Data recorded in Table (9) reveal that the carotenoids production of *R. glutinis* 32 on different raw materials was very low as compared with that produced by modified med. 3. The highest production of carotenoids was observed on molasses followed by glucose syrup treatment. These results are in line with those obtained by Mahattanatavee and Kulprecha (1991) who stated that the carotene content of yeast strains is less in molasses at 3% reducing sugars than that produced from 3.5 % glucose.

II- Carotenoids production by *R.glutinis* 32 using shake flasks as a two stage batch culture

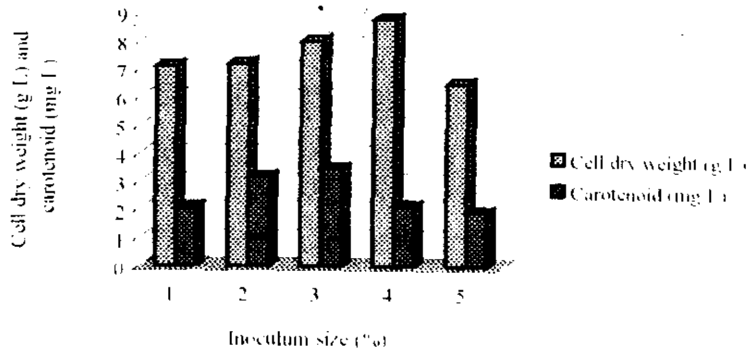
Two-stage flask cultures were created to examine the effect of different treatments of growth phase (first stage) on carotenoids productivity during production phase (second stage). Results of cell dry weight of *R. glutinis* 32 and carotenoids produced from two stage were tabulated in Table (10). Data show that the highest cell dry weight and carotenoids concentration were obtained at the end of first stage of cultivation on modified med. 3 being 7.5 gl^{-1} & 3.39 mgl^{-1} after 72 hrs fermentation period then followed after 48 hrs being 6.4 gl^{-1} & 2.7 mgl^{-1}, respectively. The corresponding figures of carotenoids yield coefficient ($Y_{c/x}$) were 0.452 and 0.422 mg g^{-1} dried cells, respectively. Also, it could be noticed that the highest carotenoids content being 0.051 % was formed by *R. glutinis* 32 grown on productive medium for 24 during first stage of cultivation. So, these cells gave the highest carotenoids production after 72 hrs incubation period of second stage on complete productive medium (modified med. 3), and recorded 4.23 mgl^{-1}, 0.529 mgg^{-1}, 0.053 %, 0.0058 % and 0.044 $mgl^{-1}h^{-1}$ for carotenoids

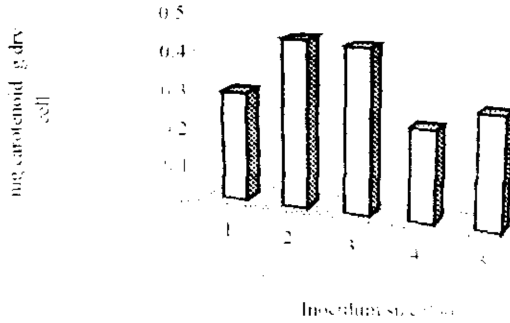
concentration, yield coefficient, content, yield and productivity, respectively. The highest cell dry of *R. glutinis* 32 was obtained at the end of second stage on modified med. 3 and nitrogen free medium 3 (9.8 and 8.8 gl^{-1}), when these cells were grown on *Saccharomyces rouxii* medium (nutrient rich medium) during the first stage. Slight increase in carotenoids production was produced on modified med. 3 than nitrogen free med. 3 when the first stage was carried out in modified medium 3 for 24, 48 or 72 hrs, whereas vice versa is true by using *Saccharomyces rouxii* medium during first stage of cultivation.

From the above mentioned results, it could be concluded that using shake flasks as a two stage batch culture on modified med. 3 for 24 and 72 hrs of first and second step, respectively, increased the carotenoids concentration and content by *R. glutinis* 32 to 1.22 and 1.23 fold, respectively as compared with that obtained on batch culture (Fig.2), whereas the carotenoids yield and productivity decreased from 0.0087 % & 0.048 $mgl^{-1}h^{-1}$ on batch culture to 0.0058 % & 0.044 $mgl^{-1}h^{-1}$ on two stage batch culture, respectively.

Table (9): Production of carotenoid pigments by *Rhodotorula glutinis* 32 as affected by some industrial wastes and by-product after 72 hours at 30°C using shake flasks as a batch culture.

Treatments	Carotenoids concentration (mgl^{-1})	Productivity ($mgl^{-1}h^{-1}$)
Crude sweet whey (4 % sugar)	0.92	0.013
Crude sweet whey (4 % sugar) + (Med.3 – glycerol)	0.42	0.006
Black strap molasses (4 % sugar)	1.16	0.016
Black strap molasses (4 % sugar) + (Med.3 – glycerol)	1.49	0.021
Glucose syrup (4 % sugar)	0.67	0.009
Glucose syrup (4 % sugar) + (Med.3 – glycerol)	1.07	0.015
Potato starchy waste (4 % sugar)	0.37	0.005
Potato starchy waste (4 % sugar) + (Med.3 – glycerol)	0.15	0.002
Modified Med.3	3.396	0.047





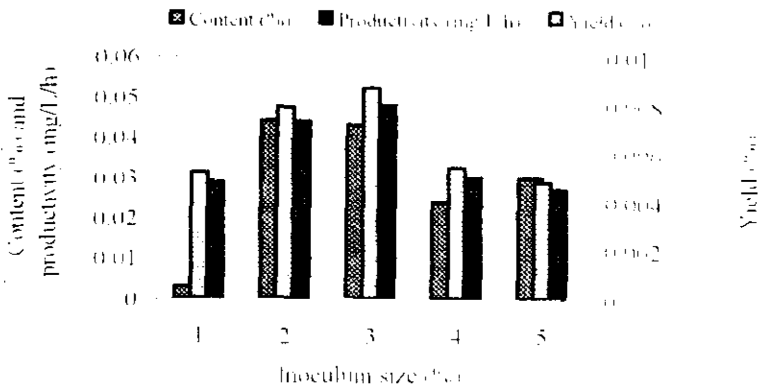


Fig.(2):Effect of inoculum size on carotenoids production by *Rhodotorula glutinis* 32 after 3 days incubation period at 30°C using shake flasks as a batch culture.

Table(10): Carotenoids production by *Rhodotorula glutinis* 32 grown on modified med.4 at 30°C for 72 hours incubation period using shake flasks as a two stage batch culture.

First stage medium	DW (gl^{-1})	Carotenoids produced			Second stage treatments	DW (gl^{-1})	Carotenoids produced after 72 hours				
		Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1})	Content (%)			Cons. (mgl^{-1})	$Y_{c/x}$ (mgg^{-1})	Content (%)	Y (%)	P ($mgl^{-1}h^{-1}$)
24 hrs on *Sacchromyces rouxii* medium	4.85	0.28	0.058	0.006	Complete productive med.3	9.8	3.48	0.355	0.036	0.0080	0.036
					Nitrogen free med.3	8.8	3.61	0.410	0.041	0.0083	0.038
24 hrs on productive medium 3	3.80	1.93	0.508	0.051	Complete productive med.3	8.0	4.23	0.529	0.053	0.0058	0.044
					Nitrogen free med.	6.5	3.29	0.506	0.051	0.0034	0.034
48 hrs on productive medium 3	6.40	2.70	0.422	0.042	Complete productive med.3	7.5	3.63	0.484	0.048	0.0023	0.030
					Nitrogen free med.3	6.4	2.58	0.403	0.040	-	0.022
72 hrs on productive medium 3	7.50	3.39	0.452	0.045	Complete productive med.3	7.0	2.924	0.418	0.042	-	0.020
					Nitrogen free med.3	5.6	2.43	0.434	0.043	-	0.017

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العوامل البيئية والغذائية التى تتحكم فى انتاج الكاروتينات بواسطة خميرة *Rhodotorula* واستخدامها كاضافات للاغذية
١- استخدام تكنيك الدوارق المهتزة كمزرعة دفعة واحدة او ذات الدفعتين

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قسم الميكروبيولوجيا الزراعية-كلية الزراعة-جامعة عين شمس

تم دراسة انتاج الكاروتينات بواسطة *Rhodotorula glutinis* 32 باستخدام تكنيك الدوارق المهتزة كدفعة واحدة او ذات المرحلتين وذلك لمدة ٣ ايام على درجة حرارة ٣٠م وكان اعلى انتاج من الكاروتينات والخلايا على البئية المعدلة والتى تحتوى على ٤٠ جرام/لتر جليسرول كمصدر للكروبون، ٠,١١١ جرام/لتر نترات امونيوم و ١,٠٣٣ جرام/لتر مستخلص خميرة كمصدر نيتروجين، ٦,٢٥ جرام/لتر فوسفات احادى بوتاسيوم، ٠,٢ جرام/لتر كبريتات ماغنسيوم، ٠,٧٥ جرام/لتر كلوريد الصوديوم، ٠,٠٠٥ جرام/لتر كبريتات حديدوز كاملاح معدنية وكان رقم الحموضة ٥.
زادت كمية الكاروتينات المنتجه وكذلك محصولها بالنسبة للخلايا بمعدل ٤,٣٤ و ٣,٨ مرة بالمقارنة بالانتاج على البيئة الاساسية. كما تم انتاج الكاروتينات باستخدام اربع مواد خام كمصدر للكروبون.وتم دراسة انتاج الكاروتينات باستخدام مزارع الدفعة الواحدة ذات المرحلتين وكان اعلى انتاج من الكاروتينات على بيئة الانتاج المعدلة خلال ٢٤، ٧٢ ساعة فى المرحلة الاولى والمرحلة الثانية من التنمية على الترتيب. وقد لوحظ زيادة فى تركيز الكاروتينات ومحتوى الخلايا منه ومحصوله الى ١,٢٢، ١,٢٣ و ١,٢٦ ضعف على الترتيب بالمقارنة بالنتائج المتحصل عليها عند استخدام المزارع ذات الدفعة الواحدة.