Improvement of Carotenoid Pigments Produced by *Rhodotorula glutinis*

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Abstract: Wild strain of the red yeast *Rhodotorula glutinis* was isolated from different food sources (dates, milk, sausage, and sugarcane). Trails for studying the carotenoids productivity of this strain [volumetric production (µg/l) and cellular carotenoids (µg/g)] were carried out using different carbon sources, nitrogen sources, organic acids and mineral salts. The major carotenoid pigments comprising ß-carotene, torulene and torularhodi. It was found that at 2% glucose the wild strain of *R. glutinis* gave highest volumetric production (165 µg/l). The wild strain of *R. glutinis* was subjected to mutagenesis using U.V radiation (254 nm) for two minutes. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) were 369 µg/l and 46 µg/g after 96 and 24 hours; respectively.
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1. Introduction:

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 animals. Carotenoids usually consists of 40 carbon atoms and their colour range from yellow to reddish with variations to brown and purple.Two major biological roles have been assigned to carotenoids in plants and prokaryotic. In photosynthetic organisms, these pigments are involved in trapping light energy. A more general role applicable to both photosynthetic and non-photosynthetic cell is protection from photodynamic action, protective agent against lethal photooxidation, i.e., quenching of singlet oxygen and inhibition of free radical reaction (Omenn *et al.,* 1996). In mammals carotenoids are precurosor of vitamin A; and are oxygen radical quenchers with potential anti-cancer activities (Nishino *et al.,* 1999).

ß-carotene and lycopene are among the most abundant carotenes in nature (Goodwin and Britton, 1988). Industerially, carotenoids such as ß-carotene are utilized as food and feed supplements, pharmaceutical purposes, as food colorants.

Carotenoids are important natural pigments produced by many microorganisms and plants. Microbial synthesis offers a promising method for production of carotenoids (Johnson and Schroeder, 1995). This explains the increasing interest in the production of microbial carotenoids as an alternative for synthetic food colorants (De Haan *et al.,* 1991 and Vandamme, 1993).

Yeasts of the genera *Phaffia*, *Rhodotorula* and *Sporobolomyces*, fungi like *Blakeslea trispora*, and algae such as *Dunaliella* and *Haematococcus*, and bacteria as *Flavobacterium* and *Micrococcus* are reported to produce carotenoids (Nelis and De Leenheer, 1991). 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. Certain yeast species of the genera *Rhdotorula*, *Cryptococcus*, *Sporobolomyces* and *Phaffia,* produce characteristic carotenoids such as torulene and torularhodin, the main carotenoids of *Rhodotorula* (Simpson *et al.,* 1964) and astaxanthin, the major pigment synthesized by *Phaffia* (Andrewes *et al.,* 1976). Costa *et al.* (1987) investigated the production of ß-carotene from *Rhodotorula glutinis*, during the stationary phase of growth and in non-proliferating conditions. They found that when the cells were transferred to distilled water, the fraction of ß-carotene produced increased from 130 to 630 µg per gram of dried cells. It is suggested that the cells are synthetising carotenoids in distilled water, lacking any substrate or some factor, the hydrogenation of γ-carotene to torulene is blocked, and the carotenogenesis follows a side way, with cyclization to give ß-carotene.

Rhodotorula glutinis is one of the most important yeasts characterized by pigment formation. This strain

had a unique exopolysaccharide and exolipid synthesizing ability. The red yeast *R. glutinis* is one of the best potential candidates for biotechnological production (Frengova *et al.,* 1994). Perrier *et al.* (1995) reported that *R. glutinis* produce characteristic carotenoids (torulene, torularhodin, γ-carotene and ß-carotene) in various proportions. The total carotenoid content varied widley in the studied strains from 10 µg/g dry weight in *R. bogoriensis* to 100 µg /g dry weight in *R. armeniaca*, *R. aurantica* and *R. mucilaginosa*. ß-carotene represented an average of 70% of the total carotenoid and was thus the major pigment. Buzzini and Martini (1999) investigated the production of carotenoids by strains of *Rhodotorula glutinis*. Maximum yield (5.95 mg total carotenoid/L culture fluid) and 630 µg carotenoid/g dry cell weight were obtained with a particular strain of *Rhodotorula glutinis* after a batch culture of 120 hours in a substrate containing concentrated retfied grape musts as the sole carbohydrate source. In all experiments, the major pigments forming carotenoids (ß-carotene, torulene, torularhodin) were quantified. Naidu *et al.* (1999) and Vaskivnyuk and Kvasinkov (1968) found that *R. gracilis* produced red pigments besides a high percentage of lipids. The pigments were a composite mixture of carotenoids containing rhodoxanthin, rhodotorulene, torularhodin and ß-carotene. Bhosale and Gadre (2001c) studied the wild strains of *Rhodotorula glutinis* concerning its carotenoid production, proportion of ß-carotene and cell mass yield. *R. glutinis* NCIM 3353 produced 2.2 mg carotenoid/L in 72 h; and the amount of ß-carotene was 14% (w/w) of the total carotenoid content (17 µg/g dry cell weight). Emilina *et al.* (2003) indicated the associated growth of *Rhodotorula rubra* GED2 and *Lactobacillus casei* subsp. casei in cheese whey ultrafiltrate. Maximum concentration of carotenoids in the cell was 0.49 mg/g dry cell. An important characteristic of carotenogenesis by *Rhodotorula rubra* GED2 + *Lactobacillus casei* subsp. *casei* was established. Abd El-Razek (2004) isolated sixty six cultures of *R. glutinis* capable of carotenoid production. The colour of pigmented yeast isolates ranged from dark orange - red to yellow. The percentage of pigments were 60.63%, 31.36%, 1.39% and 6.61% for ß-carotene, torulene, torulene like and torularhodin, respectively.

The objectives of this study was concerned on the productivity of carotenoid pigments (ß-carotene, torulene and torularhodin) of the wild type yeast strain *Rhodotorula. glutinis* using different carbon sources, organic acids, nitrogen sources and mineral salts. Also improving the productivity of the wild strain by mutagenesis treatment of the wild strain using U.V radiation.

3. Materials and Methods

3.1. Microorganisms:

Rhodotorula glutinins locally isolated from dates, milk, sausage and sugar cane by Yehia, (1998) was used throughout this investigation. The yeast culture was maintained on yeast extract malt extract agar medium. Culture was stored in a refrigerator at 5ºC after incubation at 30ºC (24-72 hours). Purity of the culture was regulatory checked. The yeast strain was monthly activated.

3.1.2. Media

The following media were used throughout the present study:

3.1.2.1. Maintaining media

Med. 1: Yeast extract malt extract agar medium (YMA)

It was recommended by Bhosale and Gadre (2001c) and used for preserving and maintaining of yeast. The medium has the following composition (g/l):

-Glucose (35.0), Malt extract (3.0), Yeast extract (2.0), K_2HPO_4 (3.0), KH_2PO_4 (3.0), $MgSO_4.7H_2O$ (0.2) Agar (20.0), pH 6.0

3.1.2.2. Growth media

Med. 2: The liquid basal medium

A basal medium recommended by Bhosale and Gadre (2001c), was used for assessing yeast growth and production of carotenoids. Its composition was as the following (g/l): Glucose (25.0), Yeast extract, (10.0) K_2HPO_4 (2.0), KH_2PO_4 (2.0), pH 6.0. The medium was prepared and autoclaved at 121°C for 20 minutes. After cooling to 50°C, the medium was aseptically supplemented with 1 ml of 100 mg/ml ampicilline stock solution (final concentration 100 µg/ml).

All media used throughout this study were dissolved in distilled water and autoclaved for 20 minutes at 121°C.

3.2. Methods

3.2.1. Cultivation of yeast for carotenoid production

3.2.1.1. Inoculum preparation

Yeast inoculum was prepared using the liquid basal medium (med.2). A loopfull of an active (24 hours) yeast culture slant was used to inoculate 5 ml of the sterile medium in 50 ml falcon tube. The inoculated tubes were incubated at 25°C for 18 hour on an orbital shaker (200 rpm). The obtained culture was then used as an inoculum (Bhosale and Gadre 2001c).

3.2.1.2. Cultivation

The liquid basal medium (med. 2) was used for assessing yeast growth and carotenoid production. Conical flasks (500 ml) containing 100 ml of the liquid basal medium were inoculated (5% inoculum) using the previously prepared inoculum. The flasks were incubated at 25°C for 5 days on an orbital shaker (250 rpm). Samples were daily withdrawn for

determination of cell growth (O.D), dry cell mass (g/l) carotenoids concentration.

3.3. Nutritional requirements of Rhodotorula glutinins

3.3.1. Carbon sources:

Glucose, fructose and sucrose, were used to study their effect on yeast growth and carotenoid production. Each carbon source was separately added to the basal medium (containing no glucose) at different concentration 2, 5, 10 and 20%, where the C/N ratio was 2:1, 5:1, 10:1 and 20:1. Also, lactose, maltose, raffinose, starch, glycerol and ethanol were tested at the concentration of 2%, where the C/N ratio was 2:1.

3.3.2. Organic acids:

Acetic acid (0.5 and 1%) was used for illustrating its effect on yeast growth and carotenoid production.

3.3.3. Nitrogen sources:

Two nitrogen sources, i.e., yeast extract and tryptone, were separately added to the basal (containing no yeast extract) medium at concentration of 1% to study their effect on growth and production of carotenoids by *R. glutinis*.

3.3.4. Mineral salts:

To study the effect of mineral salts on carotenoids production by *Rhodotorula glutinins*, calcium chloride and magnesium chloride were separately added to the growth medium at concentration of 0.2% for each individual salt.

3.4.1. Cell growth (O.D):

The yeast growth in the presence of the tested materials was recorded spectrophotometrically by measuring the optical density of yeast growth at 600 nm (Ultraspec 3000, Pharmacia Biotec., Germany)

3.4.2. Dry cell mass (g/l):

The dry weight of yeast was taken as a parameter for its growth. Aliquots (1ml) of yeast suspension were centrifuged for 5 min. at 13.000 rpm (17.000 xg), washed twice with distilled water, and then dried at 105°C till constant weight (Simova *et al.,* 2003).

3.4.3. Carotenoid assay

3.4.3.1. Extraction

Carotenoid was extracted from yeast cells according to the method described by Bhosale and Gadre (2001c). One ml aliquot of culture broth was centrifuged for 5 min at 13.000 rpm (~17.000 xg). The pellet was washed twice with distilled water and subsequently disintegrated in a homogenizer plastic tips (Omim tips) with 1 ml of acetonitril: isopropanol: and ethylacetate (40:40:20, v/v), for pigment extraction. The extract was centrifuged and the supernatant was subjected to HPLC analysis (High performance liquid chromatography) for determination of carotenoid pigments.

3.4.3.2. Separation

Thin layer chromatography (TLC) for carotenoid separation was performed with silica gel 60 plates (Merck-5721-7) and carried out generally in n-hexan: ethyacetate (7:1). The references carotenoids ß-carotene was pruchased from Roche. The chromatographed pigments were identified from their Rate of Flow (R_f). The spots were also eluted in the solvent mixture composed of acetonitril, isopropanol and ethylacetate (40: 40: 20; v/v) and used as references for HPLC analysis (Bhosale and Gadre, 2001c).

3.4.3.3. Determination

Carotenoids determination was carried out using HPLC. The analysis were performed on a reserved phase C18 analytical column (LichroCART, Lichrospher 250-4 (Merck, 1.50845.001). The HPLC (Scientific industries Merck, Munich, Germany) instrument equipped with an on line solvent degasser, low pressure quaternary gradient pump, and manual injector with 40-µl loop was used through this work. U.V-visible detector. The mobile phase was composed of acetonitril, isopropanol and ethylacetate (40: 40: 20%) and had flow rate of 0.7 ml/min the column thermostate was set at 25 °C. The detector wavelength was operated at 450 nm (Bhosale and Gadre, 2001c)

3.4.3.4. The Calibration curve of ß-carotene

The quantity of ß-carotenoid were determined according to the method described by Tzeng et al, (2004), after injection ß-carotene into HPLC, the calibration curve was made by plotting peak area against concentrations. High correlation coeffecients (R^2= 0.9653) were obtained for ß-carotene, (Fig. 5). The amount of carotenoids was calculated from the following regression equation:

Y=8E+07X

Where; Y denotes peak area,
X denotes concentration.
E denotes base

Total carotenoid (as ß-carotene) µg/l = Dry cell mass (g/l) × Total carotenoid content µg/g cells.

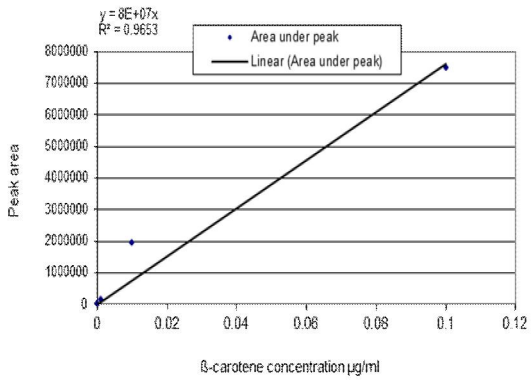

Fig. (5) Calibration curve of ß-carotene

3.5. Mutagensis

To improve the production of carotenoids by *R. glutinis* the mutagenesis with ultraviolet radiation was carried out at 254 nm. Culture of the cells in the mid-logarithmic phase (48 hours) were suspended in physiological saline (0.85% w/v, NaCl) and shaken gently to separate mature daughter cells from the mother cells. The suspension was then placed in sterile petri dishes and irridiated with U.V light (254 nm) at a distance of 10 cm for time intervals ranginig from 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8 minutes. After irridiation, the suspension was appropriately diluted and spread onto yeast extract glucose agar plates. The plates were then incubted in the dark. Exposure time was selected to get 10% survival (Bhosale and Gadre, 2001c). The cells were allowed to grow in a basal medium (med. 2) to test for carotenoid production.

4-Results and Discussion

4.1. Characteristics of yeast strain Rhodotorula glutinis

Rhodotorula is a member of the family Cryptococcaceae, subfamily Rhodotorulodeae. *Rhodotorula glutinis* and *Rhodotorula rubra* are the most common *Rhodotorula spp.,* are saprophytes and widespread in nature (Massimilliano *et al.,* 2001). *Rhodotorula glutinis* is one of the most important yeast. This strain had a unique exopolysaccharide and exolipid synthesizing abilility (Park & Chae (1998); Cho and Kim, (2001)) Hence, the red yeast *R. glutinis* is one of the best potential candidates for the biotechnological production (Frengova *et al.,* 1994).

Yehia (1998) isolated *R. glutinis* from different foods (dates, milk, sausage and sugar cane juice). The morphological and physiological proporties of *R. glutinis* was studied according to Lodder and Kregar-van Rij (1952), Kregar-van Rij (1984) and Barnett *et al.*(1990). *Rhodotorula glutinis* was form round to oval cell smooth, red colored colonies as shown in Fig. (6) reffered to carotenoid pigment, and under microscpical examination had a multilateral budding, no pseudohyphae and no ascospores.

The present investigation aimed to study the production of carotenoid pigments by the locally isolated *R. glutinis* as, ß-carotene, torulene, and torularhodin, and trails for improvement the volumetric production and cellular carotenoids by using different carbon sources, nitrogen sources, mineral salts, organic acids, and carotenoid activitors.

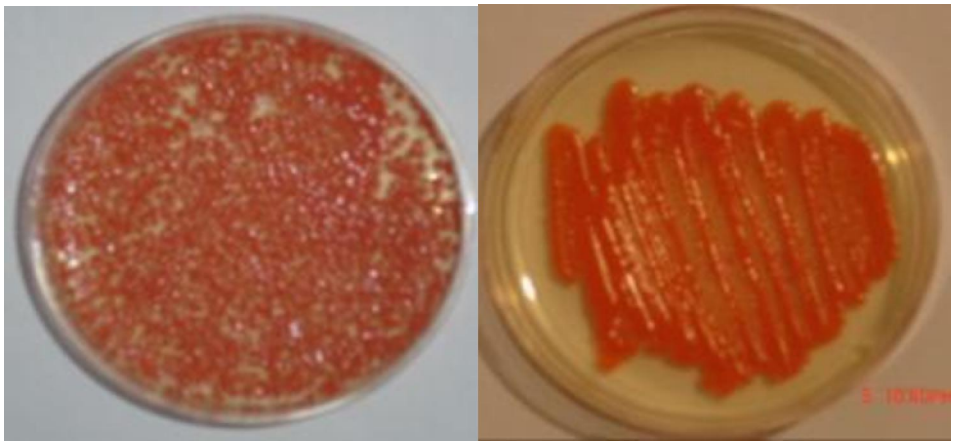

Fig. (6): Growth of *Rhodotorula glutinis* on YMA agar medium (med. 1).

4.2.1. Effect of carbon source

Varying the carbon source in the culture medium affected carotenoid production by the yeast strain of Rhodotorula glutinis. The carbon sources are the most important variable in the carotenoid production fermentation. To determine the most suitable carbon source for carotenoid production, 9 different carbon sources (glucose, fructose, sucrose, maltose, lactose raffinose, starch, glycerol and ethanol) were studied. *R. glutinis* was grown in a basal medium (med.2) containing different concentration of sugars, 2, 5, 10 and 20% in a batch culture (shake flasks, 250 rpm) at 25 ºC for 120 hours (5 days) of incubation time, the C/N ratio was 2:1, 5:1, 10:1 and 20:1, pH was adjusted to 6 using 2M NaOH or 2M HCl. The cell density (O.D) was determined spectrophotometrically at 600 nm, dry cell mass (g/l) and total carotenoids (volumetric carotenoids (µg/l) and cellular carotenoids (µg/g) were also determined.

4.2.1.1. Effect of glucose

Data in Table (1) and declare the highest cell density 2.90 (O.D) and dry cell mass 9 g/l after 120 hours, respectively at 2% glucose. The lowest figures at this level of glucose were 2.48 (O.D) and 1.6 g/l after 24 hours respectively. The highest volumetric production (165µg/l) and cellular carotenoid accumulation (18.3 µg/g) observed after 120 hours.

The lowest volumetric production (17 µg/l) and cellular the lowest carotenoid accumulation (4.89µg/g) were noticed after 24 and 48 hours respectively. With respect to, the highest proportion (%) of ß-carotene was 100%. Torulene and torularhodin were not observed. *R. glutinins* was highly varied in its ability to grow at 5% glucose where the highest cell density 2.74 (O.D) and dry cell mass 6.6 g/l after were obtained 120 and 96 hours, respectively. The lowest figures at this level of glucose were 2.66 (O.D) and 0.3 g/l after 96 and 24 hours, respectively. On the other hand the highest volumetric production 102 µg/l and cellular carotenoid accumulation 30µg/g were recorded after 72 and 24 hours, respectively. The lowest volumetric production 9 µg/l and cellular carotenoid accumulation 4.42µg/g were found after 24 and 48 hours respectively. ß-carotene recorded the highest proportion (100%), while torulene and torularhodin were not observed. At 10% glucose, the highest cell density 2.83 (O.D) and dry cell mass 8.90 g/l were achieved after 120 hours respectively. The lowest Figures at this level of glucose were 2.19 (O.D) and 2.4 g/l after 24 hours respectively. The highest volumetric production 47µg/l and cellular carotenoid accumulation 10.4µg/g observed were and after 120 and 24 hours respectively. The lowest volumetric production 20 µg/l and cellular carotenoid accumulation 2.68µg/g after 72 and 96 hours respectively. The highest proportion (%) of ß-carotene was 100%. Torulene and torularhodin not observed. At 20% glucose, the highest cell density 2.58 (O.D) and dry cell mass 3.9 g/l which after 120 and 72 hours. The lowest figures at this level of glucose were 1.97 (O.D) and 0.8 g/l after 24 hours, respectively. The highest volumetric production 23 µg/l and cellular carotenoid accumulation 5.9 µg/g after 72 hours, respectively. The lowest volumetric production 0 µg/l and cellular carotenoid accumulation 0 µg/g after 24 hours, respectively. The highest proportion (%) of ß-carotene was 100%. Torulene and torularhodin were not observed.

Statistical analysis the highest volumetric carotenoid production was 165µg/l at 2% glucose followed by 102 µg/l at 5% then 47 µg/l at 10% and 23 µg/l at 20%.

Table (1): Effect of different concentrations of glucose (2, 5,10 and 20%) on the growth of *R. glutinis* and total carotenoids production.

			Total Carotenoids		Proportion (%)
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Glucose 2%					
24	2.48 ^{f}	1.6 ^{n}	17 ^{h}	10.2 ^{e}	100:00:00
48	2.71 ^{cd}	4.7 ^{i}	23 ^{f}	4.89 ^{h}	100:00:00
72	2.82 ^{b}	7.4 ^{d}	46 ^{e}	6.22 ^{g}	100:00:00
96	2.78 ^{cd}	8.4 ^{b}	66 ^{d}	7.96 ^{f}	100:00:00
120	2.91 ^{a}	9 ^{a}	165 ^{a}	18.3 ^{c}	100:00:00
Glucose 5%					
24	2.73 ^{cd}	0.3 ^{p}	9 ^{i}	30 ^{a}	100:00:00
48	2.72 ^{cd}	4.3 ^{J}	19 ^{gh}	4.42 ^{i}	100:00:00
72	2.71 ^{cd}	5.4 ^{g}	102 ^{b}	18.88 ^{b}	100:00:00
96	2.66 ^{g}	6.6 ^{e}	67 ^{c}	10.15 ^{e}	100:00:00
120	2.74 ^{g}	5.1 ^{h}	65 ^{cd}	12.47 ^{d}	100:00:00
Glucose 10%					
24	2.19 ^{h}	2.4 ^{m}	25 ^{f}	10.4 ^{e}	100:00:00
48	2.35 ^{de}	5.6 ^{f}	25 ^{f}	4.46 ^{j}	100:00:00
72	2.62 ^{cb}	6.7 ^{e}	20 ^{gh}	2.98 ^{j}	100:00:00
96	2.71 ^{cd}	8.2 ^{c}	22 ^{fg}	2.68 ^{j}	100:00:00
120	2.83 ^{b}	8.9 ^{a}	47 ^{e}	5.28 ^{h}	100:00:00
Glucose 20%					
24	1.97 ^{i}	0.8 ^{o}	0 ^{j}	0 ^{k}	100:00:00
48	2.28 ^{cd}	1.7 ^{n}	7 ^{i}	4.12 ^{i}	100:00:00
72	2.43 ^{f}	3.9 ^{k}	23 ^{f}	5.90 ^{g}	100:00:00
96	2.48 ^{f}	3.7 ^{l}	19 ^{gh}	5.13 ^{h}	100:00:00
120	2.58 ^{e}	3.6 ^{l}	19 ^{gh}	5.20 ^{h}	100:00:00
SE	0.03	0.06	0.09	0.14	

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

Results clearly show that all Rhodotorula strain grow exponentially during the first 48 hours of incubation period in all different concentration of glucose, thereafter the growth rate decreased gradually (phase of decelerating growth, during 48-120 hrs) to be more constant (stationary phase) during the last three days of incubation. It is also interesting to notice that the best concentration of glucose which gave the highest cell density, dry cell mass, volumetric production and also cellular carotenoid accumulation, was 2% followed by glucose 5%. This result was in line with Perrier *et al.* (1995) who dealt with different species of Rhodotorula, they noticed that the total carotenoid content was 100 µg/g dry cells and obtained by both *R. armeniaca* and *R. mucilaginosa* on synthetic medium containing 10 g/l glucose. Also Bhosale and Gadre (2001c and d) explained that glucose yielded a proportionatly higher percentage of ß-carotene with a minor quantity of torulene followed by torularhodin. Costa *et al.* (1987) and Martelli *et al.* (1990) mentioned that the percentage of total carotenoids (ß-carotene) which produced by Rhodotorula glutinis reached 100%, and this results are approaches with our results. Abd El-Razek (2004) reported that the percentage of ß-carotene produced by the mutant 0:20:13 increased to 100%.

4.2.1.2. Effect of fructose

At 2% fructose, the data presented in Table (2) and Figs.(15 and 16) show that the highest cell density (O.D) and dry cell mass (g/l) were 2.88 and 5.4 after 120 hours, respectively. The lowest values at this level of fructose were 2.44 (O.D) and 2.2 g/l after 24 hours, respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) were 74 µg/l and 16.44 µg/g after 96 hours, respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 16 µg/l and 7.7µg/g after 24 hours, respectively.The highest proportion (%) of ß-carotene was 100%. Torulene and torularhodin were not observed.

At 5% fructose, the highest cell density (O.D) and dry cell mass (g/l) which were 2.97 (O.D) and 9.1 g/l after 72 and 120 hours, respectively. The lowest figures at this level of fructose were 2.34 (O.D) and 2.2 g/l after 24 hours respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 69µg/l and 11.81µg/g after 96 and 24 hours, respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 26 µg/l and 3.02µg/g after 24 and 72 hours, respectively. The highest proportion (%) of ß-carotene was 79% after 72 hours. Torulene and torularhodin were 38 and 27% after 48 and 24 hours, respectively.

The highest cell density 3.00 (O.D) and dry cell mass 11.9 g/l after 72 and 120 hours respectively at 10% fructose. The lowest figures at this level of fructose were 2.47 (O.D) and 1.6 g/l after 24 hours, respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 98µg/l and 49.46µg/g after 48 and 24 hours, respectively. The lowest volumetric production 17 µg/l and cellular carotenoid accumulation 1.89 µg/g after 72 hours respectively. The highest proportion (%) of ß-carotene was 100% at 48, 72, 96 and 120 hours. The lowest proportion (%) of ß-carotene was 55% after 24 hour. Torulene and torularhodin were 27 and 23% after 24 hours, respectively.

At 20% fructose, the highest cell density (O.D) and dry cell mass (g/l) were 2.83 (O.D) and 10.3 g/l were obtained after 72 and 120 hours, respectively. The lowest figures at this level of fructose were 1.84 (O.D) and 4 g/l after 24 hours, respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 98 µg/l and 12.6 µg/g after 120 and 48 hours, respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 33 µg/l and 4.24 µg/g after 72 hours, respectively. The highest proportion (%) of ß-carotene was 100% at all hours of growth. Torulene and torularhodin were not observed.

The highest cell density, dry cell mass, also highest volumetric production, and cellular carotenoid production at 10 % fructose, followed by 20% fructose. Bhosale and gadre (2001c) mentioned that fructose yielded a comparable cell mass 9.27 g/l, volumetric production 19 mg/l and cellular carotenoid 2.8 mg/g after 72 hours. They also added that at 2.5% fructose, the proportion (%) of ß-carotene, torulene and torularhodin were 36, 63 and 1 after 72 hours, respectively. Park and Kim (2004) reported that, the cell mass, volumteric carotenoid and cellular carotenoid were 6.78 g/l, 0.08 mg/l and 285.09 µg/g yeast, respectively, at 2% fructose.

4.2.1.3. Effect of sucrose

Data presented in Table (3) show the highest cell density 2.21 (O.D) and dry cell mass 4 g/l after 24 hours, respectively at 2% sucrose. The lowest figures at this level of sucrose were 1.57 (O.D) and 2 g/l after 120 hours, respectively. The highest volumetric production 34 µg/ml (µg/l) and cellular carotenoid accumulation 17.08 µg/g after 120 hours, respectively. The lowest volumetric production 10.8 µg/ml and cellular carotenoid accumulation 2.71µg/g after 24 hours, respectively.The highest proportion (%) of ß-carotene was 100% after 48, 72, 96, and 120 hours. The lowest proportion (%) of ß-carotene was 69% and observed after 24 hours. The highest proportion (%) of torulene and torularhodin were21 and 10 % after 24 hours, respectively.

Table (2): Effect of different concentration of fructose (2, 5,10 and 20%) on the growth of *R. glutinis* and total carotenoids production (Mean ±SE).

			Total Carotenoid	Proportion (%)	
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Fructose 2%					
24	2.44^{fg}	2.2^{l}	16^{k}	7.70^{J}	100:00:00
48	2.87^{bc}	3.9^{k}	30^{h}	7.90^{J}	100:00:00
72	2.83^{bc}	4.7^{hi}	74^{d}	15.74^{d}	1000:0:00
96	2.87^{bc}	4.5^{ij}	74^{d}	16.44^{l}	100:00:00
120	2.89^{ab}	5.4^{g}	46^{f}	8.58^{i}	100:00:00
Fructose 5%					
24	2.34^{g}	2.2^{l}	26^{i}	11.81^{f}	43:30:27
48	2.96^{a}	2.6^{l}	26^{i}	10.00^{g}	54:38:08
72	2.98^{ab}	8.6^{c}	26^{i}	3.02^{l}	79:11:10
96	2.75^{cd}	7.6^{de}	69^{e}	9.18^{h}	60:20:20
120 Fructose10%	2.87^{bc}	9.1^{c}	30^{h}	3.20^{n}	40:35:25
24	2.47^{ef}	1.6^{m}	79^{b}	49.46^{a}	55:27:23
48	2.95^{ab}	5.2^{h}	98^{a}	18.93^{b}	100:00:00
72	3.00^{a}	9.1^{c}	17^{k}	1.89^{o}	100:00:00
96	2.58^{de}	7.1^{e}	22^{J}	3.05^{n}	100:00:00
120	2.81^{bc}	11.9^{a}	27^{i}	2.28^{o}	100:00:00
Fructose20%					
24	1.84^{h}	4.0^{ijk}	47^{f}	5.17^{l}	100:00:00
48	2.72^{cd}	6.0^{f}	76^{c}	12.6^{e}	100:00:00
72	2.84^{bc}	7.8^{d}	33^{g}	4.24^{m}	100:00:00
96	2.33^{g}	7.6^{de}	46^{f}	6.11^{k}	100:00:00
120	2.54^{ef}	10.3^{b}	98^{a}	9.52^{h}	100:00:00
SE	0.068	0.20	0.58	0.14	

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

At 5% sucrose, results show the highest cell density 2.17 (O.D) and dry cell mass 4.1 g/l after 24 and 120 hours, respectively. The lowest figures at this level of sucrose were 1.83 (O.D) and 2.6 g/l after 120 and 72 hours, respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) were 30µg/l and 5.78µg/g after 72 and 48 hours, respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 9 µg/l and 2.07 µg/g after 24 and 72 hours, respectively.The highest proportion (%) of ß-carotene was 100% at all hours of growth. Torulene and torularhodin were not observed.

The highest cell density (O.D) and dry cell mass (g/l) which were 2.11(O.D) and 5.7 g/l after 48 hours respectively at 10% sucrose. The lowest figures at this level of sucrose were 1.96 (O.D) and 1.3 g/l after 120 and 24 hours respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 28 µg/l and 13.53 µg/g after 96 and 120 hours respectively.The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 10 µg/l and 4.5 µg/g after 24 and 48 hours respectively.The highest proportion (%) of ß-carotene was 100% at all hours of growth. Torulene and torularhodin were not observed.

The highest cell density 2.46 (O.D) and dry cell mass 9.5 (g/l) at 20% sucrose after 120 hours, respectively. The lowest figures at this level of sucrose were 1.56 (O.D) and 4 g/l after 24 hours respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 46µg/l and 5.78 µg/g after 96 and 48 hours respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 7 µg/l and 1.75µg/g after 24 hours respectively.The highest proportion (%) of ß-carotene was 100% at all hours of growth. Torulene and torularhodin were not observed.

The highest volumetric production was obtained (46 µg/l) at 20% sucrose. Bhosale and gadre (2001c) mentioned that sucrose yielded a comparable cell mass, volumetric production and cellular carotenoid accumulation which were 11.6 g/l, 23.3 mg/l and 2

mg/g after 72 hours, respectively at 2% sucrose, also, the proportion (%) of ß-carotene, torulene and torularhodin were 38, 59 and 2 after 72 hours, respectively. Park and Kim (2004) reported that, the cell mass, volumteric carotenoid and cellular carotenoid were 5.77 g/l, 1.50 mg/l and 260.15µg/g yeast, respectively, at 2% sucrose.

By comparison between the three sugars used, Table (4) cleary explain that glucose is the best carbon source at 2% followed by glucose 5% then fructose 10% and sucrose 2%.

Table (3): Effect of different concentration of sucrose (2, 5,10 and 20%) on the growth of *R.glutinis* and total carotenoids production (Mean ±SE).

			Total Carotenoids		Proportion (%)
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Sucrose 2%					
24	2.21^{bc}	4.0^{e}	10.8^{l}	2.71^{m}	69:21:10
48	2.06^{cd}	4.0^{e}	30^{d}	7.40^{g}	100:00:00
72	1.97^{cd}	2.6^{gh}	25^{hi}	9.61^{e}	100:00:00
96	1.73^{ef}	2.6^{gh}	27^{fg}	10.49^{d}	100:00:00
120	1.57^{g}	2.0^{i}	34^{c}	17.08^{a}	100:00:00
Sucrose 5%					
24	2.17^{bc}	3.3^{f}	9^{l}	3.16^{k}	100:00:00
48	2.05^{cd}	3.1^{f}	21^{k}	5.78^{h}	100:00:00
72	2.03^{cd}	2.6^{gh}	30^{d}	2.07^{l}	100:00:00
96	1.99^{cd}	2.9^{fg}	24^{ij}	5.76^{h}	100:00:00
120	1.84^{e}	4.1^{e}	30^{b}	3.01^{l}	100:00:00
Sucrose 10%					
24	2.06^{cd}	1.3^{J}	10^{l}	7.73^{f}	100:00:00
48	2.11^{cd}	5.7^{d}	26^{gh}	4.50^{J}	100:00:00
72	2.04^{cd}	4.4^{e}	23^{J}	5.51^{i}	100:00:00
96	2.04^{cd}	2.3^{hi}	28^{ef}	12.34^{c}	100:00:00
120	1.97^{d}	2.0^{i}	27^{fg}	13.53^{d}	100:00:00
Sucrose 20%					
24	1.57^{fg}	4.0^{e}	7^{m}	1.75^{o}	100:00:00
48	2.07^{cd}	7.0^{c}	40^{b}	5.78^{h}	100:00:00
72	2.29^{ab}	9.4^{a}	19^{k}	2.07^{l}	100:00:00
96	2.43^{a}	8.1^{b}	46^{a}	5.76^{h}	100:00:00
120	2.46^{a}	9.5^{a}	29^{de}	3.01^{l}	100:00:00
SE	0.05	0.14	0.51	0.06	SE

Different superscripted letters indicate significant differences (P<0.05) among the observed valueswithin columns.

Table (4): Volumetric carotenoid production (µg/l) by *R. glutinis* at different concentrations of glucose, fructose and sucrose during the growth for 120 hours (Mean ±SE)..

		2%			**5%**			**10%**			**20%**	
Cultivation time (h)	Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose	Sucrose
24	17^{v}	16^{v}	10.8^{w}	9^{w}	26^{pq}	9^{w}	25^{qr}	79^{d}	10^{w}	0^{y}	47^{J}	7^{x}
48	23^{st}	30^{m}	30^{m}	19^{u}	26^{pq}	20^{u}	25^{qr}	98^{c}	26^{pq}	7^{x}	76^{e}	40^{k}
72	46^{J}	74^{f}	25^{qr}	102^{b}	26^{pq}	30^{m}	20^{u}	17^{v}	23^{st}	23^{st}	33^{l}	19^{u}
96	66^{hi}	74^{f}	27^{op}	67^{hi}	69^{g}	24^{rs}	22^{t}	22^{t}	28^{no}	19^{u}	46^{J}	46^{J}
120	165^{a}	46^{J}	34^{l}	65^{i}	30^{m}	30^{m}	47^{J}	27^{op}	27^{op}	19^{u}	98^{c}	29^{mn}
SE	0.54											

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

4.2.2. Effect of organic acids

Organic acids i.e. acetic acid, at different concentrations, 0.5, 1, 1.5 and 2% were individually used as a carbon source for production of carotenoids using R. glutinis. The C/N ratio was 0.5:1, 1:1, 1.5:1 and 2:1.

4.2.2.1. Acetic acid

Results in Table (5) and at acetic acid 0.5% indicated that the highest cell density 2.70 (O.D) and dry cell mass 9.1 g/l were observed after 96 and 120 hours, respectively. The lowest figures were, 2.00 (O.D) and 4.4 g/l after 24 hours. The highest volumetric production and cellular carotenoid accumulation were 97µg/l and 10.65µg/g after 120 hours. The lowest volumetric production and cellular carotenoid accumulation were 14.6 µg/l and 2.54 µg/g after 24 and 48 hours, respectively. The highest proportion (%) of ß-carotene, was 100 % at all hours of growth. Torulene and torularhodin were not observed. At 1% acetic acid, the highest cell density 2.784 (O.D) and dry cell mass 9.2 g/l were observed after 96 hours. The lowest figures were 2.1 (O.D) and 3.4 g/l after 24 hours, respectively. The highest volumetric production was 115µg/l and cellular carotenoid accumulation was 15.35µg/g after 120 hours. The lowest volumetric production 14.2 µg/l and cellular carotenoid accumulation 4.19 µg/g were observed after 24 hours, respectively. The highest proportion (%) of ß-carotene, was 100 % at all hours of growth. Torulene was 42% after 24 hour and torularhodin was not observed. No response was observed for the growth of yeast strain at 1.5 and 2% of acetic acid.

Statistical analysis clearly show that the highest volumetric carotenoid production was 115µg/l at 1% acetic acid and 97µg/l at 0.5% acetic acid.

Table (5): Effect of different concentration of acetic acid, on the growth of yeast strain *R. glutinis* and total carotenoids production (Mean ±SE).

			Total Carotenoids		Proportion (%)
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Acetic acid 0.5%					
24	2.00^{d}	4.4^{9}	14.6^{i}	3.32^{h}	100:00:00
48	2.081^{cd}	6.1^{f}	18^{h}	2.54^{i}	100:00:00
72	2.61^{b}	$7.o^{de}$	50^{f}	7.16^{d}	100:00:00
96	2.70^{ab}	7.8^{c}	59^{e}	7.16^{d}	100:00:00
120	2.69^{ab}	9.1^{a}	97^{b}	10.65^{b}	100:00:00
Acetic acid 1%					
24	2.10^{cd}	3.4^{h}	14.2^{i}	4.19^{g}	58:42:00
48	2.19^{c}	6.8^{e}	30^{g}	4.43^{f}	100:00:00
72	2.73^{ab}	8.5^{b}	70^{c}	8.06^{c}	100:00:00
96	2.78^{a}	9.2^{a}	63^{d}	6.84^{e}	100:00:00
120	2.78a	7.5^{cd}	115^{a}	15.35^{a}	100:00:00
SE	0.06	0.19	0.52	0.06	

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

4.2.3. Effect of nitrogen sources

For studying the effect of nitrogen sources on carotenoid production by R. glutinis, the yeast extract of basal medium (med.2) was replaced by different nitrogen source as tryptone, peptone, glycine and casein, at a concentration of 1% glucose was used as the sole carbon source. The amount of glucose and each nitrogen source were adjusted to give C/N ratio of 2:1 in the cultivation medium as a batch culture (shake flasks, 250 rpm) at 25 ºC for 120 hours (5 days) of incubation time, the pH was adjusted to 6 using 2M NaOH or 2M HCl. The cell density (O.D) was determined by turbidity measurements at 600 nm spectrophotometrically. Dry cell mass (g/l), total carotenoids [volumetric carotenoid (µg/l) and cellular carotenoid (µg/g)] were also determined.

4.2.3.1. Effect of tryptone

The effect of tryptone (1%) as a nitrogen source on the carotenoid production by *R. glutinis* was studied. Data in Table (6) clearly show that the highest cell density 2.53 (O.D) and dry cell mass 1 g/l were obtained after 96 and 24 hours. The lowest figures were 2.09 (O.D) and 0.4 g/l after 24 and 48 hours, respectively. The highest volumetric production 13 µg/l and cellular carotenoid accumulation 26 µg/g were observed after 96 hours. The lowest volumetric production 6 µg/l and cellular carotenoid accumulation 6 µg/g were recorded after 24 hours. The highest proportion (%) of ß-carotene, and torulene being 68 and 40% were observed after 48 and 96 hours, respectively. The lowest proportion (%) of ß-carotene, and torulene were 60 and 31 after 96 and 48 hours respectively. Torularhodin was not detected along cultivation time. By using tryptone as a nitrogen

source, Bhosale and Gadre (2001c) noticed that the dry cell mass was 12.5 g/l, volumetric production and cellular carotenoid accumulation were 26 mg/l and 2 mg/g, in succession. Also, Park and Kim (2004) dealt with peptone stated that cell mass was 8.33 g/l, cellular carotenoid and volumetric carotenoid were 491.36 µg/g yeast and 4.09 mg/l, respectively.

Table (6): Effect of different nitrogen sources on the growth *R.glutinis* and total carotenoids production (Mean ±SE).

			Total Carotenoids		Proportion (%)
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Yeast extract					
24	2.48 ^{f}	1.6 ^{n}	17 ^{e}	10.2 ^{g}	100:00:00
48	2.71 ^{cd}	4.7 ^{i}	23 ^{d}	4.89 ^{h}	100:00:00
72	2.82 ^{b}	7.4 ^{d}	46 ^{c}	6.22 ^{h}	100:00:00
96	2.78 ^{cd}	8.4 ^{b}	66 ^{b}	7.96 ^{gh}	100:00:00
120	2.91 ^{a}	9 ^{a}	165 ^{a}	18.3 ^{d}	100:00:00
Tryptone1%					
24	2.092 ^{def}	1.0 ^{hi}	6 ^{k}	6 ^{h}	65:35:00
48	2.388 ^{bc}	0.4 ^{kl}	8 ^{j}	20 ^{c}	68:31:00
72	2.521 ^{b}	0.5 ^{kl}	12 ^{f}	24 ^{b}	63:37:00
96	2.536 ^{b}	0.5 ^{kl}	13 ^{f}	26 ^{a}	60:40:00
120	2.500 ^{b}	0.5 ^{kl}	13 ^{e}	26 ^{a}	60:40:00
SE	0.06	0.09	0.53	0.35	

4.2.4. Effect of mineral salts

The yeast strain *R. glutinis* was grown in medium (Med.2), containing 2% glucose and 1% yeast extract, C/N ratio was 2:1. The effect of different minerals on growth and carotenoids production addition was examined at a concentration of 0.2% for each individual salt. The mineral salts used in these study were, calcium chloride and magnesium chloride.

4.2.4.1. Effect of calcium chloride

Table (7) show the highest cell density 2.84 (O.D) and dry cell mass 6.8 g/l (g/l) after 72 hours respectively. The lowest figures were 2.56 (O.D) and 3.9 g/l after 24 hours. The highest volumetric production 110 µg/l and cellular carotenoid accumulation 27.06 µg/g were obtained after 120 hours, respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation were 10 µg/l and 2.59 µg/g after 24 hours respectively. The highest proportion (%) of ß-carotene, torulene, and torularhodin were 88, 34 and 23% after 120, 24 and 96 hours respectively. The lowest proportion (%) of ß-carotene, torulene, and torularhodin were 48, 8 and 0% after 24, 96 and 120 hours, respectively. Bhosale and Gadre (2001c) noticed that the dry cell mass was 11.4 g/l, volumetric production and cellular carotenoid accumulation were 52mg/l and 4.6 mg/g, respectively.

4.2.4.2. Effect of magnesium chloride

Table (7) show the highest cell density 1.98×10 (O.D) dry cell mass 10.3 g/l after 72 hours respectively. The lowest figures were 2.75 (O.D) and 1.5 g/l after 24 hours respectively. The highest volumetric production 123 µg/l and cellular carotenoid accumulation 20.24µg/g were obtained after 120 hours respectively. The lowest volumetric production 9 µg/l and cellular carotenoid accumulation 4.62 µg/g after 24 and 48 hours.The highest proportion (%) of ß carotene, torulene, and torularhodin were 87, 23 and 56% after 120, 48 and 24 hours respectively.The lowest proportion (%) of ß-carotene, torulene, and torularhodin were, 23, 8 and 4% after 24, 96 and 120 hours.

Statistical analysis of the effect of different mineral salts in Table (7) showed that magnesium chloride was the best mineral salt used and gave the highest volumetric production followed by calcium chloride, magnesium chloride, sodium sulphite and potassium chloride.

4.3. Mutagenesis

Rhodotorula glutinis produced low yield of carotenoids when grown in basal medium (med.2) at 25°C for 5 days. To improve the production of carotenoids by *R. glutinis* the mutagenesis with ultraviolet radiation was carried out at 254 nm. Overnight culture of the cells were suspended in 0.85% (w/v) physiological saline and shaken gently to separate mature daughter cells from the mother cells. The suspension was then placed in sterile petri dishes and irridiated with U.V light (254 nm) at a distance of 10 cm for time intervals ranging from 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8 minutes. After irradiation, the suspension was diluted appropriately and spread onto yeast extract malt extract agar medium (med.1). The plates were incubated in the

dark; and exposure time was selected to get 10% survival (Bhosale and Gadre, 2001c). The cells were grown in a basal medium (med. 2) for check its carotenoid production.

Table (7): Effect of different mineral salts (0.2%) on the growth of yeast strain *R.glutinis* and total carotenoids production (Mean ±SE).

			Total Carotenoids		Proportion (%)
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Calcium chloride					
24	2.56^{g}	3.9^{k}	10^{s}	2.59^{m}	48:34:19
48	2.74^{g}	4.5^{i}	15^{qr}	3.45^{m}	61:31:08
72	2.84^{g}	6.8^{e}	42^{n}	6.22^{k}	73:25:02
96	2.75^{g}	5.2^{gh}	44^{m}	8.49^{i}	69:08:23
120	2.71^{g}	4.1^{ijk}	110^{b}	27.06^{b}	88:12:00
Magnesium chloride					
24	2.75^{g}	1.5^{m}	9^{s}	5.66^{k}	23:21:56
48	17.77^{c}	10.1^{a}	47^{l}	4.62^{l}	71:23:06
72	19.81^{a}	10.3^{a}	89^{e}	8.66^{i}	84:11:05
96	18.40^{b}	9.7^{b}	104^{c}	10.68^{g}	82:08:10
120	18.21^{b}	6.1^{f}	123^{a}	20.24^{d}	87:09:04
SE	0.05	0.29	0.56	0.14	

After 2 minutes of U.V radiation (254 nm), Results in Table (8) indicated that the highest cell density (O.D) and dry cell mass (g/l) were 2.58 (O.D) and 8 g/l after 120 and 96 hours respectively. The lowest figures were 2.32 (O.D) and 5 (g/l) after 24 hours, respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) were 368 µg/l and 46 µg/g respectively after 96 hours. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 150 µg/l and 30 µg/g in succession after 24 hours. The highest proportion (%) of carotene, torulene and torularhodin were 84, 86 and 39 after 72, 48 and 24 hours, respectively. The lowest proportion (%) of carotene, torulene and torularhodin were 9, 9 and 5 after 48, 72 and 48 hours, respectively.

After 3 minutes of U.V radiation (254 nm), results indicat that the highest cell density (O.D) and dry cell mass (g/l) were 2.80 (O.D) and 8 g/l after 120 hours, respectively. The lowest figures were 2.30 (O.D) and 2 (g/l) after 24 hours. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) were 325µg/l and 46.5µg/g after 96 hours. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 24 µg/ml and 12 µg/g, respectively after 24 hours.The highest proportion (%) of ß-carotene, torulene and torularhodin were 86, 94 and 29% after 96, 120 and 24 hours respectively, while the lowest proportion (%) of ß-carotene, torulene and torularhodin were 0, 4 and 0 after120, 96 and 96 hours, in succession.

U.V radiation (254 nm) for 3.5 minutes, Results show that highest cell density (O.D) and dry cell mass (g/l) were 2.62 (O.D) and 4.1 g/l, respectively after 120 hours. The lowest figures were 2.32 (O.D) and 2 (g/l) after 24 hours respectively. The highest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) were 138 µg/l and 46 µg/g after 72 hours respectively. The lowest volumetric production (µg/l) and cellular carotenoid accumulation (µg/g) observed were 12.9 µg/l and 6.45 µg/g, respectively after 24 hours.
The highest proportion (%) of ß-carotene, torulene and torularhodin were 82, 94 and 21% after 72, 120 and 24 hours, respectively. The lowest proportion (%) of ß-carotene, torulene and torularhodin were 0, 7 and 6 after 96, 72 and 48 hours, respectively.

From the brevious results it clearly show that the total carotenoids produced by the wild strain of *R. glutinis* (165µg/l) was less than those produced by the mutant strain which irradiated by U.V for 2 minutes (368µg/l) followed by that irradiated by U.V for 3 minutes (325 µg/l). However the total carotenoids amount produced by the mutant strain which irradiated by U.V for 3.5 minutes reached (138µg/l). This amount was less than that obtained by the wild strain.

Statistical analysis (Table (8) clearly show that the highest volumetric production of carotenoids was observed after 2 minutes subjection to U.V radiation of *R. glutinis* followed by 3 minutes and 3.5 minutes subjections.

Bhosale and Gadre (2001c) mentioned that *R. glutinis* NCIM 3353 produced 2.2 mg carotenoid/l

and ß-carotene was 14% of the total carotenoid in 72 hours. After the strain was subjected to mutagenesis using U.V radiation, the mutant produced 120- fold more ß-carotene, which was 82% (w/w) of the total carotenid than the parent culture after 36 hours. Abd El-Razek (2004) reported that, the total carotenoid contents of *R. glutinis* var. *glutinis* (parent strain) produced 255.54 µg/g and 1.480 mg/l and this was the highest followed by mutants 0:20:13 which gave 227.60 µg/g, 1.224 mg/l then mutant 0:20:13:7 gave 200.29µg/g, 1.170 mg/l and finally mutant 0:29:18:11 gave 166.79 µg/g, 0.203 mg/l.

Table (8): Effect of U.V radiation (254 nm for 2, 3 and 3.5 minutes) on the growth of *R. glutinis* and total carotenoids production (Mean ±SE)..

			Total Carotenoids		Proportion (%)
Cultivation time (h)	Cell density (O.D)	Dry cell mass (g/l)	µg/l	µg/g	(ß-carotene:torulene: torularhodin)
Exposure to U.V for 2min.					
24	2.33^{d}	5.0^{cd}	150^{h}	30^{de}	50:11:39
48	2.46^{bc}	6.0^{c}	216^{e}	36^{b}	09:86:05
72	2.48^{bc}	6.7^{abc}	221^{d}	33^{c}	84:09:07
96	2.51^{bc}	8.0^{a}	368^{a}	46a	80:09:10
120	2.58^{b}	7.0^{ab}	203^{g}	29^{e}	74:11:15
Exposure to U.V for 3min.					
24	2.31^{d}	2^{f}	24^{n}	12.0^{h}	56:15:29
48	2.43^{cd}	5^{cd}	132^{j}	26.7^{f}	74:12:14
72	2.46^{bc}	7^{ab}	210^{f}	30.0^{de}	84:05:11
96	2.56^{b}	7^{ab}	325^{b}	46.5^{a}	86:04:00
120 Exposure to U.V for 3.5min.	2.80^{a}	8^{a}	290^{c}	36.0^{b}	00:94:07
24	2.33^{d}	2.0^{f}	12.9^{o}	6.45^{i}	62:17:21
48	2.50^{bc}	2.5^{cd}	75.0^{m}	30.0^{de}	81:13:06
72	2.522^{b}	3.0^{ef}	138.0^{i}	46.0^{a}	82:07:12
96	2.61^{b}	4.0^{de}	78.0^{l}	19.5^{g}	00:93:07
120	2.63^{b}	4.1^{de}	127.0^{k}	31.0^{d}	00:94:07
SE	0.06	0.54	0.56	0.47	-

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

4.4. Extraction, Separation and HPLC analysis of carotenoid pigments

4.4.1. Extraction of carotenoids

Bhosale and Gadre (2001c) declared that, the cells of *Rhodotorula glutinis* were disintegrated in a mini- bead beater with a mixture of acetonitril, isopropanol and ethylacetate (40:40:20, v/v) for pigment extraction. The extract was centrifuged and the supernatant was filtered through a 0.45 µm membrane filter and subjected to analysis. Also, Bhosale and Gadre (2002) extracted the carotenoids from *R. glutinis* by acetone. They suspended 500 mg spray-dried cell mass in 20 ml acetone and disintegrated in a Braun MSK cell homogenizer (Braun, Melssungen, Germany). Acetone was removed under vacuum, residue dissolved in mobile phase and subjected to analysis by using HPLC.

The strain of *R. glutinis* (wild strain) was grown in shake flasks (250 rpm) at 25 ºC for 120 hours using a basal medium (med.3) containing 2% glucose and 1% yeast extract (C/N ratio 2) and grown as a batch culture. The cells were washed twice using in distilled water and disintegrated using homogenizer (Labordorf) in the presence of a mixture of acetonitril, isopropanol and ethylacetate (40:40:20, v/v) to obtain the the crude carotenoid extract and subjected to separation using thin layer chromatography.

4.4.2. Separation of carotenoids

Figure (1) shows the thin layer chromatogram for carotenoid separation on silica gel 60A plates (merck) and in n-hexan: ethylacetate (7:1) as chromatographic solvent system. The pigments were identified from their R_f value (x100) as follows:

R_{f1} = 8.5/13.5 = 0.64 x 100 = 64
R_{f2} = 7.5/13.5 = 0.55 x 100 = 55
R_{f3} = 0.8/13.5 = 0.06 x 100 = 6

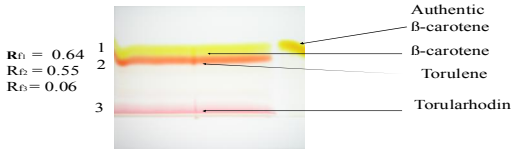

Fig (1).Thin layer chromatography (TLC) of separated carotenoid pigments on silica gel plates 60A (20x20 cm).

The crude carotenoid extract of *R. glutinis* was separated by TLC gave three main components which were identified by their R_f values. R_{f1} value identical to that of ß-carotene standard (authentic) and the colour of band was yellow, R_{f2} was orange and identified as torulene, and R_{f3} was fuchia and identified as torularhodin.

Abd El-Razek *et al.* (2004) separated three components when they used diethyl ether (25%) in petroleum ether (b.p. 40-60ºC, silica gel G60). The three components were identified as ß-carotene, torulene and torularhodin (R_f values were 93.2, 60.5, and 25.9, respectively). On the other hand they mentioned that four components were obtained when [benzene 5% in petroleum ether (b.p. 80-100°C), silica gel G60] were used. The four components were identified as ß-carotene, torulene, torulene like and torularhodin (R_f values were 95, 54, 18 and 2.7, respectively) and its colour were dark yellow, orange red, pink and fuchia, respectively.

Perrier *et al.* (1995) reported that one to ten carotenoid pigments were separated by TLC, depending on the strain of *Rhodotorula.*

4.4.3. HPLC analysis of carotenoid pigments

Three layers of purified carotenoids separated by TLC and resupended in a mixture of acetonitril, isopropanol and ethylactate (40:40:20 v/v) were used for HPLC analysis (Fig. 2). Analyses were performed on a reversed-phase C_{18} analytical column (LiChrCART, LiChrospher 125-4). The HPLC instrument equipped with an on line solvent degasser, low pressure quaternary gradient pump, manual injector with 40µl loop. The flow rate was 0.7 ml/min. The detector was operated at 450 nm. The concentration of carotenoids were calculated from the calibration curve (3.5.5) according to the following equation:

$$Y = 8E+07X$$

Where; Y denotes peak area,
X denotes concentration.
E denotes base

Data in Fig.(3) show HPLC chromatogram of carotenoid pigments which were separated from *R. glutinis.* Data indicated the presence of three peaks: torularhodin, torulene and ß-carotene at retention time of 2.27, 3.21, and 3.64 min, respectively. Bhosale and Gadre (2001c) reported that the carotenoids, torularhodin, torulene, and ß-carotene eluted at 2.39, 3.39 and 3.49 min, retention time, respectively. The three pigments were identical to those published earlier (Perrier *et al.,* 1995) and which confirmed the identification by Bhosale and Gadre (2001c). Taylor *et al.*(1990) explained that, the High performance liquid chromatography (HPLC) has been established during the past 10 years as one of the primary methods for the separation and purification of carotenoids and retenoids. The rapid analysis times (10 to 45 min), high sensitivity (low ng), high resolving power, high recovery and non destructive conditions of HPLC make it an ideal method for carotenoid and retinoid analysis. Frengova et al, (1994) declared that, the HPLC permitting stimultaneous determination of major carotenoid pigments. The three main pigments (torularhodin, ß-carotene and torulene) were analyzed and found to be 182, 43.9, 23 µg/g dry cells, respectively.

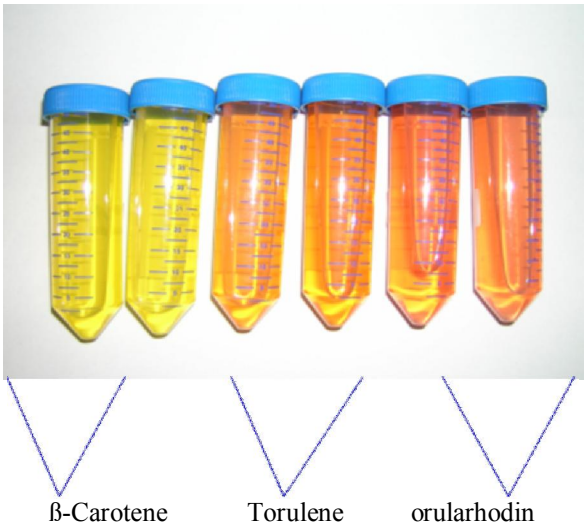

Fig. (2): The three layers of carotenoid pigments separated on Silica gel 60 A (20×20) and dissolved in a mixture of acetonitril, isopropanol and ethylactate (40:40:20 v/v), the yellow colour pigment identified as ß-carotene, the orange colour pigment identified as torulene, and the fuchia colur pigment adentified as torularhodin.

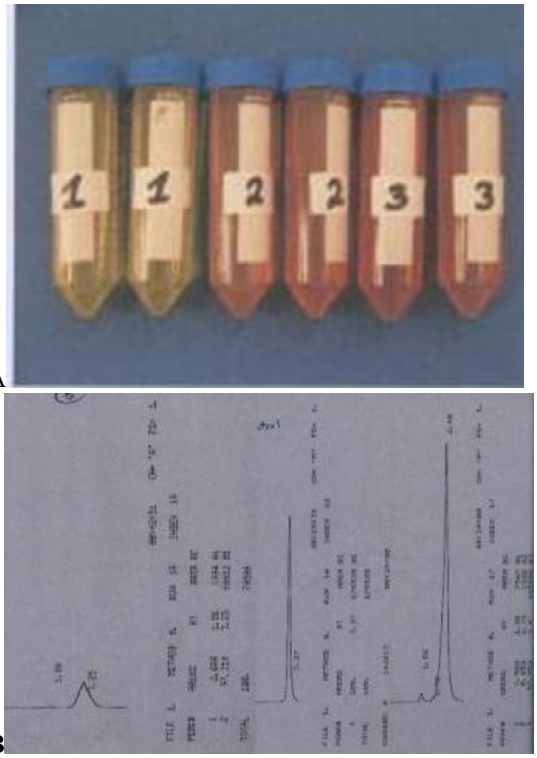

Fig. (3): (A) photomicrograph of the three layers extracted from the silica gel plate (B) HPLC chromatogram of carotenoids from *Rhodotorula glutinins*, torularhodin, torulene and ß-carotene.

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