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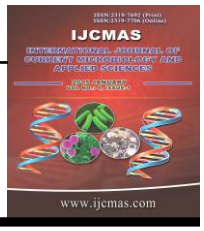

Original Research Article

Optimization of Carotenoids production by yeast strains of *Rhodotorula* using salted cheese whey

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Keywords

Rhodotorula glutinis, *R. mucilagenosa* Carotenoids-factor affecting carotenoids production, Salted whey

A B S T R A C T

There is an increased interest in caroteniod as natural antioxidant pigments with many important biological activities which can reduce chronic diseases. A new yeast strain was isolated from salted cheese whey as carotenoid producing strain and identified according to its morphological, cultural and biochemical characteristics as *Rhodotorula* spp. For conclusive identification of *Rhodotorula* spp., the genus-specific 18s rRNA gene analysis was carried out. Its intracellular and extra cellular extracts showed no influence on mice survival. The identified strain and the reference strain *Rhodotorula glutinis* were grown to study the effect of NaCl concentration, incubation temperature, initial pH and incubation period on dry biomass and carotenoid production. The maximum biomass (13.95 g/l) and volumetric carotenoid production (6.544 mg/l) were scored by the reference strain *R. glutinis* after incubation for 120 hr at 30 °C and pH 6.6 in a medium containing 3% NaCl. the isolated strain showed its maximum biomass (9.02 g/l) in a medium containing 10% NaCl while the highest amount of volumetric Carotenoid (5.044 mg/l) were obtained in a medium containing 6% NaCl after 120 hr incubated at 30 °C and pH 6.6. The obtained result showed that *R. mucilagenosa* will be a promising microorganism for commercial production of carotenoid.

Introduction

Carotenoids are the most widespread natural lipid-soluble pigments with many important biological activities and industrial applications (Marova *et al*, 2004). Due to the growing demand for such compounds in pharmaceutical, cosmetic, food, and feed industries, there is increasing interest in carotenoids naturally obtained by biotechnological processes (Aksu and Eren, 2005).

Carotenoids are important natural pigments, displaying yellow, orange, and red color, found widely in microorganisms and plants. Carotenoid pigments such as carotene and astaxanthin, are used as natural food

colorants or feed additives in aquacultures. Several studies have shown that carotenoids combat various types of cancer and other diseases because of their antioxidant and/or provitamin A potential (Valduga *et al.* 2009). Also, Marova *et al.* (2012) reported that, carotenoid have important biological activity due to their provitamin A activity and properties resulting in potential health benefits such as strengthening the immune system and decreasing the risks of degenerative diseases Furthermore, carotenoids have antioxidant action by neutralizing free radicals as electron donors. Thus, carotenoids as antioxidants prevent the damage caused on living cells by free radicals and have ability to reduce chronic diseases.

Carotenoids are produced primarily by filamentous fungi and yeasts and by some species of bacteria, algae and lichens. Among microbial sources of carotenoids, specific yeasts strains takes advantage of the utilization of the whole biomass, efficiently enriched for particular metabolites .yeasts such as *Phafia rhodozyma* and *Rhodotorula glutinis* are of commercial interest (*Martines et al. 2009)* Carotenoid production by fermentation can become industrially feasible if the cost of production can be minimized by use of cheap industrial by-products as nutrient sources. A number of studies have been carried out in recent years on the fermentation of various agricultural wastes of (oats, wheat, barley, corn, rice, sugar cane molasses, grape must and cheese whey) to produce carotenoid by different strains in shake flask fermentation (*Certik et al., 2009*).Whey is a by-product of the cheese industry contains approximately 4.5% lactose, 0.8% protein, 1% salt, and 0.1– 0.8% lactic acid. Furthermore, the high chemical oxygen demand (COD) (50 kg O_2/t permeate) of whey makes its disposal a pollution problem. Therefore, the use of whey in an inexpensive fermentation medium has long been of industrial interest. Lactose in whey is a suitable carbon source for many microorganisms. In addition, whey is rich in minerals and contains vitamins, which may provide valuable nutrients to stimulate cell growth and product formation. One of the promising ways to use lactose in whey is to use it as a low-cost carbon source for the production of carotenoid by fermentation (Frengova,*et al.*, 1994).

Rhodotorula yeasts produce characteristic carotenoid, such as β-carotene, torulene, and torularhodin in various proportions (Frengova *et al*., 2004). This study aims to investigate growth and carotenoid production by isolated and reference strains of *Rhodotoruola* using salted cheese whey on a batch-scale fermentation level. Further more, optimization of carotenoid production conditions by *Rhodotoruola* was studied

Materials and Methods

Raw material: salted Cheese whey (NaCl 8% or 1%) was obtained from The Dairy Plant of Agricultural Research Center, Giza, Egypt.

Microorganisms: *Rhodotoruola glutinis* was obtained from Fungal Center of Al-Azher University. The strain was subcultured and maintained on a potato dextrose agar at 4 °C.

Chemical analysis of cheese whey sample:

Different chemical analysis i.e., moisture (drying at 105 °C), total solid, ash, total nitrogen (micro kjeldahl) and protein content were determined and calculated according to AOAC (2000). Lactose content was estimated by phenol-sulfuirc acid method (Dubois *et al*., 1956), and fat content was determined according to Mistry

and Hassan (1992). The pH of samples was measured by a pH-meter (Orion SA720, U.S.A.)

Microbiological analysis of cheese whey

Total bacterial counts and total spore forming bacteria were determined on nutrient agar medium according to Harring and MacCance (1976), yeast and molds were determined on Potatoes dextrose agar medium according to Barnett *et al.* (2000), lactic acid bacteria was counting on MRS medium according to De Man *et al*. (1960), coliform group were determined on MacConkey agar medium according to Holt and Krieg (1994) and halotlerant bacteria was determined on plate count agar medium (Difco Manual, 1984) contained diffident concentrations of NaCl 4%, 5%, 6%, 7%, 8% and 9%.

Isolation and identification of carotenogenic yeast strain: For isolation of yeast strain, Ten ml of whey were inoculated in 250 ml conical flask containing 100 ml of Potato Dextrose broth (potatoes extract 20%, dextrose 2%) (Barnett *et al.*,2000) and incubated at 25-30 ºC for 48 hr. Loop full of cultures which give good growth was streaked on Potato Dextrose agar (PDA) plates and incubated at 25-30 ºC for 72 hr, then well separated, yellow to red colonies were isolated. Cultures that exhibited the good coloration were subcultured and repeatedly streaked on PDA for purification. The purity of the isolated strains was verified by microscopic examination, the isolates were identified morphologically and biochemically (Barnett *et. al.*, 2000) and genetically by PCR and sequencing of Sigma (Boom *et al*., 1990 and Mokhtari *et al*., 2011).

Biochemical tests for identification: For fermentation test of carbohydrates, purple broth base (Difco, 1984) medium was recommended for carbohydrates fermentation necessary for the identification of pure cultures. About 1% (W/V) of the tested carbohydrates (such as; soluble starch, sucrose, Manitol, sorbitol, lactose, Fructose, Mannose) was also added to the medium with inverted Durham tubes (autoclaved at 121ºC for 15 min). Tubes were inoculated with the pure strain and incubated at 30 ºC for 18-48 hr. yellow color is indicated for acid production while bubbles in Durham tube indicated for gas production.

Also, grow of isolated strain was tested in Nutrient broth medium (APHA, 1971) contained 10 and 16% NaCl and 50 and 60% glucose.

Molecular identification

Yeast genomic DNA isolation: Yeast colonies were picked from complete medium (CM) selective plates after two days growth at 30°C. Alternatively, single colonies were picked from those plates and inoculated into 2 ml of rich medium Yeast Peptone Dextrose (YPD), and grown in a shaking water bath at 30°C for approximately 24 hours. Cells from 1.5 ml of the overnight cultures were pelleted in a micro-centrifuge tube and the cell pellets were resuspended in 200 µl of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)](Hoffman and Winston, 1987). The oligonucleotide concentration was determined spectrophotometericaly by O.D. reading at wave length 260 nm and 280 nm. The DNA purity was determined using both O.D. ratio; 260 / 280 nm (1.6-2) which indicates that the DNA solutions is well purified (Charles, 1970). Primer selection was performed according to the primer analysis software Oligo 4.1 (National Biosciences Inc., Plymouth, MN, USA).The oligonucleotides were synthesized on an

ABI392 DNA\RNA synthesizer (Brown and Grzybowski, 1995). The two oligonucleotide sequences used as primers in this study were: ITS1 5' TC. C.GTAGGTGAAC. C.TGCGG 3' (sense) and ITS4 5' TC. C.TC. C.GCTTATTGATATGC 3' (antisense) characterized as described by Gene JET™ PCR Purification Kit (Thermo) # K0701. These primers are capable of amplifying nearly DNA lenght (approximately 850-bp) fragments from many microbial genera.

PCR amplification for identification and phylogenetic analysis: Thermocycling amplification by PCR was made by using Maxima Hot Start PCR Master Mix (Thermo#K0221). PCR was optimized by using the following for 25µl master mix: 500ng of DNA as a template,2.5mM MgCl2, 0.3µM of each primer, 12.5µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) containing 25mM concentration of each deoxynucleoside triphosphate, and 1U of Taq polymerase per ml and complete the volume to 25µl by Water, nuclease-free. The PCR conditions consisted of initial denturation 95 °C for 10 min, then 35 cycles each consisted of denaturation 95°C for 30 sec., annealing 52°C for 1 min, extension 72°C for 1 min, and Final extension step 72°C for 15 min. The amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The amplicons were stored at 4ºC until electrophoresis was carried out. The molecular weights of the PCR products were determined by electrophoresis in 0.8% agrose gel and staining with eithidium bromide. Product sizes were determined by reference to 850pb molecular size ladder. The tree generated from PhyML program was displayed using iTOL/ interactive tree of life online display tool.

Toxicity assay of the isolated strain: After five days of incubation on 120 rpm at 30 °C in Potatoes Dextrose broth medium; the biomass was collected by centrifugation at 10000 rpm for 5 minutes. The supernatant was used as source of exotoxins.

The endotoxins of *Rhodotoruola* strain: with different concentration of biomass (250,500,1000,1500,2000 ppm dry weight) were extracted with 5% acetic acid using ultrasonic cell disrupter equipped with microtip probe of 400 Watt (ULTRASONIC Gex 750). Disrupted cells were examined microscopically to ensure complete rupture of cells. Disrupted cells were then centrifuged at 5000 rpm for 5 minutes and the supernatant was retained for toxicity test. To determine the dose response curve, male Albino Swiss mice weighting 20±2 g were obtained from animal Housing Division, National Cancer Institute, Cairo, Egypt. Potency was expressed as mice units (MU), where 1 MU is defined as amount of toxins required for killing 20 g mice in 15 minutes. The survival time was measured from the completion of the intraperitonial (i.p.) injection to the last breath (AOAC, 2000). To adjust the survival time around 5-8 minutes, a preliminary toxicity determination was performed using 3 mice for each sample preparation dose. The mice were previously blocked for weight. Yeast extract was intraperitonial (i.p.) injected into 20±2 g male mice to detect any toxic activity in the crude extract of the collected samples. One ml of toxins samples were injected into 20±2 g male mice to detect any toxic activity (Agrawal et al., 2012). Toxicity was observed and death times were recorded.

Production of carotenoid: production of carotenoid pigment was studied using yeast strain isolated from salted cheese whey and reference stain of *Rhodotoruola glutinis* which was obtained from fungal center of

Al-Azher University for comparison.

Preparation of inoculum: Pure cultures of the examined yeast from PDA slants (24 hr) were transferred into conical flasks containing 100 ml of P D Broth. Flasks were incubated at 30°C for 24 hr in water bath shaker (Lab line, shaker bath, USA) at 120 rpm (Bhosale and Gradre, 2001).

Treatment of salted cheese whey

Salted Whey was boiled for 20 min and cooled, clear filtrate was obtained by filtering it through muslin cloth. The whey obtained was sterilized by autoclaving at 121°C for 15 min (Gupte and Nair, 2010).

Experiment: Reference strain of *Rhodotorula glutenis* and the isolated strain were cultivated in 250 ml conical flasks containing 100 ml of cheese whey with 8% NaCl. Each flask was inoculated with 3 %, (v/v) and incubated at 30 ºC for 5 days in water bath shaker at 120 rpm. During incubation, samples were withdrawn every 24 hr, for determination of cell growth as dry cell biomass (g/l), volumetric carotenoid (mg / l) and cellular carotenoid (µg/g dry yeast). All shacked flask experiments were carried out in triplicates.

Optimization of growth parameters for production of carotenoid by the yeast strains

Determination of incubation period for production of carotenoid: *Rhodotorula glutenis* and the isolated strain were cultivated in 250 ml conical flasks containing 100 ml of cheese whey with 8% NaCl. Each flask was inoculated with 3 % (v/v) of inoculum and incubated at 30 ºC for 5 days on water bath shaker at 120 rpm. During incubation, samples were withdrawn every 24 hr for determination of cell growth as dry cell biomass (g/l), volumetric carotenoid (mg / l) and cellular carotenoid (µg/g dry yeast). All shake flask experiments were carried out in triplicate.

Effect of NaCl: To study the effect of NaCl on yeast growth and carotenoid production by the red isolated yeast and the reference strain, different concentration of NaCl (1%, 3%, 6%, 8%, 9% and10%) were separately added to the sterilized cheese whey which inoculated by strain cultures (24 hr) and incubated at 30ºC on a shaker water bath at 120 rpm for 5 days. At different time intervals (24, 48, 72, 96 and 120 hr), dry cell mass, and carotenoid concentrations were determined.

Incubation temperature: The growth medium (cheese whey contained the optimum NaCl concentration) (3%-6%) was inoculated and incubated at different temperatures (25, 30, 35 and 40 ºC) on a shaker water bath at 120 rpm for 5 days. the media were tested for dry cell mass and carotenoid concentration every 24 hr.

Effect of initial pH: The growth medium (obtained after studying the different NaCl concentration) (3%-6%) was adjusted at pH values (4, 5.2, 6.6, 7.2 and 7.8) ,then inoculated and incubated at optimum temperature (30ºC) on a shaker water bath at 120 rpm for 5 days. The media were tested for dry cell mass and carotenoid concentration during the incubation period.

Analytical methods

Cell yield (Biomass) determination: Cell dry weight was determined by first harvesting after centrifugation of growth medium (ca 10 ml) at 5000 rpm for 10 min and washed twice with distilled water. The cells (triplicate samples) were then dried at 105 ºC to a constant weight (Frengova *et al.*,

1994).

Determination of pH values: pH values of the samples were determined by using the method of Tansey, (1973) in 10 ml of growth medium with a pH meter (Jenway, 3510, UK). Three readings were taken per a sample.

Determination of carotenoid: To extract carotenoid, 10 ml of sample were taken from each flask at time intervals. Cells were harvested by centrifugation (4000 rpm for 10 min), the cell pellet was washed three times with distilled water resuspended in one ml dimethyl sulfoxide (DMSO) preheated to 55ºC and the mixture was vortexed for 10 sec. After centrifugation, the pigment DMSO solution was pipetted off and the DMSO extraction was repeated three times. The total carotenoid was determined according to (Sedmak *et al*., 1990 and Mokhtari *et al*., 2011) by measuring the optical density using T60u spectrophotometer PG instruments LTD Germany at 501nm. The total carotenoid content of the yeast cells was calculated and expressed as cellular Carotenoids (µg\g dry yeast) and as volumetric carotenids (mg / l) of culture. The extinction coefficient ($E_{1cm}^{1\%}$) 2040was used, according to the following equation:

$$\text{Volumetric carotenids (mg /l)} = \frac{A.\ V.\ 10^6}{E_{1cm}^{1\%}.100}$$

Where:

A: absorbance at 501 nm V: Total volume
$E_{1cm}^{1\%}$: extinction coefficient
Cellular Carotenoids (mg /g dry cells) = carotenoids/l
Dry cell weight g/l

All the above operations were performed under subdued light in order to avoid pigment degradation.

Statistical analysis: The layout of experiment was determined as complete randomized block design (CRBD) with three replicates of each treatment. All percentages were transformed to arcsine to be analyzed. Data were subjected to convenient statistical analysis methods for calculations of means using MSTATC software. Mean separations was estimated by calculating LSD values at alpha 5% according to Snedecor and Cochran (1980).

Results and Discussion

Chemical composition and microbiological load of the salted cheese whey samples were determined before using it as a culture medium for production of carotenoid.

Chemical composition of cheese whey sample: Chemical analysis results of cheese whey sample indicated that, whey sample contained total solid 11% mainly; lactose 4.6%, protein 0.7%, fat 0.11% and ash 0.09%. Minerals were 1.3 mg, 4.8 Na, 0.8 K, 0.3 Fe and 0.08Zn ppm. In this study salted cheese whey was used as a culture medium for production of carotenoid by yeast.

In this respect Marova *et. al.(2012)* found that, the highest yield of carotenoid were obtained by *R.glutinis* cells cultivated in whey medium, where addition of whey substrate into production medium led to 3-5 times increased production of beta-carotene without substantial changes in biomass. Also, Marova *et. al.* (2004) reported that, salt stress is the major component that influences pigment production in *Rhodotorula*. However, Aksu and Eren (2007) indicated that, production of carotenoid in molasses sucrose containing medium was faster and higher than of glucose and whey lactose media for *R.glutinis* in the same direction, El-Banna

et. al. (2012) noted that, the highest cellular and volumetric carotenoid produced by *R.glutinis* were obtained in yeast culture containing 0.4 ppm Zn.

Incidence of microbial groups in salted cheese whey: Obtained data of microbial analysis indicated that, the examined salted cheese whey contained the following microbial groups (cfu/ml); total viable bacterial count $4.0x10^6$, total mold and yeast $7x10^5$, lactic acid bacteria $9.2x10^6$, spore forming bacteria $4.7x10^5$, and total coliform bacteria $1.3x10^4$.

In concern to halotolerant bacteria, total viable bacterial counts were determined using plate count agar media contained different concentrations of salt, the results show that, viable bacterial count decreased from $3.6x10^6$, $3.2x10^6$, $2.6x10^6$, $2.4x10^6$ and $1.7x10^6$ cfu/ml as the salt concentration increased from 4,5,6 ,7, 8 and 9% NaCl respectively.

Morphological, cultural and biochemical characterization of pigmented yeast: The genus *Rhodotorula* includes three active species; *Rhodotorula glutinis*, *Rhodotorula minuta* and *Rhodotorula mucilaginosa* (formerly known as *Rhodotorula rubra*) (Hoog *et a*l., 2001). The isolated yeast colonies were fast growing, giving red smooth and mucoid colonies on PDA. Hyphae, pseudomycelium, ascospores and ballistospores are absent in microscopic examination. Cell shape is oval with asexual reproduction by bipolar budding. The isolated strain can produce acid from soluble starch, sucrose, Manitol, sorbitol, lactose, Fructose and Mannose; and can grow in liquid medium contained NaCl up to 10% but could not grow at 16% NaCl and 50% glucose. According to Barnett *et. al.* (2000), the isolated strain was predicated as *Rhodotorula mucilaginosa* spp., so identification was completed by genetic analysis.

Molecular identification

From the initial A 260 / 280 ratio obtained, along with the intensity and molecular weight of DNA bands seen on the agarose gel in Fig. (1) it can be observed that all techniques used produced good quality.

Where: M = DNA marker, S= PCR product of sample

DNA Sequencing and phylogenetic analysis:

The obtained PCR product was 850-bp long which was the same size as the region on *Rhodotorula mucilaginosa* 18S rRNA gene between the two primers. An alignment of the PCR product and the *Rhodotorula mucilaginosa* 18S rRNA gene sequence showed that the two sequence are identical. Fig. (2) Shows the sequence of such 850-bp PCR amplicon.

The 18 S rRNA analysis was used to identify the isolated yeast strain at the moleculer level. The amplification of PCR amplicon was obtained with the phenotypically identified *R. mucilaginosa* strain. Fig. (3)

Generally, it could be concluded that nucleotide sequences obtained from the positive amplification products revealed that the sample showed highly significant similarity with *R. mucilagenosa* (Y 17485) when compared to known sequences in the NCBI database. These provide strong support that the samples are straining of *R. mucilagenosa* (Y 17485). Although the sequences share high homology with known strains, Fig. (2) Shows that this homology is not consistent across the seven sequences

obtained (Berry and Gascuel, 1997); (Creevey et al., 2004).

Toxicity confirmation of *R. mucilagenosa* strain (Y 17485) using mice bioassay

The mice bioassay, using intraperitoneal injuction of *R. mucilagenosa* strain (Y 17485) extracts showed that no toxicity found with *R. mucilagenosa* strain (Y 17485) concentrations.

Optimization of growth parameters for production of carotenoid by the yeast strains

Yield of carotenoid is directly related to the total biomass yield, to keep both high growth rates and high flow carbon efficiency to carotenoid be optimal cultivation conditions is essential in order to achieve the maximal pigment productivity (Marova et. al., 2012). Therefore, optimum conditions for growth and carotenoids production have to be well studied.

Effect of incubation period

Comparing growth of strains led to the conclusions that the growth and total carotenoids of tested strains were significantly increased after 120 hr and significantly decreased at 144 hr Table (1). Volumetric Carotenoids was 5.661 mg/l with *R. glutinis* and 9.26 mg/l with *R. mucilagenosa* (Y 17485) after 5 days at 30 °C in 8% NaCl salted cheese whey. Similarly, a previous study reported that, carotenoids production by *R. glutinis* started after the end of logarithmic growth phase then increased during the stationary phase to reach its maximum at the fifth day (El-Banna et. al., 2012). On the other hand, maximum production of carotenoid by *R. glutinis* was obtained after 80 hr (Marova et. al., 2012) and after 240 hr (Aksu and Eren ,2007).

Effect of NaCl concentration

There was a significant increase in both growth and total carotenoid for *R. glutinies* as NaCl concentration increased up to 3%. Also *R. mocilagenosa* (Y 17485) growth and total carotenoid significantly increased as NaCl concentration increased up to 10% and 6% respectively. The effect of NaCl on carotenoid production by yeast strains was studied in salted cheese whey (1–10%NaCl) after 120 hr incubation time at 30 °C. The obtained result Table (2) show that. The highest production of total carotenoid for *R. glutinis* was in 3% NaCl (V. C. 6.5 mg/l with C. C. 469 µg\g dry yeast) and for *R. mucilagenosa* (Y 17485) was in 6% NaCl (V. C. 5.04 mg/l with C. C. 758 µg\g dry yeast). But special concern should be given to the produced amount of carotenoid in the medium contained 10% NaCl cheese whey which indicate that high concentration of salt produces reasonable high total carotenoid, where *R. glutinis* produced V. C. 2.9 mg/l with C. C. 369 µg\g dry yeast and *R. mucilagenosa* **(**Y 17485) produced V. C. 3.08 mg/l with C. C. 342 µg\g dry yeast. Finding of *Marova et. al*.(2004) revealed that, cellular carotenoid increased 2.5-fold with *R. glutinis* and 6-fold with *R. mucilagenosa* (Y 17485) in a culture medium containing 10% NaCl, and emphasized that, salt stress is the major component that influences pigment production in these strains. In an effort to explain the reason for this observation, carotenoid act as membrane-protective antioxidants that efficiently scavenge free radicals, consequently, carotenoid biosynthesis involved in stress response mechanisms so, non-phototrophic microorganisms rely on carotenoid for protection when growing in light and air (Britton et. al.,1998). During stress different

classes of substances are overproduced; microbial cells exposed to mild stress can develop tolerance not only to higher doses of the same stress, but also to stress caused by other agents as a cross-protection phenomenon (Sigler *et. al.,* 1999). Therefore, salt stress influence carotenoid production in yeasts.

Effect of temperature:

Incubation temperature is an important impact affecting both growth and carotenoid production of yeast strains. Data presented in Table (3) show that, the growth and total carotenoid for *R. glutinis* significantly increased on the raising of temperature up to 30 ºC and significantly decreased above 30 °C probably due to the denaturation of the microbial enzyme system at higher temperature. On the other hand the growth of *R. mucilagenosa* (Y 17485) was non-significantly decreased and the total carotenoid was significantly increased with raising the temperature up to 30 ºC. It could be noticed that, after 120 hr incubation of yeast strains in salted cheese whey culture medium at 30ºC; the highest growth of yeast was 13.95 g\l for *R. glutinis* with V. C. 6.54 mg/l. On the other hand, although *R. mucilagenosa* (Y 17485) gave low growth (6.65 g/l) dry cell weight at 30 °C, high V. C. (5.04 mg/l) with the highest C. C. 758 µg\g dry yeast were obtained. A similar trend was observed by, Aksu and Eren (2005 and 2007) who reported that, the optimum temperature for total carotenoid production was at 30 ºC for *R. glutinis* and *R. mucilagenosa* **.** In contrast, El-Banna *et al.* (2012) found that, the optimal temperature for production of cellular carotenoid was 15 °C by *R. glutinis*, however, the optimal temperature for cell growth (dry biomass) and consequently production of volumetric carotenoid was 25 °C.

Effect of initial pH

Table (4) shows the growth and total carotenoid production by both yeast strains after 120 hr at 30 °C at various initial pH values ranging from 4 to 7.8. Results indicated that, with raising the pH, carotenoid production significantly increased and reached the maximum level at pH 6.6. Further increase in pH over 6.6 resulted insignificantly reduction of both growth and total carotenoid. Where, V. C. were 6.5 mg/l with C. C. 469 µg\g dry yeast for *R. glutinis* and V. C. 5.04mg/l with C. C. 758 µg\g dry yeast for *R. mucilagenosa* **(**Y 17485).

The obtained results are in consistence with previous studies Aksu and Eren (2005 and 2007) which noted that, optimum pH for total carotenoid production was 7.0 for *R. mucilagenosa* (Y 17485) and 6.0 for *R. glutinis* .

Generally, formation of carotenoid in microorganisms is dependent on the strain, the culture medium and condition cultivation (Sandmann et. al., 1999). Although, Marova *et. al.* (2012) found that, yeast strain *R. mucilagenosa* (Y 17485) exhibited in most cases similar biomass production characteristics as *R. glutinis* , while pigment production was substantially lower. However, results (table1-4) of the current study indicated that, cellular carotenoid production (ratio of carotenoids (µg)\ dry cell yeast (g)). For *R. mucilagenosa* **(**Y 17485) was manly higher than production by *R. glutinis* . In agreement with Aksu and Eren(2005) who emphasized that, *R. mucilagenosa* (Y 17485) will be one of the most promising yeast strain for the commercial production of Carotenoid from agricultural wastes as a cheap carbon sources. Results of the present study are in consistence with *Marova et. al. (2012)* who

reported that, addition of whey substrate into production medium led to 3-5 times increased production of beta-carotene by *R. glutinis* . The obtained results also, indicate that optimum conditions for biomass and carotenoid production were incubation at 30°C for 120 hr and pH6.6.

In conclusion, efficient productivity of carotenoid by yeast depends on optimization of condition of both growth rate and cellular carotenoid production. The highest biomass and carotenoid production by both strains were obtained after 120 hr at30 °C with initial pH 6.6, but NaCl concentration was 3% for *R. glutinis* and 6% for *R. mucilagenosa* (Y 17485). In fact carotenoid biosynthesis in yeast is involved in stress response mechanism, hence salt stress is considered a major component influences pigment production in Rhodotorula. The data obtained suggested *R. mucilagenosa* (Y 17485) as a promising microorganism for commercial production of carotenoid from whey cheese.

Table.1 Effect of incubation period on the production of biomass and total carotenoid

Incubation period (hr.)	*R. glutinis*			*R. mucilagenosa*		
	Dry cell Weight (g\l)	total carotenoids		Dry cell Weight (g\l)	total carotenoids	
		V. C. (mg\l)	C. C. µg\g dry yeast		V. C. (mg\l)	C. C. µg\g dry yeast
24	5.42	1.421	262	6.16	2.254	366
48	5.61	2.058	367	8.03	2.352	292
72	10.79	4.191	388	7.76	2.867	369
96	11.56	5.073	439	8.1	3.014	372
120	12.93	5.661	438	9.26	3.602	388
144	10.10	4.362	431	8.32	3.931	378

Initial pH 6.6 NaCl concentration 8% incubation temperature 30 ºC inoculum size 3%
shaking speed 120 rpm Cellular Carotenoids (c. c.) Volumetric Carotenoids (v. c.)
LSD (hr x Dry cell Weight$_{0.005)}$ = 0.1956 LSD (hr x V. C. (mg/l) $_{0.005}$) = 0.0767
LSD (hr x C. C. $_{(}$µg\g dry yeast) $_{0.005}$) = 12.61

Table.2 Effect of NaCl concentration on the production of biomass and total carotenoid

NaCl concentration	*R. glutinis*			*R. mucilagenosa*		
	Dry cell Weight (g\l)	total carotenoids		Dry cell Weight (g\l)	total carotenoids	
		V. C. (mg\l)	C. C. µg\g dry yeast		V. C. (mg\l)	C. C. µg\g dry yeast
1%	2.87	1.47	512	5.35	1.47	274
3%	13.95	6.54	469	7.03	2.352	334
6%	4.33	1.47	339	6.65	5.044	758
8%	5.42	1.421	262	6.16	2.254	366
9%	8.39	1.83	219	7.73	2.867	370
10%	7.96	2.94	369	9.02	3.088	342

Initial pH 6.6 incubation period 120 hr. Incubation temperature 30 ºC
inoculum size 3% shaking speed 120 rpm Cellular Carotenoids (c. c.)
Volumetric Carotenoids (v. c.) LSD (NaCl% x Dry cell Weight (g/l) $_{0.005}$) = 0.5667
LSD (NaCl% x V. C. (mg/l) $_{0.005}$) = 1.166 LSD (NaCl% x C. C. (µg\g dry yeast) $_{0.005}$) = 5.873

Table.3 Effect of incubation temperature on the production of biomass and total carotenoid

	**R. glutinis*		***R. mucilagenosa*	
	Dry cell	total carotenoids	Dry cell	total carotenoids

Temperature (°C)	Weight (g\l)	V. C. (mg\l)	C. C. µg\g dry yeast	Weight (g\l)	V. C. (mg\l)	C. C. µg\g dry yeast
25	8.87	2.37	267	7.17	2.13	297
30	13.95	6.54	469	6.65	5.04	758
35	8.08	2.49	308	8.53	3.51	411
40	10.01	2.12	211	5.45	1.37	251

Initial pH 6.6 incubation period 120 hr. * NaCl concentration 3% ** NaCl concentration 6% inoculum size 3% shaking speed 120 rpm Cellular Carotenoids (c. c.) Volumetric Carotenoids (v. c.)
LSD (Temp. x Dry cell Weight (g/l)$_{0.005}$) = 1.216 LSD Temp. x V. C. (mg/l) $_{0.005}$) = 1.676
LSD (Temp. x C. C. (µg\g dry yeast) $_{0.005}$) = 9.240

Table.4 Effect of initial pH on the production of biomass and total carotenoid

pH	**R. glutinis*			***R. mucilagenosa*		
	Dry cell Weight (g\l)	Total carotenoids		Dry cell Weight (g\l)	Total carotenoids	
		V. C. (mg\l)	C. C. µg\g dry yeast		V. C. (mg\l)	C. C. µg\g dry yeast
4	8.02	1.23	145.5	8.63	0.2145	24.5
5.4	9.82	2.45	249.5	8.61	2.087	242
6.6	13.95	6.54	469	6.65	5.044	758
7.2	9.06	3.42	378	8.58	2.518	293
7.8	8.16	3.87	474.5	5.31	1.266	237.5

Incubation period 120 hr. * NaCl concentration 3% ** NaCl concentration 6% inoculum size 3% shaking speed 120 rpm Incubation temperature 30ºC Cellular Carotenoids (c. c.) Volumetric Carotenoids (v. c.) LSD (pH x Dry cell Weight (g/l) $_{0.005}$) = 0.006 LSD (pH x V. C. (mg/l) $_{0.005}$) = 0.005
LSD (pH x C. C. (µg\g dry yeast) $_{0.005}$) = 113.914

Fig.1

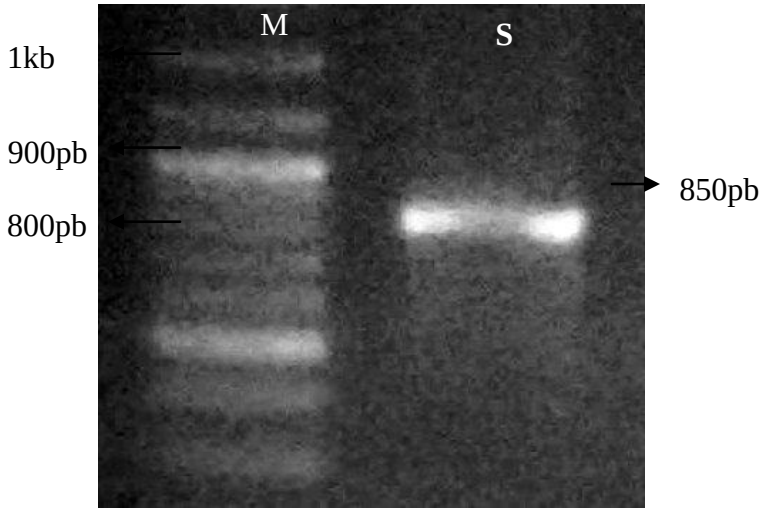
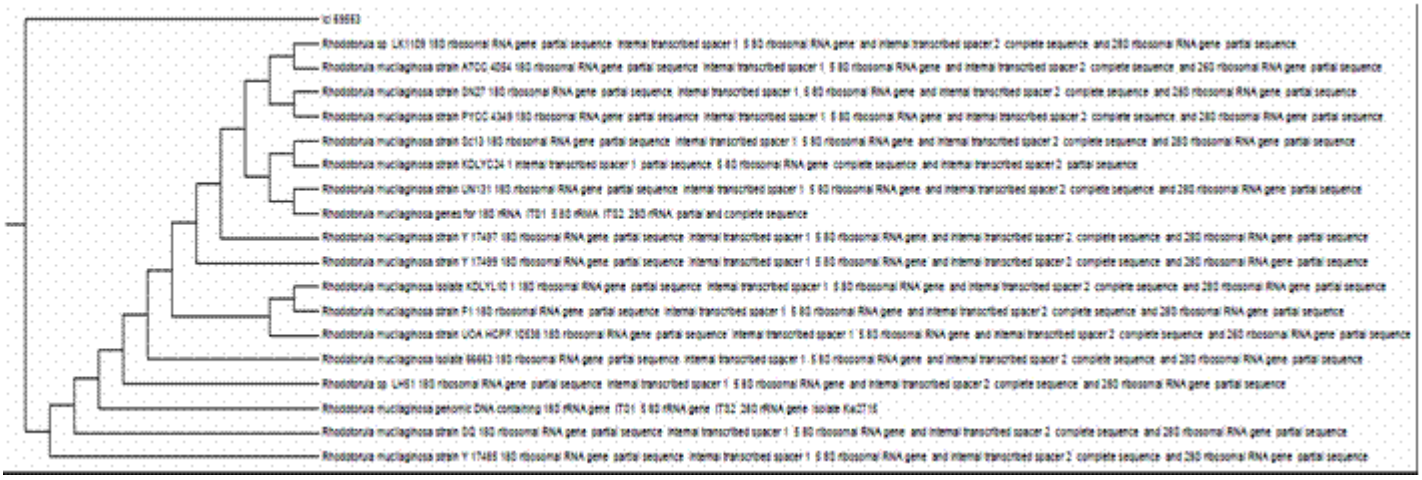


Fig.1 Agarose gel electrophoresis of PCR product 18S rRNA analysis of *Rhodotorula*

Fig.2 Sequence of 18S rRNA from *Rhodotorula*

NNNNNNNNNNNNNCCTGNTTTGAGATCTAATCTTANATGTAGACATTCTGATTAGAAGCTTCCTTTAACCCAACCCGGCTCTAGTCCGAAGACT
AGAATTCCTCAGCGAATAGTCTATTACGCCAAGTCAATCCGAAGTTCGATTGCGGATGCTAATGCATTACGAACGAGCTAGACCGTAAAGGCC
AGCAGCGCTCAGAAACCAAACACCTCTTCAATCATTAAGAAAGAGGAGGGTTGAAGTATTCATGACACTCAAACAGGCATGCTCCACGGAATA
CCATGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCG
AGAGCCAAGAGATCCGTTGTTGAAAGTTTTATTTTGTTATAAAATTTAATACATTCATAGACTTTGTGTTTATAAGTGAATAGGAGTTCGCTCTC
TTGCGAGAGTTACTATCCCAAACAAGTGCACAGGGTTAGAAAGTGAGAGTTCGGACTCCAAGTTAAGTTGGACGTCCTATATTCACTAATGATC
CTTCCGCAGGTTCACCNNNANNNAAGNGATCATTNGTGAATATAGGACGTCNANCTTNNCTTGGNGTCNGAACTCTCACTTTCTAACCCTGTGC
ACTTGTTTGGGATAGTAACTCTCGCANGAGAGCGNACTCNNATTCTCTTATCAACACGNCNGTNNATGTATGTANNTTTTTTTNACAAAANAAN
ACTTNNNNCAACGNACGCTNGNNNNCTGNNTCGATNAANNACGNNCGAANTGCGTGAAGTNNTNNGNAATTGNNANANNCGGNGAANNCNG
NATCNTTNNANGNCCTNGGNNNCNGNNNTNCGNNNANNAGGNNNNTNNNAGGNNGANNANNNNNNNNCNNNCCNNNCTNTANNNNNNNAA
NNNNNNTNNNNNCGNANCGCNGNNNNNCNTNNNNNNNNNNNNNNNNANNNANNNNNNCNNNNCNNNNGNNNNNNNGGGNNANNNNNNNNN
NNNNNNTCNNNNNNNAANNNNNGNNNNNNNNNNNNNNNNCNNTNNANNNNNNNNNNNNANNNCNNANTNANNNNNNGNNNAAN

Fig.3 phylogenetic dendogram constructed from 18S rRNA sequence of *Rhodotorula* isolate and their related strains in GenBank



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