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Agro-industrial Orange Waste as a Low Cost Substrate for Carotenoids Production by *Rhodotorula mucilagenosa*

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Abstract

Carotenoids have many important biological activities, which can be used as food colorants and as a natural antioxidant pigments. A local yeast strain was isolated from karish cheese and identified as *R. mucilagenosa.* The efficiency of carotenoids production by *R. mucilagenosa* was tested by using orange waste extract (OWE) as an agro-industrial waste to minimize carotenoids production cost. To optimize the fermentation conditions, OWE medium was supplemented with different carbon and nitrogen sources at concentration 1% and 1-6% NaCl. The highest carotenoids production and cell dry weight (7.2 mg/l and 13.75 g/l respectively) were obtained with OWE medium supplemented with 1% glucose, 1% yeast extract and 1% NaCl, at optimal pH 6, temperature 25°C and shaking at 150 rpm for five days.

Keywords: *Carotenoids, Rhodotorula and Orange waste.*

Introduction

Carotenoids are yellow to orange-red pigments, which widely distributed in nature. These pigments comprise around 700 different chemical structures. They can be found in algae, filamentous fungi, yeasts, bacteria and plants. However, animals are not able to synthesize carotenoids (Stafsnes *et al*., 2010).

Carotenoids have an important biological activity due to their provitamin A activity, antioxidant action by neutralizing free radicals as electron donor and anti-carcinogenic properties (Young and Lowe 2001).

There is increasing interest in naturally obtained carotenoids by biotechnological processes because of their growing demand in pharmaceutical, cosmetic, food, and feed industries, (Aksu and Eren 2005).

Microbial activities offer a promising method for carotenoids production (Johnson and Schroeder, 1995). Yeasts are better than algae or fungi for large scale production of carotenoids in fermenters, because of their unicellular nature and high growth rate (Baraka *et al*., 2014). Various genera of red yeasts, such as *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces* and *Xanthophyllomyces* can produce and accumulate carotenoids in their cells (Hennekens 1997).

Among different yeasts, *Rhodotorula* sp. gave a favorable high yield producer of carotenoids (Frengova and Beshkova 2009). *Rhodotorula* has the ability to grow in various cheap agricultural raw materials such as sugar cane juice, peat extract, whey, grape must, beet molasses, and sugar cane molasses, so it is potentially useful for carotenoid production industry (Aksu and Eren 2005; Bhosale and Gadre 2001a; Buzzini and Martini 1999; Park *et al*., 2005 and Simova *et al*., 2004).

Citrus fruit is one of the commercial crops in the Egyptian mar-

kets. About 50-60% of citrus fruit is transformed into waste by citrus processing industry (Wilkins *et al*., 2007). This results in accumulation of large quantities of citrus waste. This waste represents a major challenge in the citrus processing industries in Egypt and worldwide.

In this study, orange waste extract (OWE) was chosen as affordable and suitable substrate for carotenoids production by *Rhodotorula* with a view to reduce production costs and make the product economical.

Materials and Methods

Preparation of orange waste extract medium

Orange waste was collected from fruit juice shops at Mansoura City, Egypt. Orange waste extract (OWE) was prepared by adding two liters of distilled water to one kilogram of orange waste, boiled at 100 °C for 30 minutes, and then filtered to obtain orange waste extract. The OWE was used as fermentation medium to produce carotenoids using isolated yeast.

Microorganisms

An orange-pigmented yeast was isolated from yogurt and karish cheese samples. The yeast isolate was purified on GPY agar medium (yeast extract 10 g, peptone 20 g, glucose 20 g, and one liter of distilled water). The purity of the obtained isolate was verified microscopically. Isolate was subcultured and maintained on GPY agar medium at 4°C. The isolate was identified by Sigma Scientific Services Co., using 18s rRNA gene (Kanzy *et al*., (2015).

Inoculum preparation

Five ml of sterilized distilled water were added into 48 h. yeast slant grown on GPY agar medium. The growth was scratched and homogenized well, then transferred into in 250 ml Erlenmeyer flask contain 50 ml of GPY broth medium. Flask was incubated for 24 h. at 25°C with shaking at 150 rpm. inoculum contains 10^6 CFU/ml.

Production of carotenoids on OWE medium

Fifty ml of OWE medium was inoculated with one ml of the inoculum and incubated for 5 days at 25°C with shaking at 150 rpm. Yeasts cultures were centrifuged at 10000 for 20 min. Yeast cells were collected for carotenoids determination.

Optimization of carotenoids production

Several factors including incubation period, carbon sources, nitrogen sources, salinity, inoculum size, initial pH value and temperature were optimized for carotenoids production using OWE liquid medium. Final pH values, cell dry weight (CDW) (g/l) and carotenoids production (mg / l) were determined.

Effect of incubation period

The selected isolate strain was cultivated in 250 ml Erlenmeyer flask containing 50 ml autoclaved OWE medium, the initial pH was 4.7, Each flask was inoculated with 1ml of inoculum and incubated at 25°C, with shaking speed of 150 rpm. During incubation periods, samples were taken every 24 h. for 10 successive days.

Effect of additive carbon source

To determine the best additive carbon source for carotenoids production, OWE medium were supplemented with 1% of different carbon sources; glucose, fructose, sucrose, maltose, mannitol, glycerol and

starch. Flasks were inoculated with one ml of inoculum and incubated at 25°C with shaking at 150 rpm for 5 and 6 days.

Effect of different nitrogen Sources

OWE medium, contained the best carbon source were supplemented with 1% of different nitrogen sources; yeast extract, beef extract, peptone, malt extract, ammonium phosphate, calcium nitrate and ammonium sulphate. Flasks were inoculated with one ml of inoculum and incubated at 25°C with shaking at 150 rpm for 5 and 6 days.

Effect of Initial pH

To estimate the effect of pH value on carotenoids production by the yeast strain, OWE medium (contained best carbon and nitrogen source) was adjusted at pH values; 3, 4, 5, 6, 7 and 8. Flasks were inoculated with one ml of inoculum and incubated at 25°C with shaking at 150 rpm for 5 days.

Effect of temperature

OWE medium amended with best carbon and nitrogen source and adjusted at optimum pH was inoculated with one ml of inoculum and incubated at different temperatures;22, 25, 28 and 30□ with shaking at 150 rpm for 5 days.

Effect of inoculum size

Different inoculum sizes (2-10 %) were tested to estimate the effect of inoculum size on carotenoids production. OWE medium amended with best carbon and nitrogen source and adjusted at optimum pH were inoculated. Flasks were incubated at optimum temperature with shaking at 250 rpm for 5 days.

Effect of salinity

The effect of adding different concentrations of NaCl (1-6%) on yeast growth and carotenoids production was studied. Flaks were incubated under the optimum conditions for 5 days.

Determination of total carotenoids

According to the modified method of Park *et al.* (2005), 50 ml of fermented broth culture was centrifuged at 5000 rpm for 15 min. The precipitate cells were washed twice by distilled water. After cells separation by centrifugation, 10 ml of 0.1N HCl were added, boiled in water bath for 15 min., and then cooled in ice water for 10 minutes. For extraction of the pigments, the cells suspended in acetone and stirred vigorously until cells turn colorless, then equal amount of diethyl ether were added. This mixture was transferred in separating funnel. Cold NaCl solution (15%) were added as a part of purification steps of the carotenoid pigment. To determine the total carotenoids, the diethyl ether layer was taken and assayed spectrophotometrically at 455 nm. (JENWAY 6305 UV/vis. Spectrophotometer). The standard curve was blotted using β-carotene (MP Biomedicals, LLC) as a standard substrate. Amounts of carotenoids were calculated using standard curve.

Determination of cells dry weight

50 ml of yeast culture were centrifuged for 20 min. at 10000 rpm, washed twice with distilled water and then dried at 80ºC till constant weight.

Results and Discussion

Isolation and Identification of *Rhodotorula mucilaginosa*

An orange-pigmented yeast isolate was isolated from Karish cheese. The yeast isolate gave an orange-red color on GPY agar medium. Pure culture of the isolated yeast was examined microscopically. Isolated yeast was oval, non-spore forming and non-pseudo-true- mycelium former.

The potential *R. mucilaginosa* isolate was further identified by PCR targeting the 18S rRNA gene, which confirmed its belonging to *R. mucilaginosa*, the size of PCR amplicon is 567-bp (Fig 1). The nucleotide sequence of a 567 bp internal fragment of the 18S rRNA locus was investigated in *R. mucilaginosa* isolate. NCBI blast is applied to compare the DNA sequence of 18S rRNA of isolate to other reference strains. The results revealed the sequences of PCR amplicon is highly significant similarity with *R. mucilagenosa* L4 (Fig 2). These results are consistent with Kanzy *et al*., (2015) who carried out the isolation and molecular identification of *R. mucilaginosa* strain isolated from salted cheese whey.

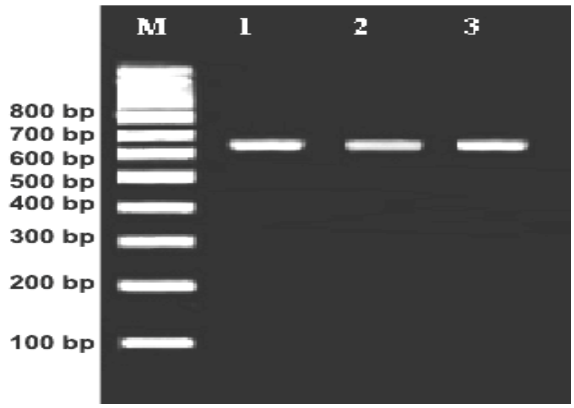

Fig. 1. Agarose gel electrophoresis (2%) of PCR amplicon amplified with ITS primer pair. Lane M: 1kb plus DNA ladder; Lane 1, 2 and 3 PCR products 18 S rRNA *R. mucilagenosa*.

Score	Expect	Identities	Gaps	Strand
1040 bits(563)	0.0	565/567(99%)	0/567(0%)	Plus/Minus

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Query  1    CCTGNTTTGAGATCTAATCTTANATGTAGACATTCTGATTAGAAGCTTCCTTTAACCCAA  60
            |||| ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  573  CCTGATTTGAGATCTAATCTTAAATGTAGACATTCTGATTAGAAGCTTCCTTTAACCCAA  514

Query  61   CCCGGCTCTAGTCCGAAGACTAGAATTCCTCAGCGAATAGTCTATTACGCCAAGTCAATC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  513  CCCGGCTCTAGTCCGAAGACTAGAATTCCTCAGCGAATAGTCTATTACGCCAAGTCAATC  454

Query  121  CGAAGTTCGATTGCGGATGCTAATGCATTACGAACGAGCTAGACCGTAAAGGCCAGCAGC  180
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            |||||||||||||||||||||||||||
Sbjct  33   CACTAATGATCCTTCCGCAGGTTCACC  7
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Fig 2: The degree of similarity between DNA sequences of 18rRNA gene of *R. mucilagenosa* to *R. mucilagenosa* L4 (reference strain), via NCBI blast.

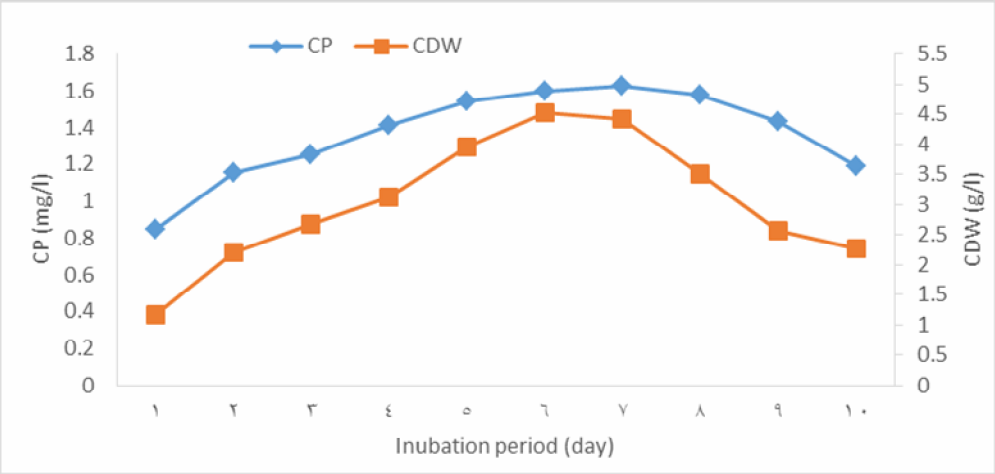
Optimization of carotenoids production

The biosynthesis of carotenoid is affected by many factors that can achieve the maximal carotenoid productivity and minimal operation cost. Therefore, the optimum conditions for growth and production of carotenoids have to be studied.

Effect of incubation period

Data presented in Fig 3. show the changes in cells dry weight (CDW) (g/l) and carotenoids production (mg/l) by *R. mucilagenosa* with time. During the first three days of incubation, *R. mucilagenosa* show exponential growth and decelerating growth until the sixth day then remained stationary, results also show that the production of carotenoids was growth correlated and directly related to the biomass yield. The maximum carotenoids production (1.63 mg/l) was achieved within seven days of incubation using OWE broth medium as a sole substrate while the maximum CDW was obtained after six days. Our results are in agreement with Bhosale and Gadre (2001b) who reported that, the carotenoids production in the yeast was growth associated. Also El Bana *et al*., (2012) stated that, the maximum yield of carotenoids achieved by *R. glutinis* at the fifth day. Korumilli and susmita (2014) who mentioned that maximum carotenoids production by *Rhodotorula* sp. was achieved at the end of logarithmic growth phase.

On the other hand, results are in contrast to those of Kanzy *et al*., (2015) who reported that, by using *R. glutinis*, the production of carotenoid started after the end of logarithmic growth phase then increased during stationary phase. Also (Marova *et. al*., 2012) reported that, maximum yield of carotenoids collected after 80 h. from *R. glutinis*.



CP: Carotenoids production; CDW: Cells dry weight

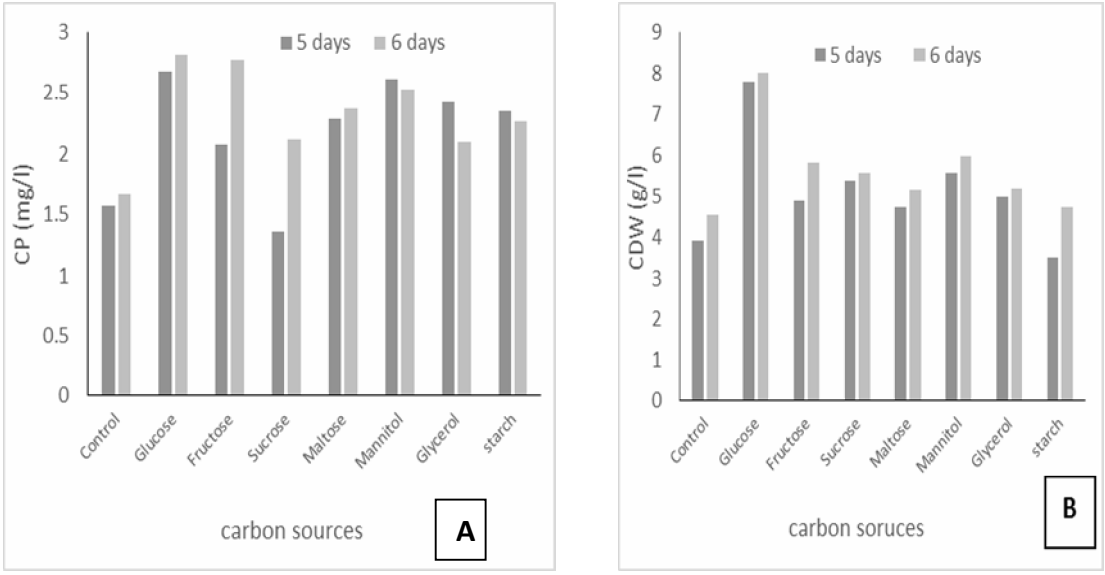
Fig. 3: Effect of incubation period on growth (g/l) and carotenoids production (mg/l) by *R. mucilagenosa*

Effect of different carbon sources

Data in Fig. 4 illustrate the effect of different carbon sources on cells dry weight (CDW) and carotenoids production by *R. mucilagenosa* after five and six days of incubation

at 25°C. Data illustrate that maximum carotenoids production (2.7 mg/l) and CDW (8.08 g/l) was observed with addition of 1% glucose to OWE medium after five days of incubation at 25 °C, followed by mannitol. While, on the sixth day the best carbon source for carotenoids production was glucose (2.8 mg/l) followed by fructose and (2.7 mg/l), res, compared with 1.6 mg/l carotenoids and 3.5 g/l CDW which were obtained by control (OWE without carbon sources).

The results exhibit the correlation between the increasing the yeast biomass and carottenoids productivity. Therefore, glucose at 1% concentration was selected as the best carbon source for further studies. These results were in line with Latha *et al*., (2005), they found that the highest pigmentation and growth by *R.glutinis* was achieved with glucose and fructose as carbon sources. Also, Baraka *et al*., (2014) stated that the best carbon sources for growth and carotenoids production by *R. glutinis* were glucose and sucrose. On the other hand, lactose was selected for *Serratia marcescens* to give the highest production of carotenoids (Wang *et a*l., 2012).



CP: Carotenoids production; CDW: Cells dry weight

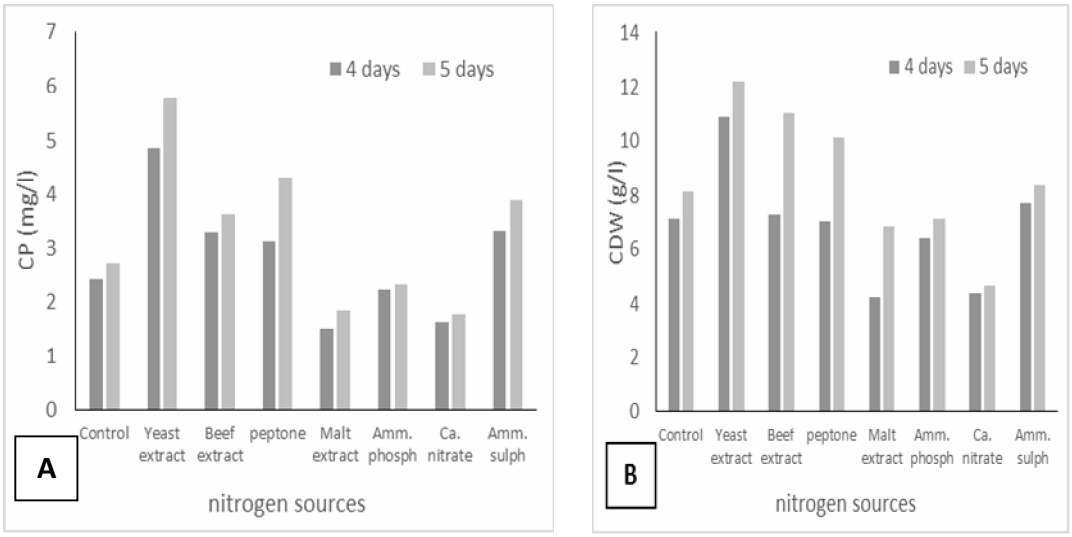
Fig. 4. Effect of carbon sources on growth (g/l) (B) and carotenoids production (mg/l) (A) by *R.mucilagenosa*

Effect of different nitrogen sources

The influence of adding different nitrogen sources to the OWE medium amended with glucose on CDW and carotenoids production was investigated after four and five days of incubation at 25°C. Data presented in Fig 5. revealed that the most tested nitrogen sources stimulated the growth and the carotenoids production by *R.mucilagenosa* compared with control (OWE amended with glucose without nitrogen sources). Also, the results show that yeast extract at 1% concentration was the best nitrogen source for carotenoids pro-

duction (5.7 mg/l) and CDW (12.1 g/l), followed by peptone (4.3 mg/l), ammonium sulphate (3.8 mg/l) and beef extract (3.6 mg/l) after five days of incubation. From the above results, it could be concluded that yeast extract is the best nitrogen source and so it is used in subsequent studies.

The results are in agreement with those obtained by Baraka *et al*., (2014) indicated that, yeast extract at a concentration of 0.75% was the best nitrogen source for carotenoid production and growth of *R. glutinis*. While Latha *et al.,* (2005) demonstrated that, maximum carotenoids and growth of *R. glutinis* was observed with sodium nitrate as nitrogen source.



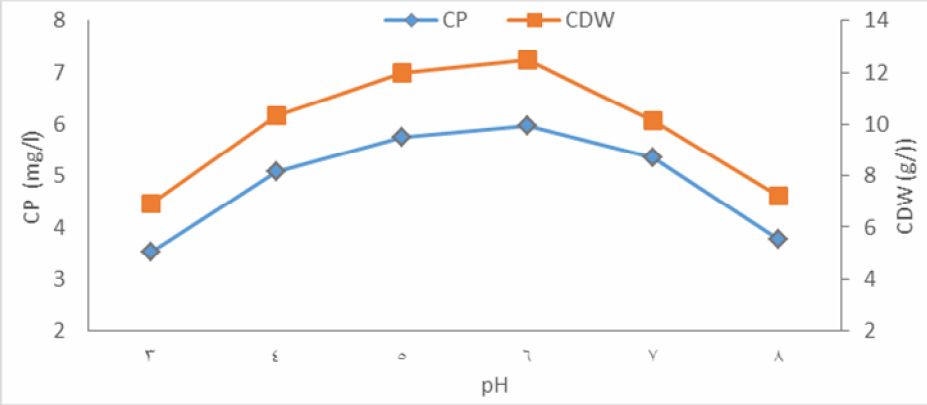
CP: Carotenoids production; CDW: Cells dry weight

Fig. 5. Effect of nitrogen sources on growth (g/l) (B) and carotenoids production (mg/l) (A) by *R. mucilagenosa*

Effect of initial pH

Fig. 6 shows the effect of initial pH values ranging from 3 to 8 on CDW and carotenoids production by *R. mucilagenosa* after 5 days of incubation at 25□. The results show that *R. mucilagenosa* had ability to grow and produce carotenoids under a wide range of pH. The highest carotenoids production (5.98 mg/l) and CDW (12.5 g/l) were obtained at initial pH 6. Therefore, pH 6 could be considered as the optimum pH for growth and carotenoids production by *R. mucilagenosa*.

Similar results were obtained by Aksu and Eren (2007) and repeated that, *R. glutinis* produced maximum yield of carotenoids at pH 6. Also Kanzy *et al*., (2015) concluded that, the maximum level of carotenoids production achieved at pH 6.6 for both *R. glutinis* and *R. mucilagenosa*.



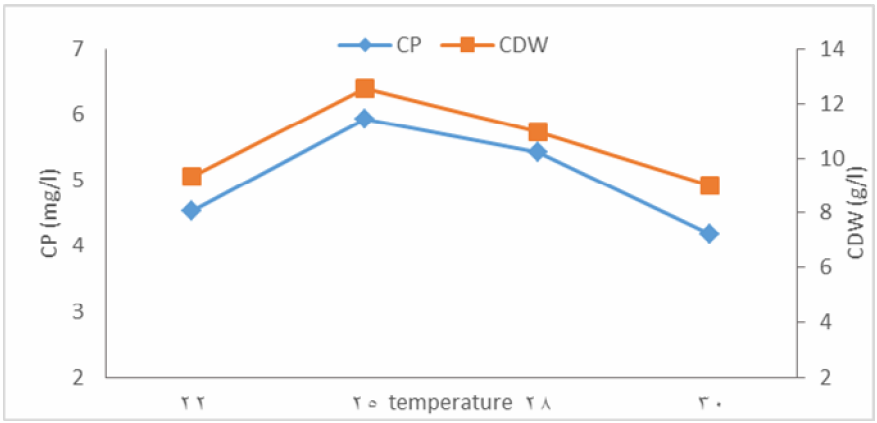
CP: Carotenoids production; CDW: Cells dry weight

Fig. 6. Effect of initial pH on growth (g/l) and carotenoids production (mg/l) by *R. mucilagenosa*

Effect of temperature

Temperature is another parameter, which affects the cell growing, and metabolites; it affects the biosynthetic pathways, including carotenogensis. Data presented in Fig. 7 show that *R. mucilagenosa* achieved highest carotenoids production (5.98 mg/l) and CDW (12.56 g/l) at 25°C after five days. Also the results show that carotenoids production and CDW decreased by decreasing temperature to 22 °C or increasing temperature to 28ºC.

Similar results were observed by El-Banna *et al*., (2012) who stated that, the optimal temperature for carotenoids production and growth by *R. glutinis* was 25°C. Also, Martin *et al.,* (1993) Reported that, the optimum temperature was 22°C for *R. rubra*. On the other hand, Latha *et al*., (2005) Recorded that, the maximum carotenoid yield by *R. glutinis* DFR-PDY was obtained at the ambient temperature 29-32°C. Also, (Aksu and Eren 2005) reported that, 30°C was the optimum for *R. mucilagenosa*. It could be noticed that the effect of temperature on biosynthesis of carotenoid depends on the species specificity and strain characteristics.

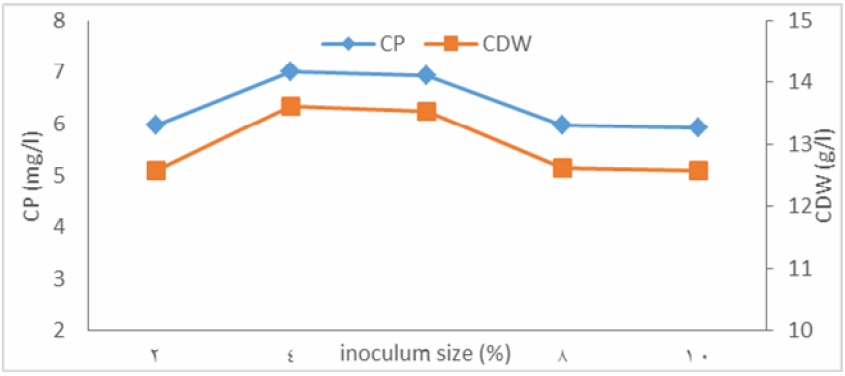


CP: Carotenoids production; CDW: Cells dry weight

Fig. 7. Effect of temperature on growth (g/l) and carotenoids production (mg/l) by *R. mucilagenosa*

Effect of inoculum size

Fig 8. shows CDW and carotenoids production by *R.mucilagenosa* after five days of incubation with different inoculum sizes (2, 4, 6, 8 and 10% (v/v)). The results indicate that yeast growth and carotenoids production increased by increasing the inoculum size to 4%, it is due to the direct relation between carotenoids production and yeast growth. With 4% (v/v) of inculum size, *R. mucilagenosa* reached the maximum carotenoids production (6.997 mg/l) and CDW (13.61 g/l). By increasing the inoculum size to 6%, a slight decrease occurred in yeast growth and carotenoids production. Data also show that at higher ratios of inoculum sizes (8 and 10%), growth and carotenoids production decreased. That would be caused by the inhibition effect of metabolites products in culture medium which increased by increasing biomass. Bhat and Marar (2015) found that, maximum pigment production by *Salinicoccus* sp. was achieved with 2% inoculum. Also**,** Ji *et al*., (2012) obtained maximum yield of pigment with 2% inoculum by *Monascus purpureus*.



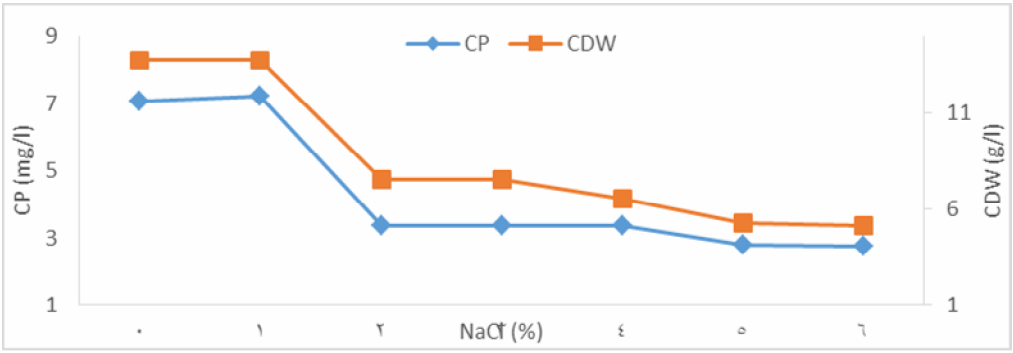
CP: Carotenoids production; CDW: Cells dry weight

Fig. 8. Effect of inoculum size on carotenoids production (mg/l) and yeast growth (g/l) by *R. mucilagenosa*

Effect of salinity on carotenoids production and yeast growth

The effect of salinity on CDW and carotenoids production by *R. mucilagenosa* was investigated in salted OWE medium (1-6 % NaCl) after five days at 25°C. The results in Fig. 9 showed that maximum carotenoids production (7.2 mg/l) and CDW (13.72 g/l) were obtained at 1% NaCl and by increasing the concentration of NaCl, growth weight and carotenoids production decreased. Therefore, the obtained results demonstrate that this strain of yeast failed to produce carotenoids at high concentration of NaCl. Mahmoud *et al*., (2014) reported similar results, which stated that there was a high significant decrease in carotenoids production by *Rhodotorula* sp. with increasing concentrations of NaCl. Also, they recorded that *Rhodotorula* sp. achieved highest values of carotenoids production with 2% of NaCl.

On contrast with our results, Kanzy *et al*., (2015) reported that the maximum carotenoid production was achieved at 6% NaCl by yeast strains of *Rhodotorula*.



CP: Carotenoids production; CDW: Cells dry weight

Fig. 9. Effect of salinity on yeast growth (g/l) and carotenoids production (mg/l) by *R. mucilagenosa*

Conclusion

In the current study, the abundantly available and non-efficiently utilized orange waste was used as a substrate for low cost production of carotenoids by *R. mucilagenosa* isolated from karish cheese. The highest carotenoids production and yeast cells dry weight was obtained after 5 days at 25 °C with initial pH 6 by adding 1% glucose, 1% yeast extract and 1% NaCl to orange waste extract. The maximal carotenoids production was 7.2 mg/l.

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استخدام مخلف عصر البرتقال كمادة خام رخيصة لإنتاج الكاروتينات بواسطة عزلة رودوتوريولا ميوسيلاجينوزا

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الملخص

تعد الكاروتينات مركبات ذات أهمية كبيرة في الأنشطة البيولوجية، حيث يمكن استخدامها كمواد ملونة في الصناعات الغذائية وكمضادات أكسدة طبيعية. في هذا البحث تم عزل سلالة خميرة من الجبن القريش تم تعريفها على أنها رودوتوريولا ميوسيلاجينوزا منتجة للكاروتينات، حيث تم استخدام مستخلص من مخلفات عصر البرتقال كمادة خام لإنتاج الكاروتينات من سلالة الخميرة المعزولة، وذلك لخفض تكلفة إنتاج الكاروتينات. ولزيادة إنتاج الميكروب من الكاروتين تم اختبار مصادر كربون ونيتروجين مختلفة لاختيار أفضلها للإنتاج. أعطت السلالة المعزولة أعلى إنتاجية للكاروتين (٧,٢ مليجرام/ لتر) وأعلى نمو ميكروبي (١٣,٧٥ جم/ لتر) بعد ٥ أيام من التنمية على مستخلص مخلف عصير البرتقال بإضافة ١% من الجلوكوز و١% من مستخلص الخميرة عند مستوى ١% من ملح كلوريد الصوديوم عند أس هيدروجيني ٦ على درجة حرارة ٢٥ درجة مئوية مع الرج على ١٥٠ لفة/ دقيقة.