PRODUCTION OF SINGLE CELL OIL FROM LOCAL ISOLATES OF RHODOTORULA GLUTINIS

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ABSTRACT

This study includes isolation and characterization of local isolates of Rhodotorula glutinis. From twenty-seven samples, three isolates showed yeast morphology and pigmented with orange-red. These isolates were subjected to biochemical tests and the results revealed that the three isolates have the characteristics of Rhodotorula glutinis. Effects of different incubation periods on growth and single cell oil (SCO) production showed that a maximum lipid content and productivity was 12.27 g/L and 64.74%, respectively after 4 days of incubation by the strain Rhodotorula glutinis WS9. A study of effect of different carbon sources revealed that the best carbon source for maximum oil production and oil productivity was glucose, which gave 12.15 g/L and 64.38%, respectively. Glucose concentration of 80 g/L gave a maximum oil content (13.76 g/L) and oil productivity (73.97%), whereas glucose 40 g/L concentration supported a maximum yeast growth (18.90 g/L). Ammonium sulfate was the best nitrogenous source to get a maximum dry biomass, lipid content and lipid productivity which reached to the 18.92 g/L, 12.21 g/L and 64.53%, respectively, 1.0% (w/v) ammonium sulfate was optimum for lipid content and lipid productivity which gave 14.96 g/L and 71.88%, respectively. Initial pH effects study revealed that the optimal initial pH was 6.5 which recorded 18.99 g/L, 12.23 g/L and 64.40% for dry biomass, lipid content and productivity, respectively.

Keywords: Production, Single Cell Oil, Local isolates, Rhodotorula glutinis

INTRODUCTION

Single cell oil (SCO) is the lipids produced by oleaginous microorganisms such as bacteria, yeasts, molds and algae that can accrue oils to more than 20% of their total cell dry weight. Rhodotorula sp. were reported to accrue intracellular lipids more than 50% of their cell dry weight (Zhu et al., 2008). The yeast Rhodotorula glutinis has been assessed with the aim of producing microbial lipids from glycerol under different aeration conditions (Kuan et al., 2018; Bento et al., 2019). It is reported that SCO eco-friendly fuels and low toxicity, biodegradability, as well as low concentration of SO2 exist in exhaust gas when used in motor vehicles (Ito et al., 2005).

Yeast Rhodotorula is wide spread saprophytic organisms that can be isolated from different sources: soil, fresh water, air samples, milk, salad, fruit juice, and tooth brushes (Tournas et al., 2006). Also, the yeast represents a part of the microbiota in all natural ecosystems, such as marine water from the ocean surface to the deep sea. Widely distributed in the nature environment, they colonize also more extreme environments such as low temperatures, low oxygen availabilities and oceanic waters (Banzatto et al., 2013). The ability to synthesize valuable metabolites such as lipids (Single Cell Oil) microbial proteins (Single Cell Protein) and carotenoids is characteristic of different microorganisms, including Rhodotorula yeast (Kot et al., 2017). It is observed that lipid content and profile differ between species in which lipid content in Rhodotorula glutinis cells reached to (72% CDW) (Beopoulos and Nicaud, 2012).

MATERIAL AND METHOD

Samples collection

Twelve soil samples and fifteen fresh flowers and fruit samples were collected from different gardens of Mosul city/ Iraq to isolate Rhodotorula glutinis yeast.

Media used in this study

Glycerol Enrichment Medium (g/L) (Pan et al., 2009)
Glycerol; 100; (NH4)2SO4; 1.0; MgSO4.7H2O; 0.5 and Yeast extract 0.2. This medium was used for isolation pigmented yeast.
Cultural Solid Medium (g/L) (Zheng et al., 2007)
Glucose; 20; (NH4)2SO4; 2; KH2PO4; 0.5 and 2% agar. This medium was used for culturing the isolated strains. After a growth period of 48 hours at 30°C, Slants were stored at 4°C. Subculturing of strains was performed every two months.
Yeast Extract Peptone Dextrose (YPD) g/L (Dai et al., 2007)
Glucose; 20; yeast extract, 20 and peptone 20. This medium was used for maintenance of R. glutinis isolated strains.
Inoculation broth medium g/L (Pan et al., 2009)
Glucose; 15; (NH4)2SO4; 5; KH2PO4; 1.0; MgSO4.7H2O; 0.5 and Yeast extract 0.5. This medium was used for preparation of inoculum.
Nitrogen Limited Medium g/L (Pan et al., 2009)
Glucose; 40; (NH4)2SO4; 2; KH2PO4; 7; NaH2PO4; 2; MgSO4.7H2O; 1.5 and Yeast extract 1.0. This medium was used as fermentation medium.

Yeast isolation

One gram from each soil sample was added in to 50 ml glycerol enrichment medium in a 250 ml Erlenmeyker flask. Incubation was done at 28±2 °C for one day with shaking at 180 rpm (Pan et al., 2009). A serial dilution of 100 to 10-4 were done from plates of pre-cultured yeast, and 0.1 ml of each dilution was spread onto cultural solid medium. The plates were incubated at 28±2 °C for 2 days. Then, the yeast colonies were isolated for screening processes (Zheng et al., 2012). Flower samples were crushed in sterilized mortor and 0.5 g was enriched in 50 ml glycerol enrichment medium for 1 day at 28±2 °C. The culture was diluted to 103, 102 and 101. 0.1 ml from each diluted culture was inoculated onto cultural solid medium for one day at 28±2 °C (Dai et al., 2007).
Identification of local isolates

The primary identification of *R. glutinis* among another yeast isolates were made according to the morphological culture, i.e., colonial feature. According to its pigmentation and shape, the yeast was examined under microscope. The secondary screening of *R. glutinis* was made to confirm the identification of the *R. glutinis* local isolated strains with biochemical tests according to Kurtzman and Fell (1998). The biochemical tests included fermentation of sugar, assimilation of nitrate and sugar, starch and acid production, pigmentation, urease activity and assimilation of inositol (Kurtzman and Fell, 1998).

Maintenance of *R. glutinis* strains

Purified local isolated strains of *R. glutinis* were streaked on slants of YPD. After a growth period of 2 days at 28±2 ºC, slants of *R. glutinis* strains were stored at 4 ºC in a refrigerator. Subculturing of the strains was done every two months.

Preparation of inoculum

A loopful of culture from strain on YPD slant was held to 50 ml inoculation broth medium in 250 ml flask. The culture was incubated at 28±2 ºC in shaker at 180 rpm for 48 hrs (Pan et al., 2009).

Nitrogen limited medium and inoculation for lipid production

2.0 ml of inoculums of yeast strain was added to 250 ml flasks containing 50 ml nitrogen limited medium. The medium was sterilized at 121 ºC for 15 min, prior to use, and the experiments were done with triplicates. Fermentation was done in shaker incubator at 28±2 ºC with shaking at 180 rpm for 1, 2, 3, 4 and 5 days, respectively.

Determination of yeast biomass

To determine yeast dry biomass for each culture, flasks were taken out from shaker incubator and cultures were harvested by centrifugation at 13000 rpm for 5 min. Harvested biomass was washed twice with 5 ml of distilled water and then dried at 105 ºC to constant mass (usually after 24 hrs) (Yehia et al., 2013).

Determination of single cell oil content

A fast procedure allowing complete lipid extraction was followed to determine the lipid content in the *R. glutinis* local strains according to Pan et al. (2009). A 50 ml of sample was centrifuged at 6000 for 100 min.; then, the pellet of yeast cells was washed twice with 50 ml of sample and centrifuged at 6000 rpm for 10 min. Then, the pellet of yeast cells was washed twice with 50 ml of distilled water. After that, centrifugation, addition of 10 ml of 4 M HCl, and incubation at 60 ºC for 1 to 2 hrs were done. The acid-hydrlyzed mass was stirred with 20 ml of chloroform and methanol mixture (1:1) at room temperature for 2 to 3 hrs. Centrifugation at 3000 rpm for 10 min. at room temperature was then performed to separate the aqueous upper phase and organic lower phase. After that, the lower phase containing lipids was recovered with Pasteur pipette. Evaporation under reduced pressure for 10 min. was done. The dry lipids were weighed.

Determination of single cell oil (SCO) productivity

Single cell oil productivity for local isolated *R. glutinis* strains was determined by the following equation (Kraistu et al., 2010):

\[
\text{SCO productivity} = \frac{\text{SCO weight (g/L)}}{\text{cell dry weight (g/L)}} \times 100.
\]

Effects of different carbon sources on growth of *R. glutinis* strains and single cell oil production

The influence of carbon sources, i.e., glucose, sucrose, maltose and xylose as carbon sources was studied. Nitrogen limited broth medium was prepared with one of the mentioned carbon sources. Carbon sources were sterilized by passing them through 0.22 mm micro filter. Forty gram per litter from each sugar was added to the medium after sterilization.

Effects of different concentration of glucose on growth of *R. glutinis* strain and single cell oil production

Effects of different glucose concentrations on growth of the yeast and single cell oil production were evaluated. Glucose with the concentrations 40, 60, 80 and 100 g/L was used in this experiment.

Effects of different nitrousous sources on growth of *R. glutinis* strains and single cell oil production

Effects of different nitrousous sources as one of nutrient compositions of nitrogen limited fermentation medium were investigated. The nitrogen sources include: \((\text{NH}_4)_2\text{SO}_4\), urea, peptone, \(\text{NaNO}_3\) and \((\text{NH}_4)_2\text{HPO}_4\) were added at 1 gram per liter.

Effects of different concentration of ammonium sulfate on growth of *R. glutinis* strains and single cell oil production

Growth of the yeast and single cell oil production were evaluated under different ammonium sulfate concentrations as follows: 0.5, 1.0, 1.5 and 2.0 g/L were added to the nitrogen limited broth medium.

Effects of different pH values on growth of *R. glutinis* strains and single cell oil production

To evaluate the effects of range of pH values on growth and single cell oil production, pH values: 6.0, 6.5 and 7.0 were applied on fermentation medium.

RESULTS AND DISCUSSION

Isolation of pigmented yeast strains

From Twelve soil samples collected, only three pure colony cultures showed the morphology typical of yeast by microscopic examination. From the three colony cultures, one colony was pigmented with orange-red. From fifteen fresh samples of flowers and fruits, only four pure colony cultures showed the morphology typical of yeast by microscopic examination. From these four pure colonies, only two were pigmented with orange-red. Sakaki et al. (2000) isolated the pigmented yeast *R. glutinis* from Japania soil. Dai et al. (2007) isolated *R. glutinis* strain T216 from flower samples in China. Pan et al. (2009) isolated *R. glutinis* yeast, which can utilize xylose from diverse soil samples in China. An attempt was made to isolate *R. sglutinis* strains from different sources (flowers, trees, exudates, fruits and cheese) in Egypt (El-Banna et al., 2012). Ibrahim et al. (2012) isolated *R. glutinis* strain chosen from 38 yeast strains isolated from garden. Enshaeieh et al. (2013) isolated Rhodotorula sp. from 34 Japania soil samples of peanut, walnuts, sunflower and almond gardens.

Identification of the pigmented yeast isolates

To identify the three pigmented yeasts, isolated strains were subjected to biochemical tests and the results revealed that the three isolated strains had the characteristics of *R. glutinis*, i.e., cell under microscopic examination were ovoid to globule and fast growing after 24 hr. The optimum growth temperature was 30 ºC and the isolates could grow at 37 ºC as well. Biochemical tests showed a positive urease reaction and assimilate nitrate. Assimilation test of the following sugars: glucose, galactose, sucrose, maltose and raffinose was positive. Biochemical tests for starch production, assimilation of inositol and gelatin liquefication were negative. Further biochemical tests were carried out and showed that the local isolated three strains had no ability to ferment glucose, galactose, sucrose, maltose, and raffinose. It can be considered that the three local isolated strains belong to *R. glutinis* according to Kurtzman and Fell (1998).
These three strains are named as follows R. glutinis WS8, WS9 and WS10, respectively. Morphological, biochemical and physiological analyses were used for the identification of the selected yeast strains of R. glutinis by the Dai et al. (2007). According to Kurtzman and Fell (1998) taxonomy, El-Banna et al. (2012) classified their isolates of pigment yeast as R. glutinis.

Effects of different incubation periods on growth of Rhodotorula glutinis and cell oil production

Results shown in Table 1 demonstrates that the increase of incubation period resulted in an increase in lipids content in the three local isolated strains of R. glutinis up to fourth day. The best lipid content and productivity percent, which are 12.27 %/ li and 64.74 %, respectively, were obtained from strain WS9 after fourth day incubation. After that, the lipid content and productivity percent decreased in the three local isolated strains. Average of dry biomass values increased up to fourth day of the incubation period for the three studied strains and reached the maximum of 18.95 g/L in WS9 strain. After the fourth incubation day, the average of dry biomass values decreased for all the studied strains which decreased to 16.52 g/L in WS8 strain after the day five of incubation. Results of incubation time agree with results of Dai et al. (2007) whom found that the optimal cultivation time is 96 hrs to get a maximal lipid productivity of 14.66 g/L by the local isolated R. glutinis strain T216, whereas Enshaeieh et al. (2013) research results showed that the best incubation time was 3 days to obtain a maximum lipid productivity (58.2 %) by the best strain Rhodotorula 110. Gonzalez-Gracia et al. (2013) found that the 6 day of incubation resulted in lipid accumulation up to 27.02 % by R. glutinis.

**Table 1 Effects of different incubation periods on growth of local isolates of Rhodotorula glutinis and single cell oil production**

| Strain No. | Incubation (Day) | Dry Biomass (g/L) | Lipid Content (g/L) | Lipid Productivity (%) |
|------------|-----------------|-------------------|--------------------|------------------------|
| WS8        | 2               | 13.14±(0.07)      | 5.91±(0.19)        | 44.97                  |
|            | 3               | 17.33±(0.01)      | 9.61±(0.08)        | 55.45                  |
|            | 4               | 18.09±(0.10)      | 10.95±(0.11)       | 60.53                  |
|            | 5               | 16.52±(0.07)      | 9.27±(0.09)        | 56.11                  |
| WS9        | 2               | 13.60±(0.02)      | 6.72±(0.14)        | 49.41                  |
|            | 3               | 17.68±(0.03)      | 10.14±(0.18)       | 57.35                  |
|            | 4               | 18.95±(0.11)      | 12.27±(0.13)       | 64.74                  |
|            | 5               | 17.60±(0.08)      | 10.03±(0.15)       | 56.98                  |
| WS10       | 2               | 13.07±(0.02)      | 5.54±(0.09)        | 42.38                  |
|            | 3               | 17.94±(0.08)      | 9.01±(0.12)        | 50.22                  |
|            | 4               | 18.51±(0.05)      | 10.43±(0.01)       | 56.34                  |
|            | 5               | 17.13±(0.03)      | 7.79±(0.23)        | 45.47                  |

* Data are presented as mean values from triplicates, ± : Slandered Deviations (S.D.).

Effect of different carbon sources on growth of local R. glutinis and single cell oil production

According to table 2, the best carbon source for best oil productivity is glucose in which average lipid content reached to 12.15 g/L and average value of oil productivity was 64.38 %. Oil productivity percentage were decreased with another carbon sources, i.e., sucrose, xylose and maltose. The average value of lipid content was 8.78, 7.15 and 6.93 g/L, respectively. The best production of dry biomass was with glucose as carbon source. The average value was 18.87 g/L, whereas the minimum average value of dry biomass was 11.76 g/L by maltose as carbon source. Results of this study coincide with Dai et al. (2007) whom found that the optimal fermentation condition were obtained by using glucose as carbon source. Amaretti et al. (2010) revealed that in the glucose-based media, cellular multiplication occurred first, while the lipogenic pathway followed whenever the culture was limited by a nutrient other than glucose. Enshaeieh et al. (2013) study revealed that lipid production in nitrogen limited medium with glucose as carbon source gave a maximum lipid productivity which reached to 36.1 %. Also, glucose supported a minimum biomass of 17.45 g/L. According to the experimental results, Sha (2013) found that the R. glutinis had the highest lipid yield in glucose sole carbon source in shake flask test.

**Table 2 Effects of different carbon sources on growth of local isolated strain Rhodotorula glutinis WS9 and single cell oil production after 4 days incubation**

| Carbon Source | Dry Biomass (g/L) | Lipid Content (g/L) | Lipid Productivity (%) |
|---------------|-------------------|---------------------|------------------------|
| Glucose       | 18.87±(0.17)      | 12.15±(0.20)        | 64.38                  |
| Sucrose       | 14.49±(0.09)      | 8.78±(0.18)         | 60.59                  |
| Maltose       | 11.76±(0.07)      | 6.93±(0.06)         | 58.92                  |
| Xylose        | 12.54±(0.13)      | 7.15±(0.11)         | 57.01                  |

* Data are presented as mean values from triplicates, ± : Slandered Deviations (S.D.).

Effects of different concentration of glucose on growth of R. glutinis and single cell oil production

The study of effects of deferent concentrations of glucose on biomass and oil production revealed that the increase of glucose concentration led to the increase of lipid content and productivity. The maximum lipid content and productivity percent were 13.76 g/L and 73.97 % respectively when used with 80 g/L. A decrease in lipid content and productivity was occurred with 100 g/L glucose. Nitrogen limited medium with 60 g/L glucose as sole carbon source supported a maximum growth of Rhodotorula glutinis strain WS9, i.e., average dry biomass value reached to 18.74 g/L. More concentrations of glucose led to decrease of biomass. Dai et al. (2007) recorded that the best concentration of glucose as carbon source for maximum lipid content was 100 g/L, whereas Amaretti et al. (2010) found that a maximum single cell productivity with 120 g/L glucose, in terms of lipid concentration 19 g/L, lipid/biomass (68 %) when using oleaginous yeast R. glutinis strain DBVP4785. Enshaeieh et al. (2013) results showed that the best grape concentration that supported a maximum lipid productivity was 75 g/L. The lipid productivity reached to 56.1 %, whereas, the glucose concentration 5 g/L gave a maximum biomass (16.21) g/L in Rhodotorula 110 strain. Results of this research work is more compatible with Enshaeieh et al. (2013) results.

**Table 3 Effects of different concentrations of glucose on growth of local isolated strain Rhodotorula glutinis WS9 and single cell oil production after 4 days incubation**

| Glucose g/L | Dry Biomass (g/L) | Lipid Content (g/L) | Lipid Productivity (%) |
|-------------|-------------------|---------------------|------------------------|
| 40          | 18.90±(0.25)      | 12.25±(0.14)        | 64.81                  |
| 60          | 18.74±(0.19)      | 12.83±(0.26)        | 68.46                  |
| 80          | 18.60±(0.18)      | 13.76±(0.14)        | 73.97                  |
| 100         | 18.53±(0.14)      | 12.88±(0.20)        | 69.50                  |

* Data are presented as mean values from triplicates, ± : Slandered Deviations (S.D.).

Effects of different nitrogenous sources on growth of Rhodotorula glutinis and single cell oil production

Effects of different nitrogenous sources revealed that the most effective one for lipid content was ammonium sulphate which gave a maximum lipid content and productivity percent 12.21 g/L and 64.53 %, respectively. Urea was a second nitrogenous source which save lipid content 11.14 g/L and productivity reached to 61.58 %. A minimum average values of lipid content was 6.98 g/L when peptone was used as nitrogenous source. Also, the lowest productivity percent (50.68) was obtained when the peptone was used as nitrogenous source as shown in Table 4. Ghanem et al. (1990) results revealed that maximal cellular lipid contents and sugar bioconversion estimates were achieved with (NH4) SO4 when R. glutinis was cultivated on beet molasses (BM), whereas Dai et al. (2007) found that the optimal fermentation conditions were obtained when yeast extract and peptone were used as nitrogenous sources. Additionally, R. glutinis accumulated lipids up to 14.66 g/L and lipid productivity reached to 49.25 % using agricultural and forestry residues as fermentation medium.
Effects of different concentrations of ammonium sulfate on growth of Rhodotorula glutinis and single cell oil production

Effects of different concentrations of ammonium sulfate on growth of local isolated strain Rhodotorula glutinis WS9 and single cell oil production after 4 days incubation showed that the maximum lipid content and productivity was obtained at 2.0% (w/v) ammonium sulfate for lipid content and productivity was 64.40 % with 1.0 g/L and 56.1%, respectively with 1.0 g/L. Increasing ammonium sulfate more than 1.0 g/L led to decreasing in lipid production and lipid productivity.

Table 5 Effects of different concentrations of ammonium sulfate on growth of local isolated strain Rhodotorula glutinis WS9 and single cell oil production after 4 days incubation

| (NH₄)₂SO₄ % (w/v) | Dry Biomass (g/L) | Lipid Content (g/L) | Lipid Productivity (%) |
|-------------------|-------------------|---------------------|------------------------|
| 0.5               | 19.34±(0.23)      | 13.01±(0.27)        | 67.26                  |
| 1.0               | 20.81±(0.16)      | 14.96±(0.11)        | 71.88                  |
| 1.5               | 20.07±(0.09)      | 13.09±(0.24)        | 65.22                  |
| 2.0               | 18.91±(0.27)      | 12.20±(0.19)        | 64.51                  |

* Data are presented as mean values from triplicates, ± : Slandered Deviations (S.D.).

Effects of different initial pH on growth of Rhodotorula glutinis and single cell oil production

Results in Table (6) shows the effects of different initial pH on growth of local isolated strain Rhodotorula glutinis WS9 and single cell oil production after 4 days incubation. A maximum lipid content was 12.25 g/L with initial pH 6.5, whereas productivity was 64.40 %. Also, this initial pH was optimum for growth, a maximum dry biomass was 18.99 g/L. Increasing initial pH to 7.0 decreases lipid content and lipid productivity which decreased to 11.91 g/L and 63.55%, respectively. Also, dry biomass decreased to 18.74 g/L. Johnson et al. (1992) results revealed that a maximum lipid production (66%) in Rhodotorula glutinis IIP-30 was at initial pH 4.0 and decreasing in initial pH led to decreasing in lipid productivity, whereas initial pH 5.0 was optimum for lipid production (10.5 g/L) and lipid productivity (64.8%) by yeast Rhodotorula glutinis TR29 in molasses medium (Taskin et al., 2016).

Table 6 Effects of different initial pH values on growth of local isolated strain Rhodotorula glutinis WS9 and single cell oil production after 4 days incubation

| Initial pH | Dry Biomass (g/L) | Lipid Content (g/L) | Lipid Productivity (%) |
|------------|-------------------|---------------------|------------------------|
| 6.0        | 18.85±(0.26)      | 12.01±(0.12)        | 63.71                  |
| 6.5        | 18.99±(0.21)      | 12.23±(0.18)        | 64.40                  |
| 7.0        | 18.74±(0.11)      | 11.91±(0.26)        | 63.55                  |

* Data are presented as mean values from triplicates, ± : Slandered Deviations (S.D.).

CONCLUSION

This research work showed the ability of local isolate of Rhodotorula glutinis strain to give a maximum lipid content 12.27 g/L with productivity 64.74 %. It is recommended that expanding the area of isolation may give better results.

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