JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

PRODUCTION OF SINGLE CELL OIL FROM LOCAL ISOLATES OF *Rhodotorula glutinis*

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doi: 10.15414/jmbfs.2020.10.2.279-283

ARTICLE INFO

Received 10. 3. 2020
Revised 13. 6. 2020
Accepted 29. 6. 2020
Published 1. 10. 2020

Regular article

OPEN ACCESS

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 SO_2 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; $(NH_4)SO_4$, 1.0; $MgSO_4.7H_2O$, 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; $(NH_4)SO_4$, 2; KH_2PO_4, 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; $(NH_4)SO_4$, 5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 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; $(NH_4)SO_4$, 2; KH_2PO_4, 7.0; NaH_2PO_4, 2; $MgSO_4.7H_2O$, 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 Erlenmeyer flask. Incubation was done at 28±2 ºC for one day with shaking at 180 rpm **(Pan *et al*., 2009)**.
A serial dilution of 10^{-2} to 10^{-8} 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 ºC 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 ºC. The culture was diluted to 10^{-1}, 10^{-2} and 10^{-3}. 0.1 ml from each diluted culture was inoculated onto cultural soild medium for one day at 28±2 ºC **(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, pigment formation, 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-hydrolyzed 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 **(Kraisitu *et al*., 2010)**:
SCO productivity = SCO weight (g/L) / cell dry weight (g/L) × 100.

Effects of different incubation periods on growth of *R. glutinis* strains and single cell oil production

Fifty milliliter of nitrogen limited broth was inoculated with 4% of inoculation broth culture of each strain. The flasks were then incubated in a rotary shaker operating at 180 rpm for 5 days at 28±2 ºC. The inoculation and incubation for each strain were done in triplicates. Dry biomass, lipid content and lipid productivity were recorded on intervals after each 24 hrs.

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. Fourty 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 nitrogenous sources on growth of *R. glutinis* strains and single cell oil production

Effects of different nitrogen sources as one of nutrient compositions of nitrogen limited fermentation medium were investigated. The nitrogen sources include: $(NH_4)_2SO_4$, Urea, peptone, $NaNO_3$ and $(NH_4)_2HPO_4$ were added at 1 gram per litter.

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 Japanian 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 Japanian 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 liquification 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 glutinins* 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 g/ 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 face 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 DBVPG4785. **Enshaeieh *et al.* (2013)** results showed that the best glucose 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 (NH_4) 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 nitrogen 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.

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

Nitrogenous Source	Dry Biomass (g/L)	Lipid Content (g/L)	Lipid Productivity (%)
$(NH_4)_2SO_4$	18.92♦(±0.14)	12.21♦(±0.17)	64.53
Urea	18.09(±0.03)	11.14(±0.13)	61.58
Peptone	13.77(±0.21)	6.98(±0.15)	50.68
$NaNO_3$	15.11(±0.17)	8.33(±0.20)	55.12
$(NH4)_2HPO_4$	16.53 (±0.09)	10.01 (±0.28)	60.55

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

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 most effective concentration of ammonium sulfate for lipid content and productivity was 1% (w/v). This concentration gave a maximum lipid content and lipid productivity, which reached to 14.96 g/L and 71.88%, respectively. Increasing the concentration of ammonium sulfate led to decrease in lipid content and oil productivity, i.e., 12.20 g/L and 64.51 %, with 2.0% (w/v) of ammonium sulfate (Table 5). The obtained results are consistent with the findings of **Erishaeieh *et al.* (2013)**. Their results gave a maximum lipid production and lipid productivity, 7.13 g/L and 56.1%, respectively with 1.0 g/L. Increasing ammonium sulfate more than 1.0 g/L let 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

$(NH_4)_2SO_4$ % (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 12.23 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 content (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.

Acknowledgments: The authors are grateful to the Northern technical University/ Technical Institute-Mosul/ Medical Laboratory Techniques Department and the University of Mosul/College of Education for Pure Sciences for their provided facilities, which helped to improve the quality of this work.

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