Isolation, screening, and extraction the more efficient local yeast isolates for biosurfactant production

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Abstract. Twelve samples of three spices of fruits (Kiwi, orange, and apple) and two spices of vegetables (Green pepper and cucumber) were collected from local shops and supermarkets to isolate the yeast using solid YEPG medium, twenty local yeast isolates were prepared from all samples, thereafter each isolate was tested for its ability to produce biosurfactant using liquid YEPG medium. Results have indicated that only three isolates had the ability to produce the biosurfactant at different abilities in growth medium depending on the emulsification index after 24 hr. They were tested for the production of extracellular biosurfactant only three isolates OS2, AS 9, and PS 14 were able to produce biosurfactant with emulsification index (E24) against machine oil 36%, 47% and 28% respectively also was capable to spread oil by 12mm, 20mm and 9mm respectively but they didn’t reduce YEPG medium surface tension significantly. The three isolates OS2, AS 9, and PS 14 were tested for intracellular biosurfactant, which gave the highest ability to emulsify both machine oil and crude oil, where the emulsification index (E24) reached 46%, 80%, and 55% towards motor oil and 50%, 100%, and 62% towards crude oil for the three isolates, respectively. According to these results, the isolate AS 9 was regarded as the best producer for biosurfactant and identified this isolate as a Saccharomyces cerevisiae. Thereafter this biosurfactant was partially purified using cold ethanol and left to dry using a Rotary evaporator and then converted into a powder by using a Freeze dryer.

Introduction
Many micro-organisms have the ability to synthesis active surface molecules to aid in the uptake of the nutrients (organic or inorganic) where these compounds are termed microbial surfactants or biosurfactants which are able to reduce the surface tension of a solution and lower the interfacial tension between two immiscible liquids. Biosurfactants are composed of two portions where one of them presented as a tail with a hydrophobic chain mostly compromised of fatty acids and the other portion is a hydrophilic head, so these biosurfactants are amphiphilic material [1].

Bacteria, fungi and yeasts have the ability to produce biosurfactants. They are classified depending on the molecular weight, origin and chemical structure. There are high molecular weight biosurfactants (polymeric biosurfactant and particulate biosurfactant) and low molecular weight biosurfactants (Glycolipids, Lipopeptides, Fatty acids, phospholipids and natural biosurfactants) [2].

Biosurfactants have been used in many applications such as in bioremediation, biodegradation, agriculture, pharmaceutical, cosmetics and medicine due to their emulsification, de-emulsification, solubilization, mobilization, less toxicity and biodegradability properties [3][4].

Biosurfactants may be secreted extracellularly or intracellular attached to the cell wall or part of the cell wall structure [1]. Studies of biosurfactant production showed that they are less toxic and have no pathogenic effects on humans [5]. Yeasts sp. are able to produce biosurfactants from renewable substrates, industrial waste products, oils, glycerol and glucose so this gives the advantage of producing biosurfactants in the most affordable methods and materials [6].

Many researchers have shown that yeasts spieces are efficient micro-organisms capable of producing biosurfactants such as Glycolipids biosurfactants from Candida bombicola URM, C. Albicans, C. glabrata and Saccharomyces cerevisiae [7-9].
Bio emulsifiers are considered as high molecular biosurfactants but not all bio emulsifiers are capable of decreasing the tension pressure of surfaces they are composed of hydrophilic parts and hydrophobic parts.

Bio emulsifiers are either considered as W/O which means water droplets are dispersed in oil or O/W which means the oil is dispersed in an aqueous solution [10]. Bio emulsifiers are produced by different types of microorganisms. The most known bio emulsifiers are mannoprotein which is produced by Saccharomycyes cerevisiae and Kluyveromyces marxianus, Liposan which is produced by Candida tropicalis, emulsan can be produced by Acinetobacter calcoaceticus and Mannosylerythritol lipids may be synthesized by Kurtzmanomyces sp., Pseudozyma rugulosa [10].

The aim of this study is to produce biosurfactants by yeasts isolated from different plants (3 fruit species and 2 vegetable species) and selecting the best isolate that produces extracellular biosurfactant and intracellular biosurfactant.

2. Materials and methods
2.1. sample collecting
Twelve samples of kiwi, orange, apple, Green pepper and cucumber with the soft structure were collected from the local market in Bab Al-Moatham, Baghdad- Iraq during January 2020.

2.2. yeast isolation
Suspensions of each sample (Kiwi, orange, apple, Green pepper and cucumber) were prepared by transferring 1 g of the samples into a tube contains 10 ml of distilled water and vortexed for a minute, then 1 ml of suspension was transferred into 250 ml flask contains 50 ml of YEPMG medium (10 g/l yeast extract, 20 g/l peptones and 20g/l glucose) supplemented with 50 mg/l of chloramphenicol and 50 mg/l of streptomycin, PH adjusted to 5 and incubated at 28°C, 150 rpm for 4 days.

For yeasts, isolation 100 μl of the growth culture transferred and spread into YEPMG agar plates (10 g/l yeast extract, 20 g/l peptones, 20g/l glucose and 20 g/l agar) supplemented with 50 mg/l of chloramphenicol and 50 mg/l of streptomycin, PH adjusted to 5 and incubated at 28°C for 24 hr.

2.3. yeast isolates selection
Colonies were observed for their color, odor and shape. yeast cells were observed under a microscope.

2.4. production of biosurfactant
The medium that used to produce biosurfactant is YEPMG medium PH adjusted to 5 yeasts colonies were harvested by a sterile loop and transferred to YEPMG medium and incubated at 28°C at 150 rpm for 24 hr.

2.5. Screening for biosurfactant
2.5.1. Emulsification activity. Emulsification index (E24) was determined for the whole culture and cell free culture by adding 2 ml of sample with 2 ml of machine oil crude oil and then vortexed for 3 minutes and let stand for 24 hr. The E24 was determined by this formula:

\[
E_{24} = \left( \text{the height of emulsion layer} / \text{the total height of liquid layer} \right) \times 100\%
\]

The heights of emulsion and total liquid were measured in millimetre [11].

2.5.2. Oil spreading method (OSM). The oil spreading method was determined by pouring 25 ml of distilling water in a petri dish then 10 μl of crude oil was added, then 10 μl of crude biosurfactant was added in the center then the spreading zone was measured by millimeter [12].

2.5.3 Surface tension measurement. The surface tension of the extracellular and intracellular biosurfactant was measured by using a tensiometer (KRUESS, Germany) with a de NUOY ring [13].
2.6. Extraction of intracellular biosurfactant
Yeast cultures were centrifuged at 4000 rpm for 15 min and washed twice with distilled water, then the cell pellets were suspended in normal saline and autoclaved for 30 minutes. The solution was centrifuged at 6000 rpm for 10 minutes and E24 and OSM were determined. [14].

2.7. Partial purification of intracellular biosurfactant
The crude biosurfactant was purified by using ethanol precipitation method, five volumes of 99% of chilled the ethanol were added on the crude biosurfactant and left for 12 hr. at 4°C, centrifuged at 6000 rpm for 10 minutes and washed twice with chilled ethanol, the precipitate dried with a rotary evaporator and freeze-dried.

2.8. FTIR characterization of crude biosurfactant
Structural characteristics of the crude biosurfactant were determined using Fourier transform infrared spectrophotometer (Bruker, Tensor 27, Germany). The measurement frequency ranged from 400 cm⁻¹ to 4000 cm⁻¹.

2.9. Identification of yeast isolates
Biosurfactant-producing yeast was identified by biochemical tests (urease test and fermentation test for glucose, maltose, sucrose and fructose) as described by [15] and Vitek 2 YST-CARD.

3. Results and Discussion
Twenty yeast isolates were obtained from fruits (Kiwi, orange and apple) and vegetables (Green pepper and cucumber), all yeasts colonies on YEPG agar showed a rounded, white to creamy in color with uniformed edges and soft texture and convexed on the surface of the plate agar and with a special ferment odor. The microscopic examination showed semi-rounded yeast cells and buds attached to yeast cells were observed, the cells were either single or piled up as described by [16].
In this study, only three yeast isolates OS2, AS9 and PS14 which were obtained from orange, apple and green pepper was able to produce biosurfactant capable of forming the highest emulsions with machine oil and they were also tested for the ability to spread the crude oil. Also, the ability to reduce surface tension was determined as shown in Table 1 and Figure 1.

| No. | Yeast Isolate | E24 % for machine oil | OSM mm | Surface tension mN/m |
|-----|---------------|-----------------------|--------|---------------------|
| 1   | OS2           | 36                    | 12     | 55                  |
| 2   | AS9           | 47                    | 20     | 50                  |
| 3   | PS14          | 28                    | 9      | 58                  |

The results showed that surface tension was slightly reduced compared to the control media (60 mN/m) which is not inoculated. A study on producing biosurfactant from S. cerevisiae 2031 produced biosurfactant by using Cooper and Paddock's medium the biosurfactant wasn’t able to reduce surface tension which meant that the biosurfactant had an emulsifier property more than a surfactant property [17].
In another study, a glycolipid biosurfactant produced from *Wickerhamomyces anomalous* CCMA 0358 reduced surface tension to 31 mN/m, the carbon source that was used is olive oil [18]. The oil spreading method is used to determine the presence of biosurfactant as shown in table 1 this test proves the yeast isolates were able to produce biosurfactants.

Extraction of intracellular biosurfactant from the three isolates OS2, AS 9, and PS 14 was done by destruction of the cell wall of yeasts by using autoclave which eases the extraction of intracellular biosurfactant under pressure and heat.

Table 2 shows the emulsification activity and oil spread method for the intercellular biosurfactant produced from the three isolates.

Intracellular biosurfactant showed a great emulsification activity against machine oil with comparing to extracellular biosurfactant and showed better activity against crude oil, showed the best results (see Figure 1 and Figure 2).

| No. | Yeast Isolate | $E_{24}$ % for machine oil | $E_{24}$ % for crude oil | OSM mm | Surface tension mN/m |
|-----|---------------|---------------------------|-------------------------|--------|---------------------|
| 1   | OS2           | 46                        | 50                      | 20     | 61                  |
| 2   | AS9           | 80                        | 100                     | 30     | 56                  |
| 3   | PS14          | 55                        | 62                      | 15     | 58                  |

Another study showed that the emulsification activity of biosurfactants produced from *B. subtilis* was higher in kerosene (46.90 %) than emulsification activity for crude oil (approximately 10 %) [19]. Furthermore, a biosurfactant produced by *Stenotrophomonas maltophilia* was able to form an emulsion against crude oil with emulsifying activity reached 70% [20].

![Figure 1. yeasts isolate (PS14, OS2 & AS9) under microscope](image1)

![Figure 2. $E_{24}$ of intracellular biosurfactant of isolate AS9](image2)

![Figure 3. biosurfactant after partial purification from AS9 isolate](image3)
The crude biosurfactant of the best isolate (AS9) was partially purified by ethanol precipitation method and dried in order to characterize its functional groups by Fourier transform infrared spectroscopy, the band centered at 3271.82 cm\(^{-1}\) was caused by O-H stretching vibration on sugar ring [21]. They peck at 2929.36 cm\(^{-1}\) could be a C-H weak stretch that may be related to polysaccharides [22]. The stretching peak of C=O carboxyl is centered at 1644.44 cm\(^{-1}\) [21]. While the peak at 1537.99 cm\(^{-1}\) peck might be the amide II N-H group which is related to protein structure [22].

The weak band at 1397.12 cm\(^{-1}\) could be due to the stretching vibration of C-H [21]. 1023.32cm\(^{-1}\) is the most intense peak is related to C-O or C-C stretching of ɑ-glucan and 917.01 cm\(^{-1}\) is related to the C-H band of β-glucan [23]. The weak stretching vibration at 808.57 cm\(^{-1}\) related to the ɑ-glycosidic bond of mannan [21] as shown in Figure 4 and Table 3.

### Table 3: FTIR wave number of the crude biosurfactant

| Band                  | Wavenumber cm\(^{-1}\) |
|-----------------------|-------------------------|
| –OH                   | 3271.82                 |
| C-H                   | 2929.36                 |
| C=O                   | 1644.44                 |
| N-H                   | 1537.99                 |
| C-H                   | 1397.12                 |
| C-O or C-C            | 1023.32                 |
| C-H                   | 917.01                  |
| ɑ-glycosidic bond     | 808.57                  |

**Figure 4.** FTIR spectrum of intracellular biosurfactant

The isolate AS9 was identified as *Saccharomyces cerevisiae* based on the biochemical tests and Vitek 2. The biochemical tests showed that *S. cerevisiae* was able to produce urease enzyme in, it was indicated by the alteration of the urea agar medium from yellowish orange to deep pinkish-red. *S. cerevisiae* was able to utilize different carbon sources glucose, maltose, sucrose and fructose, the positive result was indicated by color changes (from red to yellow) [15] as shown in table 4.
Table 4. Biochemical tests for yeast isolate AS9

| Carbone fermentation |  |
|----------------------|--|
| Urease test | + |
| Glucose | + |
| Maltose | + |
| Sucrose | + |
| Fructose | + |

Many studies confirmed that *Saccharomyces cerevisiae* is able to produce intracellular and extracellular biosurfactants such as in [24] intracellular biosurfactant was better than extracellular biosurfactant in the formation of emulsions and spreading crude oil layer. Extracellular biosurfactant didn’t form stable emulsions as the intracellular biosurfactant that was synthesized from *S. cerevisiae* 2031 also emulsifying activity was higher than extracellular biosurfactant, the intracellular biosurfactant was extracted by heat treatment and proved that the biosurfactant was associated with the yeast cell wall [25]. The biosurfactant was characterized as mannoprotein which had a high emulsification activity [26].

4. Conclusions and Recommendation

*Saccharomyces cerevisiae* isolated from the local Iraqi environment is capable to produce biosurfactants with good emulsification activity, it is recommended to study the characteristic and exact composition of the biosurfactants.

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Acknowledgments
The current study is supported by University of Technology-Iraq (UOT-Iraq).