Research Article

Production and properties of biosurfactant from the local isolation of *Candida* spp.

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ABSTRACT

Background: This study aimed to produce biosurfactants from a local isolate of *Candida* yeast and evaluate the effects of carbon and nitrogen sources. The optimum biosurfactant production conditions (including pH, temperature, fermentation time, shaker-incubator speed, and inoculum volume) were also investigated. The biosurfactants were produced from two yeast isolates, namely, *Candida albicans* and *Candida lipolytica*. **Material and Methods** The ability of isolates to produce biosurfactant was based on the estimation of emulsification activity, emulsification index, surface tension, oil displacement, and critical micelle concentration (CMC). **Results:** Results showed that the biosurfactant yield was 9 g/l, the surface tension decreased from 71 to 28 mN/m, and the CMC concentration was 1.4%. Emulsification activity depended on the determination of production capability. The quantitative and qualitative screening outputs of isolation demonstrated that the maximum activity on the wavelength of 540 nm using sunflower oil was 1.65 and that the best effect of environmental and nutritional factors on biosurfactant production was recorded by *C. lipolytica*. The model condition of production was investigated by evaluating the emulsification activity for the cell-free culture medium, which contained 1% glucose as a carbon source. Furthermore, its recorded activity was 2.251. A total of 2% urea was used as a nitrogen source with an activity of 2.141 and pH 4 in (2.185) activity and temperature 30°C with 2.285 as activity and 2.241 as activity for a period 72 h in shaking incubator under 180 rpm with activity (2.017) and inoculum volume 1% with activity (2.301) effectiveness was measured at 540 nm. **Conclusions:** This study concludes that the *C. lipolytica* isolate has a high capacity for biosurfactant production. The modification of growth medium reflects a positive effect on biosurfactant production, especially when monosaccharide and urea are used as carbon and nitrogen sources, respectively.

KEY WORDS: Biosurfactants, *Candida*, Emulsifier, Yeasts

INTRODUCTION

Biosurfactants are secondary-metabolism compounds with none or low toxicity produced by microorganisms, such as bacteria, yeast, and filamentous fungi, when grown in culture media containing organic compound as a carbon source. The microorganisms in undissolved substances, such as hydrocarbons, produce many materials, including surfactant, to spread that source inside the cell.[1-4] Some bacteria and yeast produce ionic surfactants that emulsify hydrocarbons in the growth medium. They represent a response to specific requirements as a result of exposing the microbial cells to environmental conditions.[5] Emulsifiers are amphoteric compounds with hydrophilic and hydrophobic parts. These compounds can be assembled between surfaces and minimize the surface tension between them. Furthermore, they show high emulsification activity. These compounds were produced on living surfaces, such as in microbial cell surface, or outside the cells and were classified as biosurfactants.[6]

The production of these compounds is eliciting increasing attention due to their functional properties in addition to their high range of usage in nutrition manufacturers and application in pharmaceuticals and their ability to synthesize living microorganism. Furthermore, yeast can grow on hydrocarbon and diesel with high efficiency and produce compounds with high emulsification and antibacterial activities.[7] Many biosurfactants are produced by microorganisms, such as rhamnolipids, which are made by *Pseudomonas aeruginosa* bacteria, and sophorolipids, which are

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produced by some type of *Torulopsis ssp*. Furthermore, some microorganism, such as *Candida lipolytica* and *Candida tropicalis* microorganisms, enabled lipopolysaccharides to attach to cells when they were grown (n-alkanes) and can change their wall surface.[8] Emulsifiers are capable of biodegradation and can be manufactured from wastes and cheap resources. They can increase the biodegradation of slightly soluble pollutants by increasing bioavailability.[9,10] A research made by[11] proved that *C. antarctica* yeast can make an emulsifier called mannosylerythritol lipids when it grew on a medium that was enriched with vegetable oil as a carbon source. Furthermore, Hua *et al*.[12] demonstrated that this yeast can produce biosurfactants called BS-UC when it grew on medium with C11H2O n-undecane. This study aimed to isolate and diagnose some types of *Candida* yeast from areas that were contaminated with hydrocarbons and to study their ability to produce biosurfactant, determine their best and examine the optimal conditions for production.

MATERIALS AND METHODS

Sample Collection

The present study was carried out on two yeast isolates (*Candida albicans* and *C. lipolytica*) which were isolated from soil contaminated with oil in Basra city (Nihran Omar). Soil samples were collected 5–15 cm below the surface in sterile plastic bags then stored at 4˚C and transferred to the laboratory for analysis. Soil dilution plate method was used to isolation technique that including by suspending 1 g of the soil sample in 10 ml of sterile distilled water. Dilutions of 10-2, 10-3 were used to isolate fungi to avoid over-crowding of fungal colonies, while potato dextrose agar (PDA) medium (HiMedia Co.). With 0.05 g/l chloramphenicol was prepared and sterilized by autoclave for 20 min. 0.1 ml of each concentration was added to sterile Petri dishes, in duplicates of each dilution, after cooling into 45˚C the medium was poured into Petri dishes. The plates were incubated at 28°C ± 2 °C for 3–5 days. Yeast isolates were easily isolated because they formed surface colonies that were well dispersed especially at low concentrations. Representatives of each morphologically distinct colony type were transferred into pure culture.

Yeast Identification

Yeast isolates were identified to the species level with the use of standard morphological and physiological tests. Specifically isolates were identified to the species level based on their germ tube formation test, a set of macroscopic and microscopic features and colony color on CHROM agar *Candida* medium (CAC) from HiMedia, as well as the assimilation profile by use of API 20C AUX system (Biomerieux, France) a commercial kit for the evaluation of the assimilation of 19 carbon sources.

Biosurfactants Production

Yeasts are cultured in 250 ml volumetric flask containing 50 ml of mineral salts medium that consists of the following materials g/100 ml (0.3 g NaNO3, 0.3 g KH2PO4, 0.3 g MgSo4.7H2O, and 1 yeast extract) hydrocarbons were added as a carbon source by a percentage of 4%v/v. The flasks incubated in a shaker incubator on 30°C and the speed of 200 rpm for 6 days.

Biomass Determination

For biomass determination, 5 ml samples were mixed in preweighed tubes with distilled water and centrifuged at 5000 rpm for 20 min. After two washing cycles, the cell pellet was dried in an oven at 90°C for 24 h. All the assays were carried out in triplicate.[13]

Qualitative Screening

Drop collapsing test (quantitative screening)

In this experiment, the (96-well) microtiter lids were used, 2 µl of mineral oil were added in the pores, then it has been left in a room temperature of 25°C for 1 h, after that, 5 µl of free bacterial cells supernatant was added and left for 2 min, at last, the shape of oil drops was examined.[14]

Determination of the emulsifying activity (qualitative screening)

The isolated yeasts were cultured on mineral salt medium MSM agar which is contain (Sunflower oil, Crude oil, kerosene, paraffin, Olive oil, and glycerol) as a carbon source for 6 days, than they were centrifuged at a speed of 6000 rpm for 20 min and the biosurfactant then precipitated by adding acetone 1:1 (v/v) (21). After that, they were dried under reduced pressure and were dissolution in a test tube containing Tris-HCL (PH 8) solution and 0.1 ml of diesel was added to the tubes. Then, the mixture was mixed by a shaker incubated for 20 min in 150 rpm. The mixture left four 20 min; OD values were measured at 610 nm.[15]

Emulsification index (E24) (qualitative screening)

The emulsification index was determined according to Nitschke *et al*.,[4] equal amounts of oil substrates (crude oil, sunflower oil, and diesel) and the cell-free culture broth were taken in test tubes, vortexes at high speed for 2 min and left undisturbed. Emulsion stability after 24 and 168 h was calculated as emulsification index (EI) = (height of emulsion layer/height of the total mixture) × 100.

Oil displacement test (qualitative screening)

This test was done in a Petri dish with a diameter of 150 mm, 50 ml of distilled water was added, then 50 µl

of diesel was added over the water, after then100 µl of free yeasts cells supernatant was added in the center of the membrane layer, which formed on the surface, the diameter and the area of the clear zone were evaluated after 30 s.[16]

Optimization of cultural conditions

The same media in production media were used with some modification in the quantitative screening to determine the optimal conditions. To reach the optimal conditions for the production of the surfactant from the selected isolates, the variables physical conditions that affect the production such as duration of incubation, temperature, pH, size of inoculum, agitation, and stirring. The glucose uses as a carbon source in range (0, 1, 2, 3, 4, 5, 6, and 7%) and urea as a nitrogen source in range (0, 1, 2, 3, 4, 5, 6, and 7%). Temperature: the inoculated flasks were incubated at the temperature range from 25, 28, 30, 35, 40, 42, 45, and 50°C . PH; the media pH was adjusted by (1N) NaOH or (1N) HCl to achieve pH values ranged (2, 3, 4, 5, 6, 7, 8, and 9). Size of inoculum; the different size of inoculum was used being 1, 2, 3, 4, 5, 6, and 7 ml/100 ml. Agitation and stirring: all flasks were incubated in shaker incubators using different rpm, being 100, 150, 180, 200, and 220rpm. Incubation time: all flasks were incubated for different period being 0, 24, 48, 72, 96, 120, 144, and 168h. The results were recorded.

Surface tension and critical micelle constriction determination

Surface tension of free yeast cell medium was measured after centrifugation on 5000 rpm for 20 min using a ring method using tension meter 7000 from Sigma at a room temperature and the critical micelle constriction (CMC) was evaluated after measuring the surface tension for biosurfactant diluted in distilled water, until they reached a fixed value as described by Santos *et al*.[17]

RESULTS AND DISCUSSION

In the preliminary study, 12 colonies with the typical morphology of yeasts and filamentous fungi were isolated from contaminated oil soil. Out of these isolates, two yeast isolates (i.e., *C. albicans* and *C. lipolytica*) were selected for biosurfactant production assay.

Quantitative Screening

The technology of oil displacement test and the drop-collapsing test where the components of isolated yeast created a transparent circle in high volumes when added to the oil layer have been used to detect biosurfactants. The same finding was achieved with the drop-collapsing test where the oil drop completely collapsed when the biosurfactants were added to it. This method has been officially used to reveal biosurfactants and to determine the isolates that produce it. The results showed that the isolates of *C. lipolytica* and *C. albicans* yielded a circle of a 7.5 and 4.5 cm diameter, respectively [Figure 1].

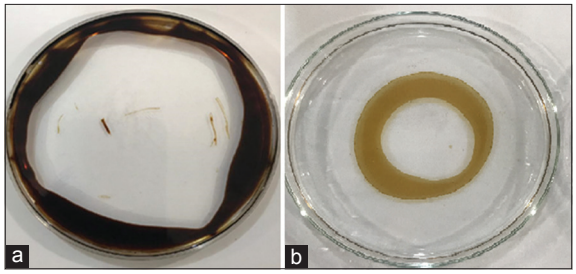

Figure 1: Circle formed on the oil layer due to the addition of biosurfactants (a: *Candida lipolytica* b: *Candida albicans*)

Qualitative Screening

A screening process in Table 1 demonstrates the emulsification activity of biosurfactants produced from yeast by measuring the emulsification activity in accordance with the method of Juwarker *et al*.[15] The results showed that the increase in the optical density at 610 nm was an indication of high emulsification activity, which was caused by the high concentration of the emulsification substance produced from the yeast isolate. The emulsification activity of biosurfactants produced from *C. lipolytica* yeast, which reached its best with sunflower oil, where the activity measured 1.650, thereby surpassing the emulsification of olive oil, which measured 1.084, whereas a very low activity was measured with paraffin and glycerol. The highest emulsification activity of biosurfactants from *C. albicans* yeast was observed in sunflower oil, reaching 1.140, which was close to the activity in olive oil. Low activity was measured with other hydrocarbons due to the concentration difference of biosurfactants in the production field, which, in turn, was caused by the difference in the ability of the isolates to produce substances in the presence of the aforementioned carbon sources.[18] Results of the statistical analysis in the SPSS program showed significant differences at the probability level of 0.01. The results showed the emulsification activity of biosurfactants from *C. lipolytica* yeast. All the other hydrocarbon sources with *C. lipolytica* and *C. albicans* had been exceeded using sunflower oil as the hydrocarbon source. Therefore, *C. lipolytica* is the best.

Table 2 demonstrates the study of carbon and nitrogen sources used to produce biosurfactants from *C. lipolytica* isolates. Results showed that the local isolate of *C. lipolytica* yielded the highest emulsification activity when glucose was used as a carbon source with most nitrogen sources after conducting quantitative and qualitative screenings on

Table 1: Screening of yeast isolates that produce the highest levels of biosurfactants

Emulsification activity at 540 nm						
Isolate	**Sunflower oil**	**Crude oil**	**Kerosene**	**Paraffin**	**Olive oil**	**Glycerol**
Candida lipolytica	1.65±0.006	0.34±0.002	0.475±0.008	0.1±0.005	1.084±0.001	0.16±0.002
Candida albicans	1.14±0.005	0.4±0.005	0.55±0.005	0.075±0.002	0.99±0.002	0.12±0.001

Table 2: Determining the best carbon and nitrogen sources to produce biosurfactants by measuring the emulsification activity

Nitrogen carbon	**Whey**	**White corn**	**Bran Wheat**	**Glutamic acid**	**Sodium nitrate**	**Ammonium nitrate**	**Urea**
Sunflower oil	1.480±0.003	0.325±0.004	1.100±0.004	1.020±0.003	0.520±0.01	0.505±0.005	0.805±0.002
Olive oil	0.770±0.005	0.500±0.007	1.090±0.004	1.100±0.002	0.330±0.003	0.390±0.003	0.850±0.005
Glycerol	0.820±0.0005	0.190±0.0000	0.450±0.0005	0.260±0.003	0.190±0.002	0.150±0.001	0.720±0.009
Dates syrup	1.280±0.001	0.090±0.4	0.690±0.004	1.310±0.005	1.230±0.005	1.165±0.002	1.280±0.001
Molasses	0.275±0.002	0.250±0.001	0.410±0.003	0.210±0.002	0.270±0.003	0.130±0.004	0.600±0.005
Glucose	2.085±0.005	1.135±0.002	1.259±0.05	1.905±0.004	1.205±0.005	2.165±0.001	2.320±0.004
Crude oil	0.780±0.003	0.205±0.003	1.120±0.003	0.890±0.003	0.910±0.0000	0.670±0.002	0.720±0.001

the isolates used in this study and determining which isolates are most active and most capable of producing biosurfactants by examining the emulsification activity and measuring the absorption level at a wavelength of 610 nm. Urea was the best nitrogen source with an emulsification activity of 2.320, followed by ammonium nitrate with an activity of 2.165 and whey protein with an activity of 2.085. Thus, the best carbon and nitrogen sources are glucose and urea, respectively. The results of the statistical analysis showed a significant difference in all of the carbon and nitrogen sources and an evident prevalence of the emulsification activity using glucose and urea as carbon and nitrogen sources. The numbers refer to the electromagnetic radiation on a wavelength of 540 nm, which corresponds to biosurfactant production

Optimum Conditions for Biosurfactant Production

Table 3 demonstrates the ideal conditions in producing biosurfactants from the local isolate *C. lipolytica*. The results showed that the optimum concentration of the carbon source glucose was 1%, which yielded the highest emulsification activity of approximately 2.251. This result is in agreement with the authors studies.[19-21] Urea was the best nitrogen source. It yielded the highest emulsification activity of 2.141 and the highest biosurfactants with a concentration of 2%, and the results matched the findings of Padmapriya and Suganthi.[23] The results in Table 3 also showed that the best pH indicator was 4, where the yeast produced its highest levels of biosurfactants with an activity of 2.185 and began to drop with the increase in the pH indicator's value toward neutral and alkaline. Furthermore, the decrease of the pH level to a value lower than 4 also reduced the activity level, thereby indicating a reduction in the production quantity. The results in the table also show that the activity increased with the temperature in the incubator, reaching its highest level at 30 °C, where the activity reached 2.285 and began to drop with the increase of temperature, reaching an activity of 1.007 at 40°C. The ideal incubation period was 72 h, which matched the results in Padmapriya and Suganthi,[23] Rufino *et al*.[23,24] The results also confirmed that the best speed for the shaker incubator was 180 s/m, where the emulsification activity reached 2.017, which was the highest value that indicated the highest production of biosurfactants. The amount of vaccine used was 1%, where the emulsification activity reached 2.301 and began to decrease with the increase of vaccine until reaching its lowest levels with vaccine amount of 7%. The statistical analysis results in Table 3 also show significant differences among all of the following treatments: Glucose concentration 1%, urea 2%, pH indicator 4, temperature 30 °C, incubation period 72 h, speed 180, and volume inoculum 1%.

The production amount of biosurfactants from the local isolate of *C. lipolytic*a reached 9 g/l after an incubation period of 72 h, which agrees with[20,25] and exceeds the findings of,[26] which produced 8 g/l after an incubation period of 72 h. The final production amount of biosurfactants from the *C. lipolytica* yeast was 8 g/l after the same incubation[24] and also exceeded the results of,[27] which obtained 8 g/l. The reason may be due to the source of the isolate and the carbon source used in the production field

Surface Tension and Critical Micelle Concentration Determination

The surface tension relies on the biosurfactant composition. The CMC is known to have lesser concentration than biosurfactants, which yield the highest reduction in the surface tension of water and the beginning of particle formation. Recent studies have shown that different kinds of *Candida* yeast can produce highly active biosurfactants, which, in turn, can reduce water surface tension to very low levels, even less than that produced by artificial active components. Figure 2 demonstrates that components

Table 3: Optimum conditions for biosurfactant production

Carbon source		Nitrogen source		Acidity		Temperature		Fermentation time		Incubator speed		Volume inoculum	
Glucose %	540 nm	Urea %	540 nm	pH	540 nm	Temperature	540 nm	Time	540 nm	Speed	540 nm	Volume %	540 nm
0	0.075±0.001	0	0.066±0.001	2	0.12±0.000	25	0.641±0.003	0	0.056±0.001	100	0.582±0.001	0	0.06±0.000
1	2.251±0.010	1	0.988±0.001	3	0.243±0.000	28	1.205±0.001	24	0.34±0.006	150	1.673±0.001	1	2.301±0.000
2	1.938±0.001	2	2.141±0.003	4	2.185±0.001	30	2.285±0.002	48	0.540±0.013	180	2.017±0.001	2	1.725±0.001
3	1.84±0.000	3	1.219±0.002	5	1.202±0.001	35	1.508±0.002	72	2.241±0.001	200	1.123±0.001	3	1.696±0.002
4	1.732±0.000	4	0.63±0.01	6	1.002±0.004	40	1.007±0.002	96	1.875±0.001	220	0.445±0.002	4	1.639±0.001
5	1.653±0.001	5	0.332±0.000	7	0.985±0.000	42	0.524±0.002	120	1.206±0.000			5	1.605±0.002
6	1.421±0.07	6	0.251±0.000	8	0.63±0.000	45	0.112±0.000	144	1.006±0.002			6	1.407±0.002
7	1.154±0.001	7	0.081±0.001	9	0.215±0.001	50	0.013±0.000	168	0.546±0.003			7	1.394±0.001

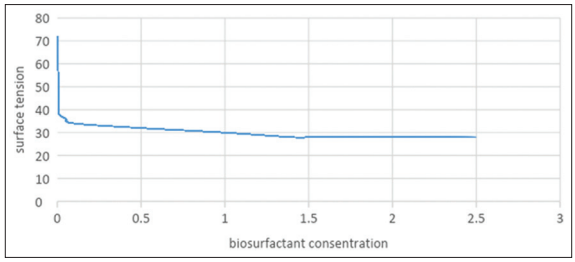

Figure 2: Surface tension and the critical micelle concentration of biosurfactants from *Candida lipolytica* yeast

produced from yeast *C. lipolytica* can reduce surface tension from 71 to 28 mN/m, and the increase in the biosurfactant concentration does not result in the reduction of surface tension at that point. Furthermore, the value of critical micelle concentration was 1.4%, thereby matching the results of,[28] which obtained a surface tension of 28.8 mN/m and surpassed the CMC results of 2%. Many other researchers achieved a surface tension value between 25 and 30.

CONCLUSION

This study concludes that the *C. lipolytica* isolate has a high capacity for biosurfactant production. The modification of the growth medium reflects a positive effect on biosurfactant production, especially when monosaccharide and urea are used as carbon and nitrogen sources, respectively.

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