

## ORIGINAL ARTICLES

### Comparative study of biosurfactants production by *Bacillus licheniformis* and *Candida albicans* for Microbial Enhanced Oil Recovery

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#### ABSTRACT

In this study, the bacterium *Bacillus licheniformis* DSM= 13 strain ATCC 14580 and the yeast *Candida albicans* IMRU 3669 were used for biosurfactants production. Surface properties of the produced biosurfactants were confirmed by determining the emulsification power as well as surface tension. The crude biosurfactants have been extracted from supernatant culture growth, the chemical structure of the produced biosurfactants were confirmed using FTIR analysis, the yields of crude biosurfactants were about 1g/l and 12g/l for *Bacillus licheniformis* and *Candida albicans* respectively. Also, the results revealed that the emulsification power has been increased up to 96% and 65 % with kerosene and the surface tension decreased from 72 to 36mN/m after 72h of incubation with *Bacillus licheniformis* and 45mN/m after 4 days of incubation with *Candida albicans*. The potential application of this bacterial species in microbial-enhanced oil recovery (MEOR) was investigated. The percent of oil recovery was 16.6 % and 8.6% for the bacterial and yeast species respectively, upon application in sand pack column designed to stimulate an oil recovery.

**Key words:** Biosurfactants, *Bacillus licheniformis*, *Candida albicans*, MEOR.

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#### Introduction

Surface active agents which produced by different groups of microorganisms are known as biosurfactants. Biosurfactants reduce surface tension in both aqueous and hydrocarbon mixtures. Biosurfactants can aggregate at interfaces between fluids having different polarities, such as water and oil, leading to the reduction of interfacial tension. Because of their efficiency in lowering interfacial tension, biosurfactants have been employed for the enhancement of oil production especially in tertiary oil recovery. Low toxicity, high biodegradability and ecological acceptability are among the main characteristics of these surface active materials (Banat, 1995; Desai and Banat, 1997; Mulligan, 2005; Nitschke and Pastore, 2006; Fang *et al.*, 2007 and Yin and Qiang, 2008). These favorable features make biosurfactants potential as one of the best alternatives of chemically synthesized surfactants in a variety of applications (Banat *et al.*, 2005 and Demin *et al.*, 1999). Biosurfactants can be categorized into four main groups: lipopeptides and lipoproteins, glycolipids, phospholipids, and polymeric surfactants (Mulligan *et al.*, 2001).

Biosurfactants are widely used in different industries, such as cosmetics, special chemicals, food, pharmaceuticals, agriculture, cleaners and microbial enhanced oil recovery (MEOR) (Sharma and Pant 2000; Daoshan *et al.*, 2004; Joshi *et al.*, 2008 and Lotfabada *et al.*, 2009). The last mentioned application has attracted more attention because only 30% of oil present in a reservoir can generally be recovered using primary and secondary recovery techniques (Banat, 1995). MEOR is considered as a tertiary recovery technique that could recover the residual oil using microorganisms or their products (biosurfactants). However, the application of biosurfactants in microbial enhanced oil recovery depends on their stability at extreme conditions of temperature, salinity and pH, or surface activities (Joshi *et al.*, 2008). Stimulation of microorganisms that produce biosurfactants and degrade heavy oil fractions in situ reduces the capillary forces that retain the oil into the reservoir and decreases oil viscosity, thus promoting its flow. As a result, oil production can be increased (Gudiña *et al.*, 2012a).

The present study aimed to investigate the potential of *Bacillus licheniformis* and *Candida albicans* in biosurfactant production and the ability of these microbes to enhance the microbial oil recovery.

#### Materials and Methods

##### *Microorganisms:*

The bacterium *Bacillus licheniformis* has been isolated from oil reservoir which located in Niage field, Badr El-din Petroleum Company, west desert, Egypt and identified as *Bacillus licheniformis* DSM= 13 strain ATCC 14580 applying the 16S rDNA sequence with 99% identity.

*Candida albicans* IMRU 3669 used in the present study were purchased from Microbiological Resource Center (MIRCEN), Faculty of Agriculture, Cairo, Egypt.

*The growth kinetic and screening for the production of biosurfactant:*

The bacterial strain *Bacillus licheniformis* was streaked on a nutrient agar slant and incubated for 24hs at 30°C. Two loops of culture were inoculated in 40 ml of nutrient broth in a 100 ml Erlenmeyer flask and incubated in a rotary shaker 150 rpm at 30°C for 8–12hs until cell numbers reached  $10^8$  CFU/ml. This was used as inoculum at the 5% (w/v) level. For biosurfactant production, a mineral salt medium with the following composition was utilized: 2.5 g/l of NaNO<sub>3</sub>, 0.1 g/l of KCl, 3.0 g/l of KH<sub>2</sub>PO<sub>4</sub>, 7.0 g/l of K<sub>2</sub>HPO<sub>4</sub>, 0.01 g/l of CaCl<sub>2</sub>, 0.5 g/l of MgSO<sub>4</sub>·7H<sub>2</sub>O, and 5 ml of a trace element solution (Haghighat *et al.*, 2008). The respective carbohydrate (glucose) was added to make a final concentration 2%. The concentration of yeast extract was 3%. Cultivation studies have been done in 500 ml flasks containing 150 ml medium at 30°C for 48hs. Experiments were conducted in three independent triplicates (Haddad *et al.*, 2008).

*Candida albicans* was cultivated on Yeast malt broth medium (Atlas, 2004) used for developing the seed culture contained (g/l) glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; and pH was adjusted to 6.0. Erlenmeyer flasks (250 ml) containing 50 ml of the seed culture medium were autoclaved at 121°C for 20 min and inoculated with a loop full of the microorganism freshly grown on yeast malt agar slant. The culture was then incubated for 24 hs at 30°C and 150 rpm in rotary shaker. The final biomass weight after 24 hs was estimated to be 10 g l<sup>-1</sup>.

For sophorolipids (SL) production by *Candida albicans* the medium composed of (g/l) Glucose, 100; sunflower oil, 100; yeast extract, 1; urea, 1, was inoculated with 5% (v/v) seed culture. Cultivation studies have been done in 2000 ml flasks containing 1000 ml medium at 26°C for 5 days and the pH was adjusted to 6.0. Samples were taken periodically for carrying out the surface properties of the medium in different time intervals (Achleshand Kannan, 2009).

*Extraction of the crude biosurfactants:*

The bacterial broth (10ml) was inoculated into the medium MSM (1000 ml) using glucose as a sole carbon source and the pH value was adjusted to 7.5. Incubation was carried out at 30°C, 150rpm, for 72hs. The extraction technique is a combination of acid precipitation and solvent extraction (Vateret *et al.*, 2002). The broth culture sample was centrifuged (at 4°C using 13000xg for 15 min). The obtained supernatant was treated by acidification to pH 2.0 using 6 M HCl, and the acidified supernatant was left overnight at 4°C for complete precipitation of the biosurfactants. Remove supernatant to obtain pellet then extracted with methanol for 2hs while stirring continuously. Filter methanol to remove remaining material and evaporate to dryness using a rotary evaporator.

Extraction of sophorolipids (SL) is carried out by solvent extraction method, but without acidification after centrifugation at 4°C using 13000 xg for 15 min (Nunez *et al.*, 2001). The obtained supernatant was extracted three times with an equal volume of ethyl acetate, shaking vigorously each time and allows the two layers to separate in a separating funnel. Transfer bottom aqueous layer and the top ethyl acetate layer to separate flasks. Re-extract the aqueous portion twice more or until no further color persists in the ethyl acetate layer. Add 0.5 g of magnesium sulfate per 100 ml of ethyl acetate portion, to remove the traces of water present, filter to remove materials other than biosurfactant then evaporate using rotary evaporator to yield a brown gum extract.

*Chemical structure of the produced biosurfactants:*

Infrared (IR) spectra of the biosurfactant (a film of each sample on KBr pellet) were obtained using a Nicolet IS-10FTIR spectrometer. IR spectra were conducted between 4,000 and 500 cm<sup>-1</sup> with a resolution of 1 cm<sup>-1</sup> (Ghojavand *et al.*, 2008).

*Surface properties:*

Surface properties including surface tension, Emulsification index (E<sub>24</sub>) and Foaming were determined as indicators of biosurfactant production these measurements were done for three replicates.

*Surface tension:*

Surface tension was measured on a ring tensiometer (Krüss-tensiometer K6) using the broth supernatant solution (20ml) at 30°C, samples were taken and tested periodically in different time intervals (Nitschke and Pastore, 2006).

*Emulsification index (E<sub>24</sub>):*

Emulsification power of the produced biosurfactant in the culture supernatant was measured by adding kerosene (6ml) to the aqueous phase (of culture supernatant) and vortexing for 2 mins, after 24hs the emulsion index (E<sub>24</sub>) was calculated according to the following equation Cooper and Goldenberg (1987):

$$(E_{24}) = 100 (\text{height of the emulsion layer/ the total height})$$

*Foaming:*

Foaming of biosurfactant in culture medium was determined by shaking vigorously the supernatant (10 ml) for 2 mins, and then foaming was calculated according to the following equation Mohammed (1997):

$$\text{Foaming} = (\text{height of foaming/ total height}) \times 100$$

*Application of the produced biosurfactant for oil recovery enhancement:*

The potential application of the biosurfactants for MEOR was evaluated using the sand pack column technique designed to stimulate an oil recovery, this technique described by [Sutharet *et al.*, (2008)]. Three symmetrical columns were used for this test, column 1 for *Bacillus licheniformis*, column 2 for *Candida albicans* and column 3 for control. The operation of the sand pack column was as follows:

1. Saturation of the sand pack with brine: The column was flooded with brine under pressure to ensure its 100% saturation with brine, pore volume of the column was calculated by measuring the volume of brine required to saturate the column (PV).
2. Saturation of the sand pack with oil: the oil was collected (from Niage 1 oilfield Badr El-din Petroleum Company oil). The oil filled in a tank was passed under pressure into the sand pack column, in the same way as brine, until residual brine saturation was reached. As oil entered into the column, brine was displaced and discharged from the pack through a tubing inserted into the bottom end of the column. Initial oil saturation (S<sub>oi</sub>) was calculated by measuring the volume of brine displaced by oil saturation, also called original oil in place (OOIP).
3. Brine flooding: the sand pack was again flooded with brine until there was no oil coming in the effluent, i.e. residual oil saturation (S<sub>or</sub>) was reached. The amount of crude oil retained in the sand pack was determined volumetrically. S<sub>or</sub> was calculated by measuring the volume of displaced oil.
4. Biosurfactants flooding: this was done in a manner similar to oil and brine floods. 0.6 Pore volume of crude biosurfactant was passed through the column at a flow rate of approximately 2.5ml/min and incubated for 24hs then; the column was again flooded with brine. Discharges from the column were collected in 25ml quantities to measure the amount of oil recovered using crude biosurfactant.

**Results and Discussion***Growth kinetic and evaluation of biosurfactant production:*

The stationary phase of *B. Licheniformis* appeared in 24 up to 72hs as shown in (Table 1). On the other hand, surface tension and the emulsification power of the supernatant obtained from the corresponding broth culture for *B. Licheniformis* and *Candida albicans* was taken as an indication of the ability to produce biosurfactants. The maximum surfactin production has been achieved in 72hs of incubation during the stationary phase of the growth curve, so that the production of biosurfactants is considered as secondary metabolites, and this was clearly evident in the results as surface tension decreased gradually to 36mN/m with increasing emulsification power up to 95%. While the maximum sophorolipids (SL) production was detected in 4 days incubation in case of *candida albicans*, where surface tension of the culture medium had been reduced to 45mN/m with increasing emulsification power to 65% (Table 2). Similar results have been reported by Daniel *et al.*, (1998) and Solaiman *et al.*, (2004). Moreover, Lin (1996) reported that most biosurfactants are considered as secondary metabolites, some may play essential roles in the survival of the producing microorganisms either through facilitating nutrient transport or microbe host interactions, or as biocides. It has been suggested that the production of biosurfactants can enhance emulsification and solubilization of hydrocarbon substrates, and therefore facilitates the growth of microorganisms on hydrocarbons. By secreting biosurfactants into the growth medium, microorganisms relying on non-polar substrates as sole carbon sources, ensure the timely supply of carbon source to maintain their survival and growth. One of the most important properties, which should be existing in biosurfactants, is the foaming power. The observed foaming due to the biosurfactant obtained in the supernatant of *Bacillus licheniformis* was found to be 51%, while that value reached 40% in the case of yeast. The stable foaming coupled with reduction in surface tension and increasing in the emulsification power of a medium is considered as a qualitative indication of biosurfactant production (Das *et al.*, 2009). It is worth to note

that the formation of foaming during enrichment of a culture in a mineral medium with glucose as carbon source was potential for application of biosurfactants in microbial enhanced oil recovery Bordoloi and Konwar, 2008).

**Table 1:** Evaluation of growth kinetic and biosurfactant production of *Bacillus licheniformis* at different time intervals.

Incubation period Hs	Log count	Emulsification power (E <sub>24</sub> ) %	Surface tension (S.T.) mN/m
Control*	0	0	59
0**	8.2	0	59
3	8.2	0	56
6	8.8	25	50
12	9.0	62	50
24	9.3	75	46
48	9.3	85	42
72	9.7	96	36
96	9.1	80	45

Control\*: Sample without inoculum 0\*\*: Inoculation time

**Table 2:** Evaluation of growth kinetic and biosurfactant production of the yeast *candida albicans* at different time intervals.

Incubation period Days	Log count	Emulsification power (E <sub>24</sub> ) %	Surface tension (S.T.) mN/m
Control*	0	0	50
0**	6	0	50
1	7	5	47
2	7.2	40	46
3	7.2	50	46
4	7.3	65	45
5	7.3	10	46

Control\*: Sample without inoculum 0\*\*: Inoculation time

#### Extraction of crude biosurfactant:

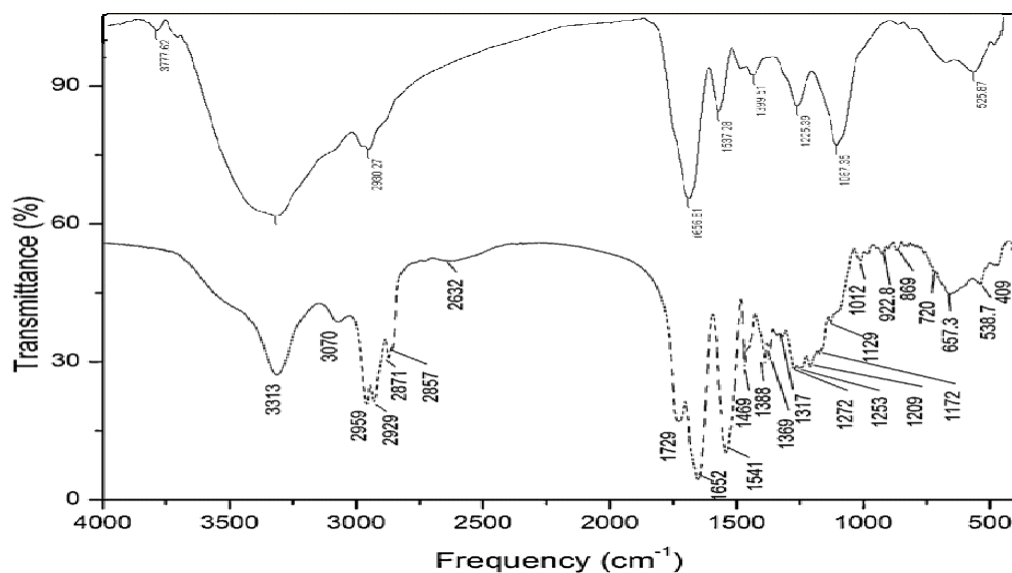
*Bacillus licheniformis* ATCC 14580 was grown under the optimum conditions and the recovery of biosurfactant from cell free culture was carried out by the classical techniques. They include solvent extraction, precipitation and crystallization. The yield of surfactin was relatively low (1g/l). In fact, modification of the succeeding fermentation process is expected to raise the production rate. This is supported by the results of (Rodrigues *et al.*, 2006) where they reported that the potential use of alternative fermentative medium instead of the synthetic medium for biosurfactant production by *Lactococcus lactis* 53 and *Streptococcus thermophilus* effectively proceeded with high yields and productivities of biosurfactant. An increase about 1.2–1.5 times the mass of the produced biosurfactant per gram cell dry weight was achieved. About 1.8 g/l dry weight of the crude bioemulsifier was obtained after the partial purification process by *B.licheniformis* K125 (Suthar *et al.*, 2008). On the other hand, the yield of sophorolipids (SL) produced from *candida albicans* IMRU 3669 was relatively high 12g/l if compared with bacterial biosurfactant. Solaiman *et al.* (2004) observed that the yeast produced about 21g/l of sophorolipids, and its yield slightly improved to a value of 53g/l when grown on medium containing only soy molasses and oleic acid.

#### Structural characterization:

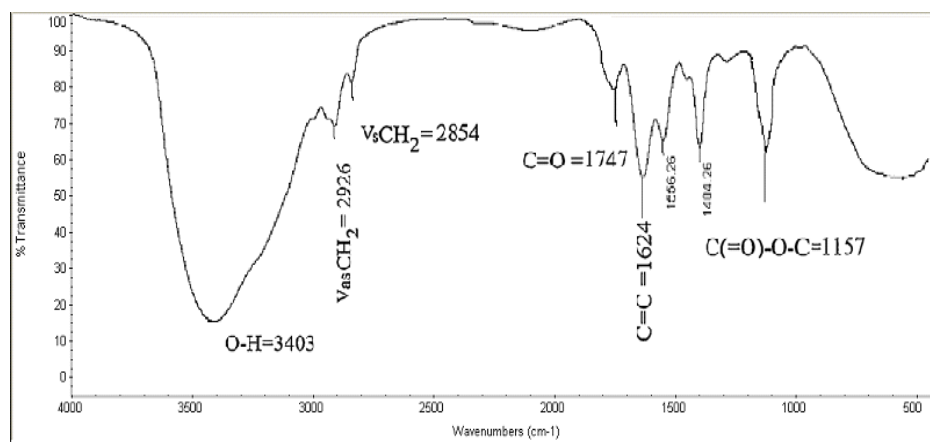
The infrared spectrum of the *Bacillus licheniformis* biosurfactant and the spectrum of a standard sample of surfactin from *B.subtilis* produced from de Oliveira *et al.*, (2013) were shown in Fig. (1). In both spectra it is possible to observe bands characteristic of peptides (wave length 3430 NH, wave length 1655 CO, and wave length 1534 CN) and aliphatic chains (wave length 3000-2800), indicating that this compound is a lipopeptide. Similar results were obtained by other authors de Oliveira *et al.*, (2013) with *B.subtilis* and Lin *et al.*, (1994). IR spectra showed no significant difference of the biosurfactant produced in this work or the standard sample.

The FTIR spectra of the sophorolipids (SL) which obtained from *candida albicans* was shown in Fig. 2, which reveals a broad band at 3403 cm<sup>-1</sup> corresponding to the O–H stretch in its structure.

The spectra also revealed that asymmetrical stretching (ν<sub>as</sub> CH<sub>2</sub>) and symmetrical stretching (ν<sub>s</sub> CH<sub>2</sub>) of methylene groups occurred at 2926 and 2854 cm<sup>-1</sup>, respectively. Further, since lactones and esters have two strong absorption bands arising from C–O and C=O stretching, the C–O absorption band at 1747 cm<sup>-1</sup> may include contributions from these groups (lactones, esters, or acids). The stretch of C–O band of C(=O)–O–C in lactones exists at 1157 cm<sup>-1</sup>. Moreover the band at 1404 cm<sup>-1</sup> corresponded to the C–O–H in-plane bending of carboxylic acid (–COOH) in the structure of the product. All these structural details of the product were found similar to those reported in the literature (Yongmei and Kwang, 2001) which therefore confirmed the fermentation product to be SL group of compounds.



**Fig. 1:** A comparison of FTIR spectroscopy of standard surfactin (in down) and crude extracellular biosurfactant (in up) produced by *Bacillus licheniformis*.



**Fig. 2:** FTIR spectroscopy of crude sophorolipids produced from *Candida albicans*.

#### Oil recovery using sand-pack column:

Microbial biosurfactants had been used to perform the oil recovery technique with crude oil using sand-pack column. Biosurfactant produced from bacteria and yeast can reduce the surface tension value up to 36mN/m and 40mN/m and emulsify hydrocarbon about 96% and 65% respectively. So, exhibit desirable properties for application in MEOR. Table (3) showed that, both *Bacillus licheniformis* and *Candida albicans* have the ability to enhance oil recovery with the sand-pack column. The pore volume (PV) of the column about 43ml, OOIP (original oil in place) of the column is 37 ml and 35 ml, after water flooding process, 32.4% and 42.8% of the oil remained trapped into the column1 and column2 respectively. When the biosurfactant of *Bacillus licheniformis* was introduced into the column1 and incubated for 24hs at 35°C, the amount of oil recovered after biosurfactant flood was 2ml. This means that additional crude oil was recovered (16.6%). also when the biosurfactant of *Candida albicans* was introduced into the column2 and incubated for 24hs at 35°C, the amount of oil recovered after biosurfactant flood was 1.3ml. This means that additional crude oil was recovered (8.6%). It can be concluded that bacterial biosurfactant more efficient than biosurfactant produced from yeast. This result is in agreement with that of (Gudiña *et al.*, 2012b and Suthar *et al.*, 2008).

**Table 3:** Summary of the results obtained in sand-pack column for crude oil recovery using *Bacillus licheniformis* and *Candida albicans*.

Parameter	Control	<i>Bacillus licheniformis</i>	<i>Candida albicans</i>
PV (ml)	45	43	43
OOIP (ml)	38	37	35
Soi (%)	84.4	86	81
Swi (%)	15.6	14	19
Sorwf(ml)	20	25	20
OOIP-Sorwf(ml)	18	12	15
Sor (%)	47.4	32.4	42.85
Sorbf(ml)	1	2	1.3
AOR (%)	5.6	16.6	8.6

Where: OOIP; Original oil in place, Soi; Initial oil saturation,

Swi; Initial water saturation, Sor: Residual oil saturation,

Sorbf::Oil recovered after biosurfactant flooding,

Sorwf: Oil recovered after water flooding

AOR: Additional Oil Recovery

Soi(%) = OOIP/PV\* 100 (Eq. 1)

Swi (%) = PV- OOIP/ PV\* 100 (Eq. 2)

Sor(%) = OOIP-Sorwf/ OOIP\* 100 (Eq. 3)

AOR (%) = Sorbf/ OOIP-Sorwf \* 100 (Eq. 4)

### Conclusion:

In the present work *Bacillus licheniformis* and *Candida albicans* were used for biosurfactants production.

The produced biosurfactants were able to decrease the surface tension, and increased the emulsification capacity; also the evaluation of those biosurfactants in microbial enhanced oil recovery was performed. It was found that the bacterial biosurfactant has the ability to recover about 16.6% of the crude oil entrapped in the sand-pack column, where the additional oil recovery was 8.6 % with yeast strain.

So we can concluded that the bacterial biosurfactant exhibit high efficiency than biosurfactant produced from yeast.

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