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Investigation of bacterial biosurfactants production using waste industrial oil as carbon source in the electric power plants.

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Abstract

The production of the biosurfactants (BS) from industrial organic waste has gaining significant interest due to its potential to reduce production costs. The production and properties of biosurfactants have been investigated from eight bacterial strains of *Bacillus sp*. and *Peribacillus frigoritolerans*. Which were previously isolated from biofouling anion exchange resin used in electric power plants (Egypt). Waste industrial oil (transformer and lubricating) was used as alternative cheap carbon sources for the biosurfactants production. Screening and selection of biosurfactant producer(s), were examined by using different parameters includes; blood hemolysis test, surface tension measurements, oil spreading test, emulsification index and biosurfactant yield. The stability values showed significant emulsification results at pH 12 resulting of *Bacillus licheniformus BaDB24* (75.6%) > *Bacillus cereus SH16* (63.05%)> *Peribacillus Frigoritolerans* (29%). The stability of temperature at 75°C that showed emulsification values resulting for *Peribacillus Frigoritolerans* (60.5%)> *Bacillus licheniformus BaDB24* (35.2%)> *Bacillus cereus SH16* (25%). The stability of salinity at 14% resulting that showed emulsification values of *Peribacillus Frigoritolerans* (60.8%)> *Bacillus licheniformus BaDB24* (58.95%)> *Bacillus cereus SH 16* (41.7%). The microorganisms studied were able to produce a biosurfactant from industrial waste oil. From tested strains, two bacteria of *Bacillus licheniformus BaDB24* and *Peribacillus frigoritolerans* were chosen to be the potential choice for biosurfactant production.

Keywords: biosurfactants, stability, lubricating oil, transformer oil.

1. Introduction

Biosurfactants are surface-active compounds possess both moieties (hydrophilic and hydrophobic) which could solubilize the organic substances by interacting with the phase boundaries in heterogeneously system [9]. Biosurfactants synthesized biologically from various microbial strains mainly extracellular or attached to cells, that utilizing hydrocarbons as carbon

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source from different substrates, increasing surface interactions, enhancing emulsification and solubility of hydrocarbons, and improving hydrocarbon bioavailability [25,13,55].

Biosurfactants have a significant importance as they are produced from natural green material, and have significant advantages over their synthetic surfactant. They are ecofriendly, high selective, biodegradable, biocompatible, bioavailable, less toxicity and ecologically acceptable, tolerated to extreme condition as pH, temperature and high salt concentrations, that they can be utilized in many industries for example: oil refinery, cosmetics, agriculture, pharmacy, food, and restoration of polluted environment [6,56].

Biosurfactants can produced by many bacteria of a wide variety of genera, as *Pseudomonas, Bacillus sp, Brevibacterium, Stenotrophomonas, Gordonia, Rhodococcus, Nocardiopsis and Alcaligenes, Pseudoxanthomonas Serratia, Aeromonas, Streptomyces,* and *Citrobact.* [39]. *Bacillus sp* was the potential biosurfactant producing bacteria, that was known to produce different lipopeptides. Several isoforms and analogs exist showing significant structural heterogeneity and possessing potentil applications [27].

[8,11] indicated that biosurfactants growing interest. A typical medium for production of biosurfactants contains pure chemicals as glucose which resulting in the high biosurfactant titres but have relatively high production costs with negative effects on the environment, so practical applications remain limited [64]. Potential solutions to this challenge include developing and scaling up technological processes and reducing expenses associated with the raw materials utilized [33].

The using of organic wastes as a primary raw material for production of biosurfactants had received significant attention due to savings its potential cost, reducing environmental waste, and promoting the integration of waste materials into recycling cycles at the same time [54,28,11].

[36,12] illustrated organic waste examples as contamination oil and oil industrial waste products like waste transformer oil, waste engine oil and waste frying oil from food industry which may cause severe harm.

The waste lubricating oil, called spent oil or used - lubricant, was obtained after servicing and subsequent draining from, generators, industrial machines and automobiles. It has long chain (C16–C36) of saturated hydrocarbons and more than 75 % cyclic alkanes which making it resistant

to microbial degradation. In addition, the presence of PAHs, toxic metals, etc. in used oil inhibits the microbial degradation process [10].

Mineral oil was the most used oil in transformers, that produced from crude oil [63]. [29] reported that the hydrocarbon of naphthenic and paraffinic were the base of all transformer mineral oils. The main reason for the degradation of the hydrocarbon base of oils is oxidation forming peroxides, alcohols, ketones and acid**.**

In the present study, eight strains previously identified were tested for their ability to synthesize biosurfactants on various carbon sources. Subsequently, the most effective microorganisms and waste industrial oil both were selected, giving the highest biosurfactant productivity on most tests, then the stability of biosurfactants was studied.

2. Materials and methods

2.1. Microorganisms:

Eight strains of *Bacillus licheniformus BaDB24*, *Bacillus pumilus SH12*, *Bacillus cereus SH16*, *Bacillus cereus SH33*, *Bacillus cereus SH42*, *Bacillus cibi SH43*, and *Bacillus thuringiensis SH116* and *Peribacillus frigoritolerans* - previously known as *Brevibacterium Frigoritolerans* SH115 [46] - used in this study was isolated from a biofilm of the anion exchange resin of Shoubra El - Khiema and Damietta electric power plants in Egypt [1].

2.2. Media and Culture Conditions:

Modified **salt peptone (SP) medium**, containing (g/L):10 g peptone,1 g yeast extract,0.5 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.7 g $CaCl_2 \cdot 3H_2O$,0.05 g $MnCl_2 \cdot 4H_2O$,0.2 g K_2HPO_4,0.2 g KH_2PO_4 was used for screening of different bacterial biosurfactants production. **Halophile moderate (HM) medium,** with the following composition (g/L): 5 g proteose peptone, 10 g yeast extract, 2 g KC1, 1 g $MgSO_4 \cdot 7H_2O$,1 g glucose, 0.36 g $CaCl_2 \cdot 3H_2O$, 0.23 g NaBr,0.2 g K_2HPO_4, 0.2 g KH_2PO_4, trace of $FeCl_3$ and 1 ml distilled water. The pH was 7.2. It was utilized for biosurfactant production from different carbon sources [37].

2.3. Carbon source:

Different carbon sources as glucose (Nasr company, Egypt), crude oil, waste transformer oil (insulating oil) from Ezz steel transform station and waste lubricating oil from Shoubra El -

Khema electric power plant (**Table 1**) was also used in selection the best bacteria strains in biosurfactants production.

Table 1: Physical and chemical properties of oil waste samples from transformer station and electric power plant:

Properties	**Standard method**	**Transformer oil**	**lubricating oil**
Specific gravity at 15 °C	ASTM D1298	0.8747	0.8962
Kinematics Viscosity at 40°C (CST)	ASTM D445	9.62	57
Color	ASTM D 1500	4	More than 8
Total acidity (mg KOH/g oil)	ASTM D974 IPI	0.14	1.2
Flash point open (°C)	ASTM D 92	-	216
Flash point closed (°C)	ASTM D 93	152	-
Impurities	-	Not present	Not present
Water content (mg/l)	-	10	-
Break down voltage (K.V)	-	70	-
Power factor (unit)	-	0.1619	-

-, Not detected.

2.4. Screening of biosurfactants production using different bacterial strains:

The biosurfactant production were studied on SP media (Salt Peptone broth) supplemented with 1% crude oil and 3% NaCl (**Kheiralla *et al.*,2013**). The medium pH was adjusted to 7.0 and autoclaved at 121 °C for15 min. Pre-inoculum of pure bacteria was prepared as one loop of bacterial culture were inoculated in 50mL of nutrient broth and incubated at 37°C for 24h in the rotary shaker at 150 rpm, this was used as a standard inoculum. Bacterial suspension (1 ml of 10^3 CFU/ml) of OD_{600} (optical density) 0.5 (Thermo scientific spectrophotometer**,** model Aquamate, England) was equal inoculated to 250 ml flask containing 50 ml media and were incubated at 37°C with 180 rpm and was incubated for 96h. The cell free supernatant was obtained by centrifugation at 6000 rpm for 15 min (4 °C) to separated bacteria [58]. The supernatants were filtered to remove excess oil and used to examine the quality and efficiency of the biosurfactants production by the following screening tests to select the most effective bacteria. The bacteria were selected based on the highest value obtained from hemolytic activity, emulsification index (EI24) and oil spreading of its biosurfactant that reduce surface tension properties.

2.4.1. Hemolytic activity

A pure bacterial culture was streaked on blood agar having 5% (v/v) human blood and was incubated for 24–48 h. at 37 °C. The diameter of clear zone around the bacterial strain was measured that confirmed biosurfactants production [47].

2.4.2. Surface tension measurement

The surface tension was measured by the ring method using a Du Nouy tensiometer (Sigma 702EI Tensiometer, KSV Instruments Ltd, Finland) at room temperature on cell free supernatant after the bacterial culture were centrifuged. Distilled water was used for the calibration of the instrument [19].

2.4.3. Emulsification Index (EI24)

The ability of bacteria strains to emulsify the hydrocarbon was examined as mentioned by [47]. Briefly equal volume of olive oil (2mL) and cell free medium (2mL) were mixed in a test tube and homogenized at high speed by vortex (VWR vortex) for 2 min. The screening tests were performed in triplicate and distilled water was used as the control. The emulsification activity (EI24%) was calculated after 24h. using the following equation by [24]:

$$\text{Emulsification index (\%)} = \frac{\text{Height of emulsion layer (cm)}}{\text{Total height (cm)}} \times 100$$

2.4.4. Oil spreading test

In the Petri plate, distilled water of 30 mL was poured then 20µl of crude oil which liquefied in diesel oil was added to the surface of distilled water forming thin film. Then, 20 µl of cell-free supernatant was put on the center of the oil film. The diameter of clear zone formed on the oil surface was measured after 30 s and compared to that of distilled water which served as a negative control [18]. The screening tests were performed in triplicate and distilled water was used as the control.

2.5. Effect of different carbon source on biosurfactant production:

Glucose was used as control and waste oil (lubricating oil and transformer oil) were used as alternative low-cost carbon source. They were supplemented in HM media with 0.1% of glucose concentration or 1% concentration of different waste oil [37]. The medium pH was adjusted to 7.0 and sterilized at 121 °C for 15 min. Optical density of overnight cultural broth media in nutrient

broth was adjusted to 0.5 at 600 nm (count of bacteria 10^5 CFU/ml) and equal volume was inoculated to 250 ml flask containing 100 ml HM media had 2%NaCl. After incubation period 72h at 37°C with 180 rpm. The cell culture was centrifuged for 20 min at 6000 rpm and at 4 °C to get cell free supernatant. The supernatant was filtered to remove excess oil and was used to examine biosurfactants production quality. The waste oil was selected based on the highest value obtained from surface tension, emulsification index (EI24), oil spreading tests and production yield.

2.6. Extraction and the recovery of the biosurfactant:

The cell culture was centrifuged at 6000 rpm on a centrifuge for 20 min at 4 °C to get the cell free supernatant. To extract the biosurfactant, the supernatant pH was adjusted to 2 by 3N HCl and was cooled at 4°C for 30-45 min to collect the biosurfactants then was centrifugated again at 6000 for 20 min to precipitate the biosurfactants as pellets, the broth was discarded [17]. The white precipitate (pellets) was washed with distilled water and dried on air and weighed. For further purification, the crude biosurfactant was mixed with equal volume of chloroform:methanol (2:1) and left on dark place overnight for evaporation and weighted as g/L [47].

2.7. Biosurfactant Stability Studies:

Biosurfactant stability was examined under different conditions and expressed as emulsification index (E24) % as described before. After bacteria was grown on HM media supplement with 1% lubricating oil as carbon source for 72h on shaker at 180 rpm at 37° C. The culture was centrifuged at 6000 rpm for 20 min (4 °C) to get the cell free supernatant. Thermal stability of biosurfactant was estimated by maintaining the cell-free supernatant at constant temperature between 25 and 120 °C for 30 min and then cooled to room temperature. The pH effect was evaluated in the range of pH 2–12 by using 1M HCl or 1M NaOH [47]. The salinity was assessed using NaCl (0–14% w/v). All assays were performed in triplicates.

2.8. Statistical analysis

Data obtained were analyzed using The Statistical Package of Social Science (SPSS) by Minitab version 18 software using one-way ANOVA to estimate the statistical parameters, followed by Duncan range test. Data were collected from three independent experiments and the results were expressed as the mean± standard deviation. A probability value of <0.05 was used as criterion for statistical significance.

3. Results and discussion:

The used of waste as a feedstock by microorganisms for production of value-added products (as biosurfactants) has opened new ways contributing to environmental sustainability which significantly lowest the overall process cost [30].

3.1. Screening bacterial strains for biosurfactant production:

Among eight bacterial strains tested, six strains were positive for hemolytic activity (**Table 2**). They showed hydrolysis zone around the colonies **(Fig. 1: a,b,c,d,e,j)**. The strains of *Bacillus licheniformus BaDB24*, *Bacillus pumilus SH12*, *Bacillus Cereus SH16* and *Peribacillus frigoritolerans* exhibited the most significant hemolytic activity as 3.5, 3.7, 3.4, 4.4 cm respectively, While *Bacillus Cereus SH 33 and Bacillus Cereus SH42* showed low values as 2.8 and 1.73 cm respectively. Two strains *Bacillus cibi SH43* and *Bacillus thuringiensis SH116* were negative, as they didn't show any hydrolysis **(Fig. 1: f,k)**. The strains which could lyse red blood cells and produced inhibition zone of hemolytic assay were directly affected by increasing concentration of biosurfactants. This ability may be due to the surface activity of biosurfactants secreted by cells.

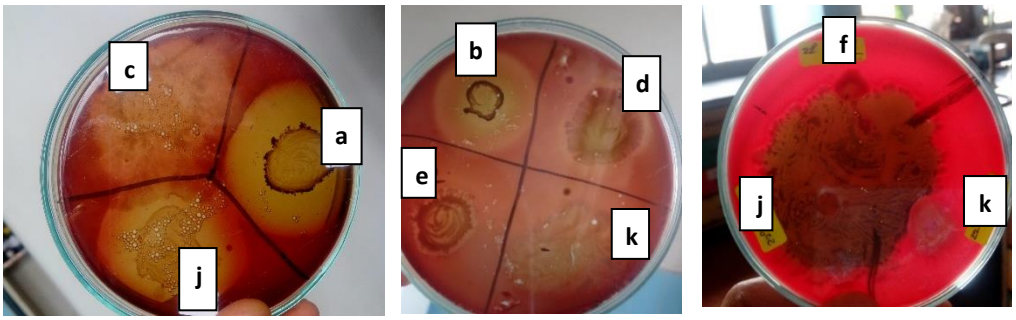

Fig. 1: Hemolytic activity of different bacteria stains studied, *Bacillus licheniformus BaDB24*, **a**; *Bacillus pumilus SH12*,**b**; *Bacillus cereus SH16*,**c**; *Bacillus Cereus SH 33*,**d**; *Bacillus Cereus SH42*, **e**; *Bacillus cibi SH43*,**f;** *Peribacillus frigoritolerans*,**j**; *Bacillus thuringiensis SH116*,**k.** showed a clear zone around bacteria a,b,c,d,e, and j. Bacteria f and k not showed clear zone around cell growth.

As previously reported by [2] several screening methods could be included in primary screening of potential biosurfactants production. Where a single method isn't confirming the determination of biosurfactants production from isolated bacteria [68,66]. [50] showed a significant relationship between the hemolytic activity and biosurfactant production, however, not

all biosurfactants have hemolytic activity and compounds other than biosurfactants may cause hemolysis [31].

The obtained results agree with [41] who mentioned that *Bacillus sp.* strain LP04 can lyse red blood cells. Also, [13] reported that a *Bacillus sp* of sample B1 and B2 exhibited the topmost hemolytic activity as 2cm zone. The increasing of biosurfactants concentration could lead to form larger hemolytic zone on blood agar around all side of colony **(Fig. 1: a,b,c,d,e,j)** as reported by [23]. The mechanism causing of the hemolysis by biosurfactant compounds can be in the form of normal membrane dissolution at high biosurfactant concentrations or increased membrane permeability to small solutes at low biosurfactant concentrations, as a result of osmotic lysis [60]. Red cells lyses caused by inducing membrane reorganization and giving changes in cell morphology. The hydrophilic part modulates the penetration of the hydrophobic part, which disrupts the cell membrane [42].

Table 2: Screening of biosurfactants production using different bacterial strains:

Bacterial isolates	Hemolytic activity cm	Surface tension mN/m	EI24 (%)	Oil spreading cm	Weight g/l
Bacillus licheniformus BaDB24	$3.5^{ab}\pm0.5$	$32.65^{a}\pm1.39$	$37.5^{b}\pm0$	$0.85^{a}\pm0.05$	$0.296^{a}\pm0.05$
Bacillus pumilus SH12	$3.7^{ab}\pm1.1$	$45.9^{b}\pm1.14$	$31.25^{c}\pm1.25$	$0.45^{cd}\pm0.05$	$0.132^{e}\pm0.05$
Bacillus cereus SH16	$3.4^{ab}\pm0.6$	$45.6^{b}\pm0.82$	$37.5^{b}\pm0$	$0.4^{d}\pm0$	$0.238^{c}\pm0.05$
Bacillus Cereus SH 33	$2.8^{b}\pm0.4$	$49.86^{d}\pm0.4$	$36.6^{b}\pm0.9$	$0.55^{b}\pm0.05$	$0.036^{f}\pm0.05$
Bacillus Cereus SH42	$1.73^{c}\pm0.07$	$45.35^{b}\pm0.42$	$37.75^{b}\pm0.25$	$0.45^{cd}\pm0.05$	$0.168^{d}\pm0.05$
Bacillus cibi SH43	0^{d}	-	-	-	-
Peribacillus frigoritolerans	$4.4^{a}\pm0.6$	$51.46^{d}\pm1.23$	$42^{a}\pm0$	$0.53^{bc}\pm0.08$	$0.276^{b}\pm0.05$
Bacillus thuringiensis SH116	0^{d}	-	-	-	-

Growth on SP media contained 1% crude oil, for 96h at 37 °C, pH 7, inoculum of 10^3 CFU/ml,3% NaCl. Presented values are the means ± standard deviations and different letters from a-f in the same columns indicate a significant difference from each other according to the analysis of variance ($p < 0.05$). -, not detected.

Primary screening of biosurfactants on SP medium using 1% crude oil as carbon source (**Table 2**) showed that *Bacillus licheniformus BaDB24* was the most statistically significant bacteria for most screening tests. The recorded results of surface tension was 32.65±1.39 mN/m, emulsification index was 37.5±0 %, Oil spreading was 0.85 cm and produce high yield was 0.296 g/l.

On the other hand, *Peribacillus frigoritolerans* have different performance that was recorded surface tension as 51.46±1.23 mN/m, emulsification index was 42±0 and oil spreading was 0.53±0.08 cm and can produce biosurfactant of 0.276 g/l.

But *Bacillus cereus* SH16 produce more biosurfactant of 0.238 g/l than *Bacillus pumilus SH12* of 0.132 g/l (**Table 2**). The biosurfactants yield showed significant effect for the three bacteria, *Bacillus licheniformus BaDB24, Bacillus cereus SH16* and *Peribacillus Frigoritolerans*.

According to the significant results of most screening tests (**Table 2**), the strains of *Bacillus licheniformus BaDB24*, *Bacillus cereus SH16* and *Peribacillus frigoritolerans* were identified as a promising biosurfactants producers and those strains were selected for further tests.

Surface tension reduction of bacterial strains studied were between (51.46 -32.65 mN/m) (**Table 2**). In the same direction [35] reported that significant surface-active properties is the reduction of surface tension to around 40 mN m^{-1}.

Bacterial strains studied were recorded emulsification activity between (31.25 - 42%) (**Table 2**). Emulsification activity is an important property of biosurfactant producing bacteria affected by properties of biosurfactants that can emulsify oils forming emulsion [37]. Similar results were mentioned by [32] who reported that in presence of crude oil the most biosurfactants producer are *Brevibacterium* sp of strains PBE178 and PBE190, recording maximum EI24 of 23.15% and 25.8% respectively. [62] mentioned that biosurfactants that have an emulsification activity value of >30% in the first screening stage were promised positive for producing significant emulsification index.

Oil spreading values were between (0.4 - 0.85 cm) by different strain studied (**Table 2**). According to [13], the positive results of oil spreading indicate that the strains can produce biosurfactants efficiently. [51] detailed that production of biosurfactant could assay by oil spreading test efficiently. [37] reported that the sensitivity of the oil displacement test enabled to assay of at least 10 μg (about 10 nmol) of biosurfactant but it is easy to implement and takes less

time. [13] showed similar results that *Bacillus sp* MN 243657 strain B1 and strain B2 recorded top results of oil displacement of 3.9 cm, and of emulsification value recorded 28 % respectively. The present results agree with [20] who showed that *Bacillus sp* had extremely positive values for various biosurfactants screening tests. In the same direction, [11] described that the most effective strains for biosurfactants production, those with weight scores surpassing 20 mg/l^{-1} as *Bacillus sp*. and *Peribacillus sp.*

3.2. Effect of different carbon sources on the biosurfactant production

The cost of substrate for biosurfactant production was a significant expense, consuming almost 30–50% of the total production cost. It could be tackled by using of waste oils like waste engine oil, waste transformer oil, and waste frying oil from the food industry [12].

From **(Table 3)** different carbon sources lubricating oil, transformer oil and glucose used for production of biosurfactants. Glucose was used as a control. The obtained results lowering surface tension values between (42.45-47.95 mN/m) for the three bacteria studied. The variation in carbon sources wasn't affected in surface tension activities of the three bacterial strains studied. Different strains have different performance across different substrates in the production of biosurfactants, whoever *Peribacillus frigoritolerans* showed a significant emulsification capacity with the highest value of 72.6 % for glucose, followed by 60 % for transformer oil and 50% for lubricating oil as carbon source. On contrast, *Bacillus licheniformus BaDB24* and *Bacillus cereus* SH16 showed significant lower emulsification values of 50 % across all tested carbon sources.

The use of lubricating oil as carbon source (**Table 3**) showed a significant ($p<0.05$) values of oil spreading for the three tested bacteria, that was 3.35 cm for *Bacillus licheniformus BaDB24,* 2.1 cm for *Bacillus cereus SH16* and 0.7 cm for *Peribacillus frigoritolerans*. While the using of transformer oil were recorded lowering oil spreading of 1.18 cm for *Bacillus licheniformus BaDB24,* 1.4 cm for *Bacillus cereus SH16* and 0.35 cm for *Peribacillus frigoritolerans*. Compared to glucose that recorded the lowest oil spreading of 0.63 cm for *Bacillus licheniformus BaDB24,* 1.03 cm for *Bacillus cereus SH16* and 0.7 cm for *Peribacillus frigoritolerans*.

Based on biosurfactants yield, glucose demonstrated the highest significant yield capability followed by lubricating oil and transformer oil as carbon source for three bacteria studied as

(1.17>0.258>0.01 g/L), (0.402>0.225>0.01 g/L), (1.304>0.54g>0.204 g/L) of *Bacillus licheniformus BaDB24, Bacillus cereus SH16* and *Peribacillus frigoritolerans* respectively (**Table 3**).

According to significant value of our results **(Table 3)**, the using of lubricating waste industrial oil was considered to be best alternative carbon source for the production of biosurfactants by the three bacterial strains studied.

Table 3: Screening of biosurfactants production using different bacterial strains:

Types of carbon source	Bacteria	Surface tension mN/m	EI24 %	Oil spreading cm	Weight g/l
Glucose 0.1%	***Bacillus licheniformus*** *BaDB24*	44.7^{b}±0	42.6^{a}±9.8	0.63^{c}±0.3	1.17^{a} ±0.05
	Bacillus cereus SH16	42.45^{a}±0.88	30.7^{b}±0	1.03^{c}±0. 08	0.402^{a} ±0.05
	Peribacillus Frigoritolerans	46.1^{b}±0.28	72.6^{a}±5.95	0.7^{a}±0	1.304^{a} ±0.05
Lubricating oil 1%	***Bacillus licheniformus*** *BaDB24*	44.02^{a}±0.02	42.1^{a}±7.9	3.35^{a}±1.17	0.258^{b} ±0.05
	Bacillus cereus SH16	47.95^{c}±0.07	35.75^{a}±2.5	2.1^{a}±0	0.225^{b} ±0.05
	Peribacillus Frigoritolerans	45.32^{a}±0.02	50^{c}±0	0.7^{a}±0.1	0.54^{b} ±0.05
Transformer oil 1%	***Bacillus licheniformus*** *BaDB24*	46.81^{b}±0.02	21.5^{b}±1.5	1.18^{b}±0.33	0.01^{c} ±0
	Bacillus cereus SH16	45.44^{b}±0.015	35.25^{a}±2.25	1.4^{b}±0	0.01^{c} ±0
	Peribacillus Frigoritolerans	46.93^{c}±0.18	60^{b}±0	0.35^{b}±0.05	0.204^{c} ±0.05

Growth on HM media with 2%NaCl, at 37 °C, p H 7 for 72 h and 10^{5}CFU/ml. Presented values are the means ± standard deviations. Different letters within the same bacteria on each tests represent different statistical group ($p<0.005$, Duncan test).

Our results agree with [37,49,67,11] whose reported that the metabolites produced by strains with high surface activity do not necessarily correlated with high emulsifying activity and the properties of resulting biosurfactants are affected by the substrate used, also the growth condition and the microbial strain used as showed by [52,48,65]. This variability might be

attributed to the individual metabolic capabilities of each specific strain to utilize different substrate for production of biosurfactants that as observed by [3]. Biosurfactant was classified into surfactants and emulsifiers, that surfactants reduce surface tension while emulsifiers share in the formation and stabilization the given emulsions [5]. This variation could be attributed to the different hydrophilic-lipophilic balances of these substrates. For example, the distinct hydrophilic-lipophilic balances of waste oil and glucose facilitated the synthesis of biosurfactants with varying molecular weight and properties, that agree with [40]. So, it was recommended that multiple screening methods should be included in the initial screening to identify all types of biosurfactants producers as reported by [59].

Also, same observation was mentioned by [43] that *Peribacillus* sp, could reduce the surface tension to 25.9-27.6 mNm^{-1}. [15] reported same findings by *Bacillus salmalaya*, that transformer oil exhibited the lowest surface tension reduction rates with value of 33.5%, while sunflower oil had the highest value of 71.1%. Additionally, surface tension showed reductions with olive oil to 59.6 %, vegetable oil to 57.37%, and glycerol to 48.6%, [10] found that *Ochrobactrum* sp. C1 could grow with waste lubricants as the sole carbon, that showed the highest EI24 value of 69.42 ± 0.32 % was obtained after 7 days incubation period at optimized culture condition that agree with our results. [61] reported high results than our results that *Bacillus licheniformis* which was isolated from oil reservoir grown on 2% glucose; exhibited higher emulsification index (up to 96%) and lower the surface tension of 36 mN/m after 72h of incubation. [16] agree with our results that *Bacillus toyonensis* which was isolated from oil-contaminated places (in Egypt) could reduce the surface tension to 47 mN m^{-1}. Same observation were reported by [11] who showed that strains of *Peribacillus* sp. 1mo, *Bacillus* sp. 1os and *Bacillus* sp. 2os were identified as the most effective strains for producing emulsification index above 50% and reduce surface tension below 40 mN m^{-1} when growing on different carbon source of glycerol, waste frying oil and sunflower cake. As reported by [7] that sugar cane molasses waste was produced a significant emulsification capacity with the highest value of 77.6 %, followed by 59.3 % for spent lubricating oil while spent lubricating generator oil produced zero EI24%.

Oil spreading assay as indirect test to screen biosurfactant production is considered more accurate and quicker method to screen the biosurfactants production [13]. [68] showed that the oil

spreading test was highly reliable method for detecting biosurfactant production by different microorganisms that agree with our finding.

Same observation was showed by [11] who showed that *Bacillus sp.* 2os strain can produce high biosurfactant yield on a medium contained glucose of 0.636 g L^{-1}, as observed by [45]. [57] observed that *Bacillus sp.* can grow in poor nutritive medium R2A broth and produce high biosurfactants yield ranged from 1-2.5 g L^{-1}. [69] observed similar results that *Bacillus atrophaeus* biosurfactants yield varying from 0.53 to 1.11 g L^{-1}, also, the diameter of oil spreading varying from 17.2 to 19.6 cm, according to the carbon source used.

3.3. Factors affecting the emulsification activity:

3.3.1. Effect of different temperatures:

The biosurfactant produced by each isolate exhibited stable emulsification activity across broad range of abiotic factors (**Fig. 2, a**,**b,c**). Stability of biosurfactant production showed the highest emulsification index of 64.6% at temperature of 25 °C for *Bacillus licheniformus BaDB24*, 43.4 % at temperature of 37°C for *Bacillus cereus* SH16 and 60.5% at temperature of 50°C for *Peribacillus frigoritolerans*, then gradually decreased for all bacterial strains except *Peribacillus frigoritolerans* showed stable EI24% to 60.5 % at 50 °C and 75 °C, however the biosurfactant maintained produced of emulsification activity between 25-120°C. Elevation of temperature to 120°C resulting in a significant decrease in the emulsification activity of all tested biosurfactants (**Fig. 2, a**).

As observed by [44] who recorded that *Bacillus licheniformus* biosurfactant was stable up to temperature of 50 °C. Also**,** [47] showed that the biosurfactants produced by *Bacillus brevis* demonstrated thermal stability between 30 °C and 80 °C. Different result was observed by [38] who reported that *Bacillus licheniformis 86* could produce stable biosurfactant in the temperatures range between 25-120ºC. Also, [53] reported that the stability of *Bacillus licheniformis DS1* could produce emulsification activity at high temperatures up to 120 °C. *B. licheniformis* W16 produced a stable biosurfactant from 40 to 160 ºC that reported by [34].

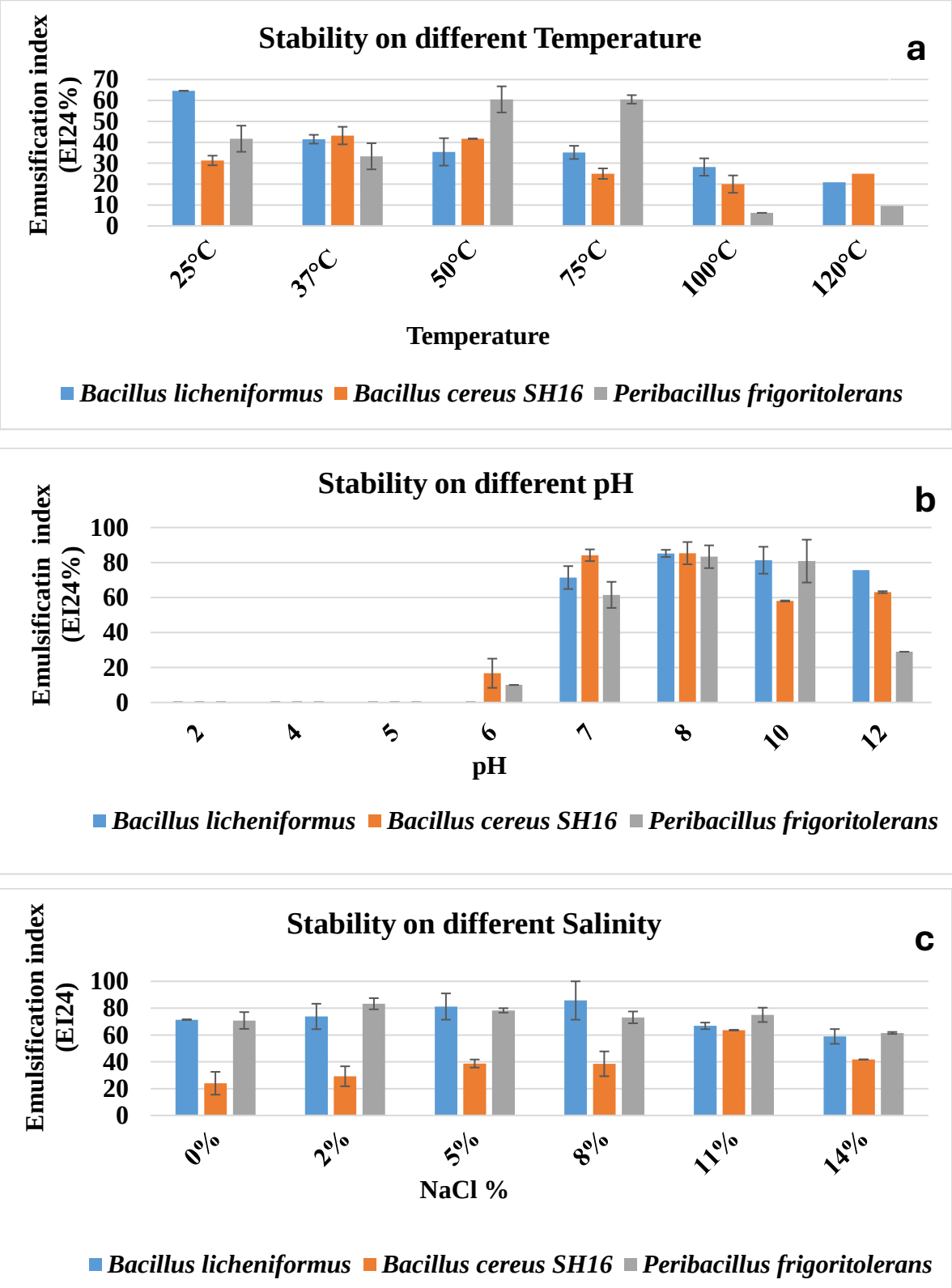

Fig. 2: Effect of different factors (**a**) temperature; (**b**) pH and (**c**) NaCl % on the emulsification activity production, the error bars representative of standard deviations.

3.3.2. Effect of pH

Regarding pH effect, the highest significant EI24 % was obtained at pH 8 of 85.2±6.5 for *Bacillus licheniformus BaDB24,* 85.35±6.35 for *Bacillus cereus SH16* and 83.3±12.5 % for *Peribacillus Frigoritolerans* and the decreasing values were observed in EI24 % at pH (7–12), but almost no observed emulsification index at pH 2–6 for the three bacterial strains because the biosurfactant was precipitated under highly acidic condition (pH ≤ 6) and the sample was turbid (**Fig. 2, b**) as reported by [22,14]. [34] showed similar results that *B. licheniformis* W16 biosurfactant maintained stability within a pH range of 6 – 12 and precipitated under highly acidic condition (pH ≤ 4.0) as observed by [4]. Different observation was showed by [53] who reported that *Bacillus licheniformis DS1* biosurfactant was stable between pH 4–10. [44] showed that the pH resistant capacity over a range of 4.5–9. [21] reported that biosurfactants of Alkaliphilic Bacterium SJS1 was stable between pH 2 -12.

3.3.3. Effect of different salt concentrations

The emulsification activity was significantly increased for the three bacteria when NaCl was 8 % for *Bacillus licheniformus BaDB24* (85.7 %), also when NaCl was 11 % for *Bacillus cereus* SH16 (63.6) and when NaCl was 2% for *Peribacillus Frigoritolerans* (83.3 %), followed by gradual decrease in activity using 14% of NaCl for the three bacteria studied (**Fig. 2, c**).

In contrast, [26] observed different results that *Bacillus cereus* strains isolated from oily polluted soil exhibited maximum biosurfactant production at 8% NaCl. Also,[21] showed that biosurfactants of Alkaliphilic bacterium SJS1 could be stable in the extreme condition of high salt concentration. Stability of biosurfactants of *Bacillus licheniformis DS1* was up to 10% NaCl (w/v) [53]. On another direction [34] showed that *B. licheniformis* W16 biosurfactant was stable up to 4% NaCl.

From (**Fig 2, a,b,c**), The stability values showed significant emulsification results at pH 12 resulting of *Bacillus licheniformus BaDB24* (75.6%) > *Bacillus cereus SH16* (63.05%)> *Peribacillus Frigoritolerans* (29%). The stability of temperature at 75°C that showed emulsification values resulting for *Peribacillus Frigoritolerans* (60.5%)> *Bacillus licheniformus BaDB24* (35.2%)> *Bacillus cereus SH16* (25%). The stability of salinity at 14% resulting that showed emulsification values of *Peribacillus Frigoritolerans* (60.8%)> *Bacillus licheniformus BaDB24* (58.95%)> *Bacillus cereus SH 16* (41.7%).

Among significant stability results of biosurfactants (**Fig. 2**, **a,b,c**), indicated that *Bacillus licheniformus BaDB24 and Peribacillus frigoritolerans* were chosen to be the most powerful biosurfactants producer.

4. Conclusions

Biosurfactant production can be produced by utilizing waste substrates as a carbon source, providing a safe, environmentally friendly and affected in its function properties. The waste industrial oil of (transformer oil or lubricating oil) appeared to be good alternative sources for biosurfactants production by *Bacillus licheniformus BaDB24*, *Bacillus cereus SH16* and *Peribacillus frigoritolerans*. The polymer produced had interesting emulsification and oil spreading properties. The observed variability in biosurfactant production was influenced by both strain and the substrate used, as a result of difference in metabolic pathways. The substrates used could reduce the biosurfactant production cost and make it more economically competitive and stable under a wide range of alkaline pH, salinity, and temperature conditions. The extreme properties of the biosurfactant such as its stability under various conditions, make it suitable for a wide range of industrial applications.

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6. Conflict of interest

All authors declare that they have no conflict of interest

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الملخص العربي

دراسة انتاج المواد البكتيرية الخافضة للتوتر السطحي باستخدام نفايات الزيوت الصناعية كمصدر كربوني يستخدم في محطات انتاج الطاقة الكهربائية

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[2].كلية البنات للآداب والعلوم والتربية، جامعة عين شمس، القاهرة، مصر.

الملخص العربي :

لقد حظي إنتاج المواد الخافضة للتوتر السطحي من النفايات العضوية الصناعية اهتمامًا كبيرًا نظرًا لإمكانياتها في توفير تكاليف الإنتاج. تم اختبار إنتاج وخصائص المواد الخافضة للتوتر السطحي المحضرة بواسطة ثماني سلالات بكتيرية من نوع *Bacillus sp.* و *Peribacillus frigoritolerans* الذين تم عزلهم سابقًا من الغشاء الحيوي لراتنج التبادل الأنيوني المستخدم في محطات إنتاج الطاقة الكهربائية (مصر). وقد تم إنتاجها باستخدام نفايات الزيوت الصناعية (زيوت المحولات وزيوت التربينات) كمصادر بديلة ورخيصة للكربون لإنتاج المواد الخافضة للتوتر السطحي. يشمل فحص واختبار منتجي المواد الخافضة للتوتر السطحي، وفقًا لمعايير مختلفة وهي: اختبار انحلال الدم، قياسات التوتر السطحي، اختبار انتشار الزيت، مؤشر الاستحلاب (EI24) وإنتاجية المواد الخافضة للتوتر السطحي. وقد أظهرت قيم الثبات نتائج استحلاب كبيرة عند درجة الأس الهيدروجيني 12 وكانت لـ *Bacillus licheniformus BaDB24* (75.6٪) > *Bacillus cereus SH16* (63.05٪) > *Peribacillus Frigoritolerans* (29٪). ووفقا لثبات درجة الحرارة عند 75 درجة مئوية فقد أظهرت قيم استحلاب ل *Peribacillus Frigoritolerans* (60.5 %) > *Bacillus licheniformus BaDB24* (35,2%) > *Bacillus cereus SH16* (25 %). كما أظهرت الملوحة عند نسبة 14% ثبات قيم الإستحلاب ل*Peribacillus frigoritolerans* (60.8%) > *Bacillus licheniformus BaDB24* (58.95%) > *Bacillus cereus SH16* (41.7%). وقد كانت الكائنات الحية الدقيقة المدروسة قادرة على إنتاج المواد الخافضة للتوتر السطحي من زيوت النفايات الصناعية. ومن بين السلالات المختبرة، تم اختيار نوعين من البكتيريا *Bacillus licheniformus BaDB24* و *Peribacillus frigoritolerans* ليكونا الخيار الافضل لإنتاج المواد الخافضة للتوتر السطحي.