The Egyptian Journal of Hospital Medicine (October 2023) Vol. 93, Page 7488- 7501

Production, Properties and Application Trends of Iturin Biosurfactant Produced from *Bacillus altitudinis A*HMNAZ2

Alaa Atef[1*], Mostafa M. Abo Elsoud[2], Heba I. Elkhouly[1] and Nagwa M. Sidkey[1]
[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University (Girls Branch), Cairo, Egypt
[2]Microbial Biotechnology Department, National Research Centre, Dokki, Giza, Egypt
***Corresponding author:** Alaa Atef, **Tel.:** 01069078438; **E-mail:** alaaatef78@yahoo.com; alaasoliman.el.8.89@azhar.edu.eg.

ABSTRACT
Background: Biosurfactants are surfactants derived from several types of microorganisms such as bacteria, yeasts and fungi as membrane components or secondary metabolites.
Objective: To improve the output of biosurfactants as a low-toxic, biocompatible alternative for chemical surfactants utilized in a wide range of applications in today's industry.
Materials and Methods: Different culture media were used in isolation of the biosurfactant (BS) producers. By using 16S rRNA, the most effective bacterial isolate was examined. For the optimization circumstances, Plackett Burman and Box-Behnken designs were employed. Purification and characterization of biosurfactant was done.
Results: Only one isolate (BS) out of 63 isolates was selected for its potential ability of biosurfactant production, which lowered the surface tension (38.1 mN/m) and exhibited excellent emulsifying index against benzene (60.92 %) as well as oil spreading activity (9.76 cm), which was analyzed as *Bacillus altitudinis* AHMNAZ2 with accession number OP807875.1. The highest biosurfactant production yield was 2570 mg/L. The biosurfactant was lipopeptide in origin, identified as an iturin. Isolated iturin can be employed as an antibacterial agent against various Gram +ve and Gram -ve bacteria as well as some fungi. It showed remarkable action against the cell lines of breast cancer (MCV- 7) and colon cancer (HCT- 116).
Conclusion: The investigation found a cheaper way of synthesis of biosurfactants from industrial wastes, delivering a twofold benefit of reducing pollution of environment and manufacturing useful biotechnological biosurfactant products, in addition to biosurfactant synthesis with high potency.
Keywords: Biosurfactant, *Bacillus altitudinis*, Iturin, Antimicrobial, Antitumor.

INTRODUCTION
Biosurfactants are microbial substances with a unique surface action. They belong to various groups, including glycolipids, fatty acids, phospholipids, lipopeptides, neutral lipids, and polysaccharide-protein complexes. These substances can play a variety of biological functions in the development and reproduction of microbes. Most biosurfactants are produced extracellularly or on cell surfaces by different microorganisms (bacteria, yeast and fungi) [(1)].

These are amphiphilic compounds that are organic and surface-active, including both hydrophobic and hydrophilic moieties. The non-polar hydrophobic part has an affinity for non-polar compounds like oils, fats, and greases, whereas the polar hydrophilic part has a strong affinity for polar solvents like water, acetic acid, methanol, etc [(2)]. Due to their capacity to aggregate between fluid phases and lower overhead or interfacial tensions, surfactants are extensively utilized in the food and beverage, cosmetics, pharmaceutical, textile, and improved oil recovery from rocks industries [(3)].

According to **Jadhav *et al.*** [(4)], many commercial surfactants in the market today are chemically produced from hydrocarbon feedstock. These surfactants frequently result in an additional source of pollution to already depleted natural habitat because they are intrinsically hazardous and non-biodegradable [(5)]. Improved biodegradability is one benefit added by biosurfactants, among others, environmentally friendly, low toxicity, non-hazardous, higher selectivity, mild production conditions using renewable materials and industrial waste/by-products as substrates, capacity to operate in harsh conditions with varying pH range and salinity levels, and modification by biotechnology and genetic engineering [(4)]. As a result, this current investigation was carried out to generate stable potent biosurfactant using different cost-lowering renewable wastes and to assess the potential of produced iturin for various applications.

MATERIALS AND METHODS
1. Isolation sources and culture media
A sum of sixteen solid samples were gathered in date of 8/2021 from different locations polluted with oil viz. (Shoubra El Kheima, Nasr City in Cairo, Elbehira, Red Sea in El Ein El Sokhna, Egypt) for isolation of the biosurfactant producers. To isolate, purify, and maintain bacterial isolates, nutrient agar medium and minimal salts medium (MSM) were utilized. After the isolates were purified, their capacity to generate biosurfactants was assessed, and one especially strong isolate was selected for further study. Modified production medium, **Bushnell and Hass**[(6)] medium-molasse (BHM-M), which contains

Received: 26/04/2023
Accepted: 22/06/2023

BHM (g/l), provided with glucose 1% and molasse 6% was used.

2. Screening methods for biosurfactant producers:
Microbial isolates have been tested for biosurfactant production by usage of: Oil displacement method [7], emulsification activity according to **Patel and Desai** [8] and emulsification index (E_{24}) [9], and surface tension (SFT) measurement using du Nouy ring method [10].

3. Molecular identification of the most effective bacterial isolate:
The most potent bacterial isolate (BS) has been identified using 16S rRNA by Sigma Company (https://sigmaeg-co.com) using eubacterial primers of 16S ribosomal RNA (5-AGAGTTTGATCCTGGCTCAG-3), (5-GGTTACCTTGTTACGACTT-3) as forward and reverse primer respectively for DNA amplification. The protocol and all the purification solution system were from **Quick-DNA™** Miniprep Plus Kits (Zymo Research) and The DNA amplification was done using Biometra Uno thermal cycler. The DNA sequence of the positive clone was subjected to a similarity search BLAST on the NCBI website (http://www.ncbi.nlm.nih.gov), and deposited into GeneBank. Evolutionary analyses were conducted in MEGA11 software.

4. Optimization of biosurfactant production by the most potent bacterial isolate:
The Plackett Burman Factorial design and Box-Behnken were developed. Data represented in **table (1)** showed the factors used in the (PB) design with their ranges and data represented in **table (2)** showed (B-B) design used for modeling biosurfactant production by the most potent isolate.

Table (1): Factors used for Plackett Burman design and their range of study:

Factor	Name	Units	Low	High
A	Sucrose	g/l	0	10
B	Lactose	g/l	0	10
C	Glucose	g/l	10	20
D	Glycerol	ml/l	0	10
E	Molasse	ml/l	0	10
F	Bagasse	g/l	0	10
G	Crude oil	ml/l	0	10
H	Ammonium sulphate	g/l	0	1
J	Sodium nitrate	g/l	0	1
K	Peptone	g/l	0	2
L	Yeast extract	g/l	0	2
M	Ammonium nitrate	g/l	0.6	1.2
N	Calcium chloride	g/l	0	0.06
O	Zinc sulphate	g/l	0	0.06
P	Copper sulphate	g/l	0	0.06
Q	Ferric chloride	g/l	0	0.06

Table (2): Box-Behnken design used for modeling biosurfactant production by the most potent isolate:

Factor	Name	Units	Low	High
A	Glycerol	ml/l	10	50
B	Molasse	ml/l	0	40
C	Bagasse	g/l	0	40
D	Crude oil	ml/l	0	40
E	Sodium nitrate	g/l	0	4
F	Yeast extract	g/l	2	10
G	Incubation period	Days	5	9
H	pH	Unit	6	8

5. Analytical methods:
A. Extraction, purification and characterization of the most potent isolate biosurfactant:
The generated biosurfactant was extracted according to **Varadavenkatesan and Murty** [11]. The culture broth was briefly spun in a chilled centrifuge (Ohaus USA Frontier 5706) at 5000 rpm (20 min, 4°C) in order to produce a cell-free filtrate. Using 6N HCl and pH 2, biosurfactant was acid precipitated overnight at 4 °C and was centrifuged at 10,000 rpm for 30 minutes at 4°C. The precipitate was extracted three times using a 2:1 v/v combination of methanol and chloroform. The organic layer was removed and evaporated at 40°C, yielding a viscous light brown substance that was relatively pure biosurfactant. After that, this material was weighed. By utilizing the elution solvents chloroform, methanol, and water (65:25:4), ninhydrin-specific spots for amino acids were revealed in TLC plat. Biosurfactant was further purified at room temperature using a silica gel column (25*2 cm) and elution by methanol and water ranging from 50 to 100 (v/v). Five milliliters of fractions were collected, and the oil displacement zone was assessed. The activity of each peak was further assessed using the displacement of oil zone, emulsion index, and surface tension of fractions. The highest peak was investigated using FTIR, GC/MS, and tandem mass spectrometry LC/MS.

B. Fourier transform infrared (FTIR) spectroscopy.
The infra-red absorption spectrum (4000-400 cm^{-1}) of purified biosurfactant was measured and recorded using Thermo Scientific company FTIR spectrophotometer (USA) model Nicolet™ iS50/iS50R FTIR Spectrometer and performed at Regional Center for Mycology and Biotechnology (RCMB), Nasr City, Cairo, Egypt.

C. Analysis of Gas chromatography–mass spectrometry (GC-MS)
The chemical constituents of biosurfactant sample were determined by usage of Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x

0.25 µm film thickness) as reported by **Abd El-Kareem *et al.*** [12].

D. Mass spectrometry

Liquid chromatography-electrospray ionization (ESI) analyses in the negative and positive ion modes were used for determining the molecular weight of the surfactant's constituent parts. The methanolic extract lipopeptides were examined by using UPLC MS/MS "Agilent" 6420 [13].

6. Applications of extracted biosurfactant.

A. Antimicrobial potency of the produced biosurfactant:

By usage of the agar well diffusion assay method, the produced biosurfactant was tested for its antimicrobial activity against the following bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (NRRL B-543) and Methicillin-Resistant *Staphylococcus aureus* (MRSA 2658RCMB), *Bacillus cereus* RCMB 027, *Enterococcus faecalis* (ATCC 29212), *Staphylococcus epidermidis* RCMB 009, *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 13315), and the fungal strains: *Aspergillus fumigatus* (RCMB 002008) and *Candida albicans* (RCMB 05031). Gentamicin (4 µg/ml) and ketoconazole (100 µg/ml) were utilized as positive standard antibacterial and antifungal agents), respectively.

B. Minimum inhibitory concentration (MIC)

The biosurfactant MIC was obtained according to **Meir *et al.*** [14].

C. Antitumor activity of the biosurfactant:

The biosurfactant that was produced was tested for its cytotoxicity against VERO (Mammalian Normal Cells from African Green Monkey Kidney) and for its antitumor activity against MCF-7 cells (a human breast cancer cell line) and HCT-116 (colon carcinoma). The tests were conducted at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

D. Viability assay for cytotoxicity evaluation:

The cytotoxicity of the biosurfactant was assessed in accordance with **Mosmann** [15]. These plots indicated the 50% inhibitory concentration (IC_{50}) and the cytotoxic concentration (CC50); the concentration needed for making toxic effects in 50% of tumor cells and intact cells, respectively.

Ethical approval

This study was approved by Al-Azhar University ethical board.

Statistical Analysis

Analysis of variance (ANOVA) analyzed the data, with p less than 0.05 designated as appropriate level for statistical significance. The data from the Box-Behnken (B-B) and Plackett Burman (PB) designs were analyzed and constructed using Design Expert® Version 7.0 software from State-Ease Inc., Minneapolis, MN, USA.

RESULTS

1. Isolation and purification of biosurfactant producers:

The isolation, purification and testing of biosurfactant producer results revealed that just one purified bacterium with the code BS was the most effective biosurfactant producer out of sixty-three (63) isolates (49 bacteria and 14 fungi). This bacterium was isolated from a cooktop of a kitchen stove, Cairo, Egypt and cultured for 48 hours at 35 °C using modified **Bushnell and Hass**[6] medium (BHM).

2. Screening methods for biosurfactant producers:

A summary of all biosurfactant screening assay techniques that are represented in **table (3)**, which showed that isolated BS was the most potent biosurfactant isolate compared with control as found by using different screening methods.

Table (3): A summary of all biosurfactant screening assay techniques for BS isolate

Different screening methods	Biosurfactant activity	Control with distilled water
Oil spreading (cm)	9.76±0.25	0
E_{24}(%)	60.92±0.55	0
Emulsification activity (EA) (EU)	1.201±0.032	0
Reduction in surface tension (mN/m)	38.1±0.17	72.0

3. Identification of the most potent biosurfactant producing isolate:

The isolate BS has been identified using 16S ribosomal RNA and analyzed by BLAST at NCBI. The phylogenetic tree was constructed as represented in **figure (1).** The isolate BS displayed the highest degree of similarity (99.56%) with *Bacillus altitudinis* based on the phylogenetic data that were acquired. *Bacillus altitudinis* strain AHMNAZ2's 16S ribosomal RNA nucleotide sequences were uploaded to the NCBI and assigned the accession number OP807875.1.

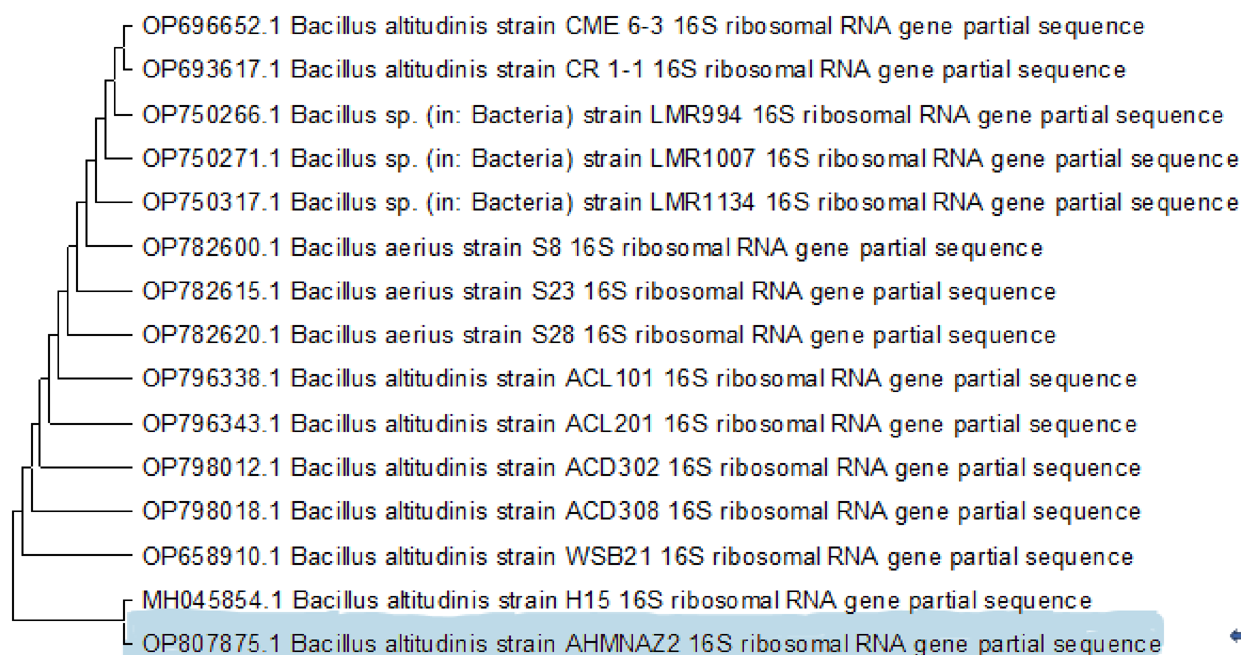

Figure (1): Phylogenetic tree showing genetic relationship between *Bacillus altitudinis* strain AHMNAZ2 with accession number OP807875.1 and other reference *Bacillus* species in gene bank.

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4. Optimization using Plackett Burman (PB) factorial design.

Implementation of PB design for biosurfactant production by *Bacillus altitudinis* resulted in data represented in **figure (2).** The predicted and actual results showed an excellent correlation coefficient (R^2) of 0.9154 and adjusted R^2 of 0.7041 in figure (3). The model "Lack of Fit" was insignificant with "F-value" of 0.21. The Adequate precision of the model which measures the signal to noise ratio, was 6.807 that indicates an adequate signal. Analysis of variance (ANOVA) of the resulted data showed that the model was significant with "F-value" of 4.33. The factors coded: B, G, J, L, M, N, AB, AC, AD were all significant model terms. The model can be utilized to navigate the biosurfactant model space, according to these results. The following regression equation represents biosurfactant production using significant factors according to Plackett Burman design.

Emulsification Activity (EA) = 1.878694 - 0.38751 * A - 0.07335 * B - 0.11186 * C + 0.079293 * D+ 0.026176 * E + 0.04864 * F + 0.067549 * G - 0.24249 * H + 1.026212 * J + 0.234741 * L - 0.75414 * M -13.9447 * N + 0.023746 * A * B + 0.023842 * A * C - 0.01791 * A * D

Where; A: Sucrose, B: Lactose, C: Glucose, D: Glycerol, E: Molasse, F: Bagasse, G: Crude oil, H: Ammonium sulphate, J: Sodium nitrate , L: Yeast extract, M: Ammonium nitrate, N: Calcium chloride.

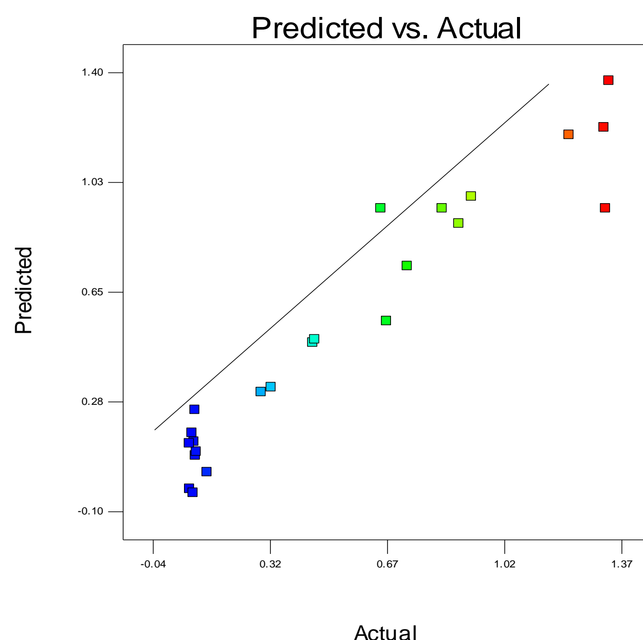

Figure (2): Relation between the actual and predicted results obtained from Plackett Burman design.

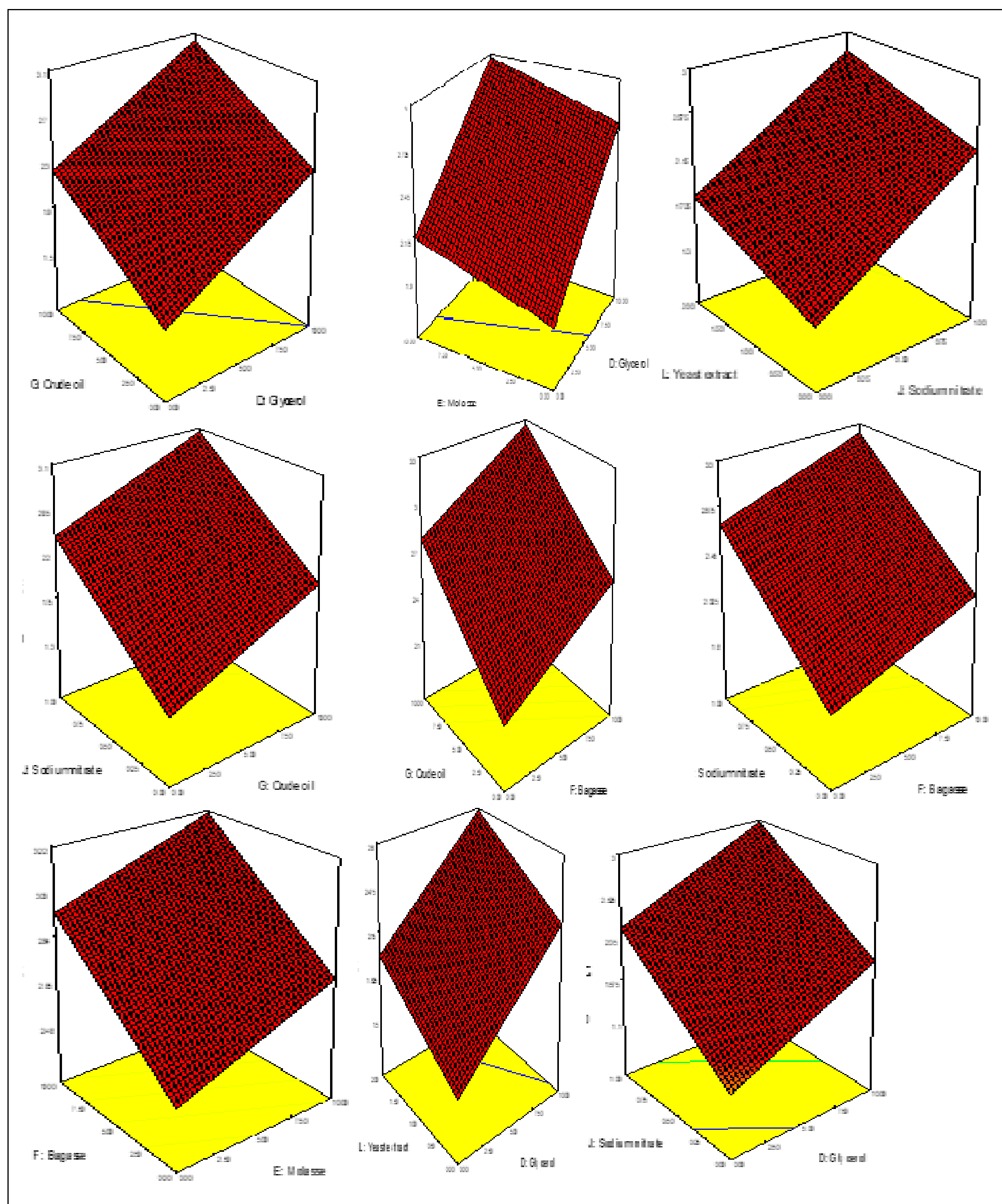

Figure (3): 3D plotting of biosurfactant production based on Plackett Burman design.

Box-Behnken design for BS

The significant factors resulted from design of Plackett Burman were used for formulation of Box-Behnken design. Implementation of Box-Behnken design resulted in the data in **figure (4).** The results showed an excellent correlation among the predicted and actual data R^2 of 0.341327, adjusted-R^2 of 0.148021.

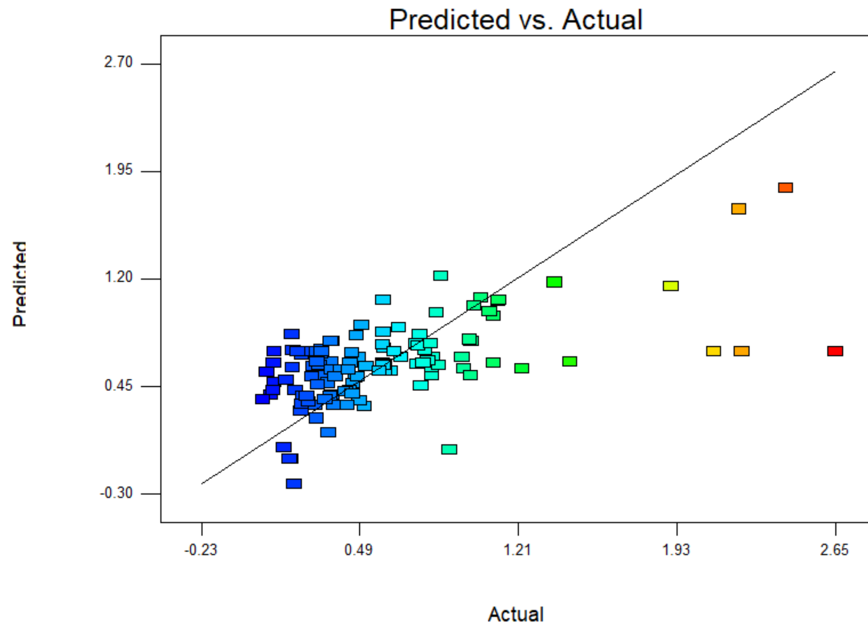

Figure (4): Relation between the actual and predicted results obtained from Box-Behnken design.

ANOVA of the data obtained from Box-Behnken design showed the significance of model with p-value of 0.0242. The model "Lack of Fit" was insignificant with p-value of 1.00. Adeq precision of 9.459515 was adequate signal. The following equation represents biosurfactant production using significant factors according to Plackett Burman design.

Emulsification Activity (EA) = -2.35773 -0.13373 * A -0.03478 * B 0.259597 * D +0.176979 * E -0.3223 * F+ 0.888703 * G+ 0.142891 * H +0.029798 * A * F +0.018088 * A * H +0.001751 * B * D +0.005286 * B * F -0.00812 * D * E -0.03134 * D * F -0.01652 * D * G -0.03668 * D * PH -0.03633 * E * F +0.036096 * F * G+ 0.0059 * F * H +0.001376 * D^2 -0.07971 * G^2 -0.00407 * A * F * H -0.0003 * B* D * F +0.001608 * D * E * F -0.00185 * D * F * G +0.006346 * D * F * PH -0.00021 * D^2 * G +0.002313 * D * G^2

Where; A: Glycerol, B: Molasse, D: Crude oil, E: Sodium nitrate, F: Yeast extract, G: Incubation period, H: pH.

Prediction of the conditions optimizing for maximum biosurfactant production:

The conditions optimizing for maximum biosurfactant productivity by *B. altitudinis* was expected from the Box-Behnken model as follows: glycerol, 50.00 ml/l; molasse, 40.00 ml/l; crude oil, 0.02 ml/l; sodium nitrate, 0.59 g/ L; yeast extract, 10.00 g/ L; incubation period, 6.38 days and pH: 6.00 to obtain maximum emulsification activity of 2.40. The data showed a significant increase in emulsification activity (2.40) compared with that obtained at the original conditions (1.20).

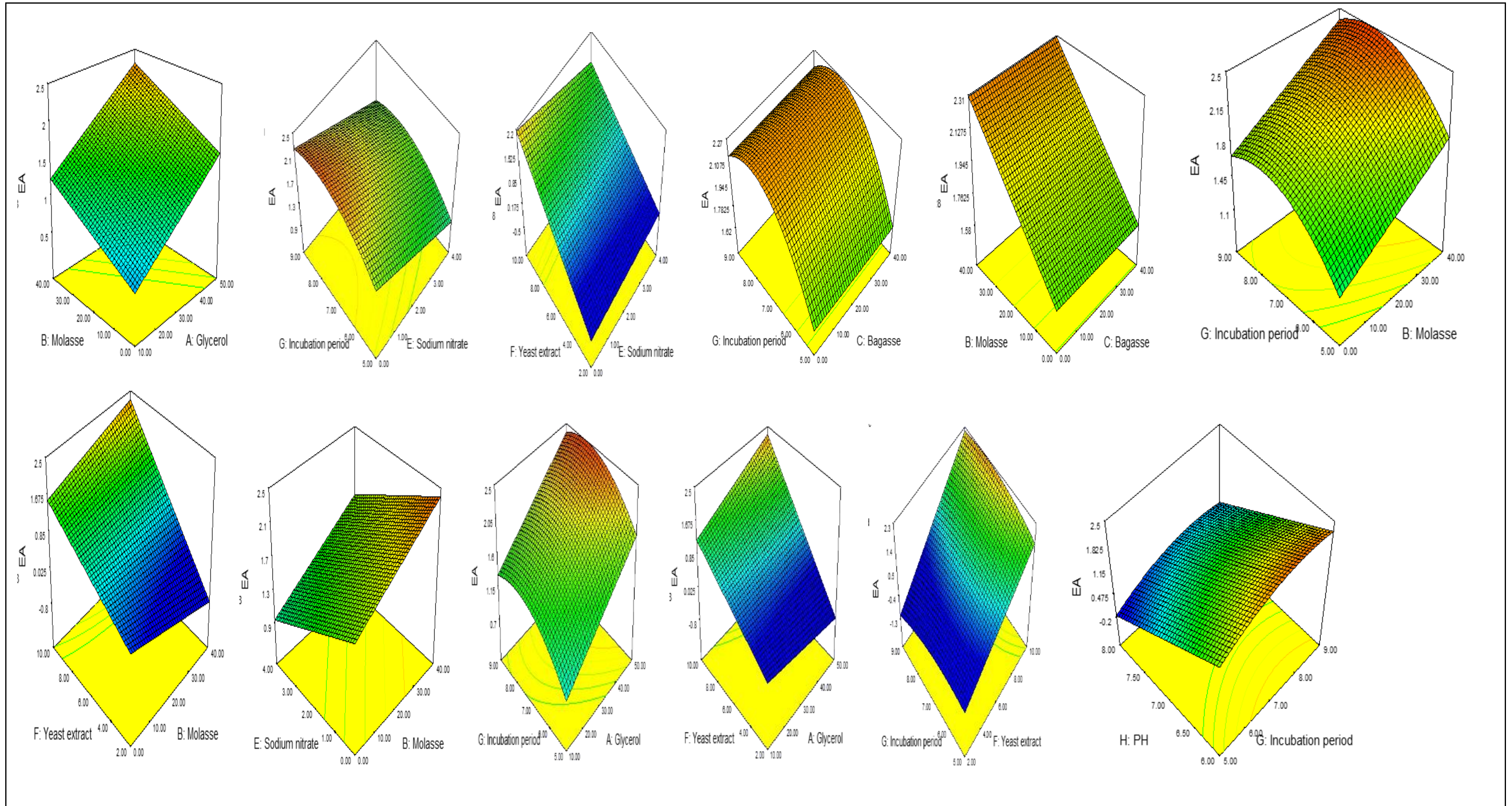

Figure (5): 3D plotting showing the effect of different variable and factor-factor interactions on biosurfactant production based on Box-Behnken design.

5. Extraction, purification and characterization of biosurfactant produced from *Bacillus altitudinis*

Extraction of biosurfactant produced from *Bacillus altitudinis* AHMNAZ2 under the optimum conditions resulted in 2570 mg/L. The generated biosurfactant was identified by thin layer chromatography (TLC) as a lipopeptide with an RF value of 0.69, which corresponded with the iturin family. The silica gel column chromatography purification of the biosurfactant resulted in the activities of fractions shown in **figure (6).**

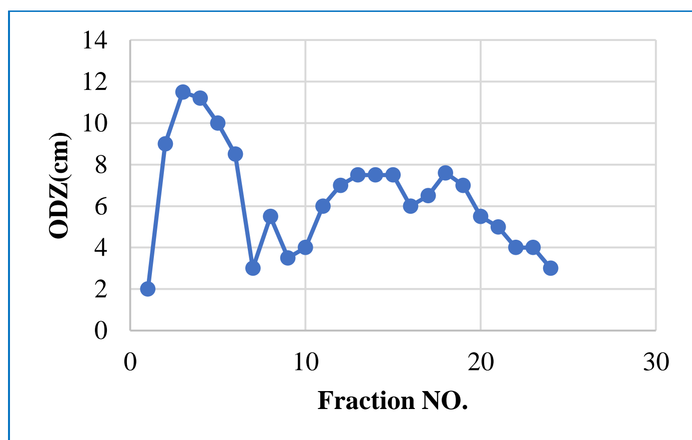

Figure (6): Typical elution profile for the chromatography of *Bacillus altitudinis* lipopeptide biosurfactant on silica gel (60) column chromatography.

In the present investigation, figure (6) showed five fractions (2:6) (peak:1) from elute of *Bacillus altitudinis* collected together in a single vial which demonstrated 10.53 cm in oil displacement zone, 62.5 % in emulsification index and lowering in surface tension of 37.16 mN/m when compared with fractions (11:16) (peak:2), which demonstrated 6.86 cm in oil displacement zone, 48.33 % in emulsification index and lowering in surface tension of 42.26 mN/m and fractions (17:19) (peak:3), which demonstrated 7.43 cm in oil displacement zone, 50.66 % in emulsification index and lowering in surface tension of 39.56 mN/m.

A. Fourier Transform Infrared Spectroscopy for biosurfactant produced by *Bacillus altitudinis*.

A strong broad absorbance peak (centered around 3456.63) with wavelength numbers ranged from 3600 cm^{-1} to 3100 cm^{-1} was found in **figure** (7) as a result of C–H stretching vibrations, N–H stretching vibrations, and hydrogen linked–OH functional groups. This is characteristic of molecules combining carbon and amino groups. The FTIR spectra revealed a prominent peak at 1050 cm^{-1}, showing the existence of C–N aliphatic amines, and a peak at 2084, representing the aliphatic group. Additionally, a prominent band was noted at 1637.01 cm^{-1}. This is because peptide components have ester carbonyl groups.

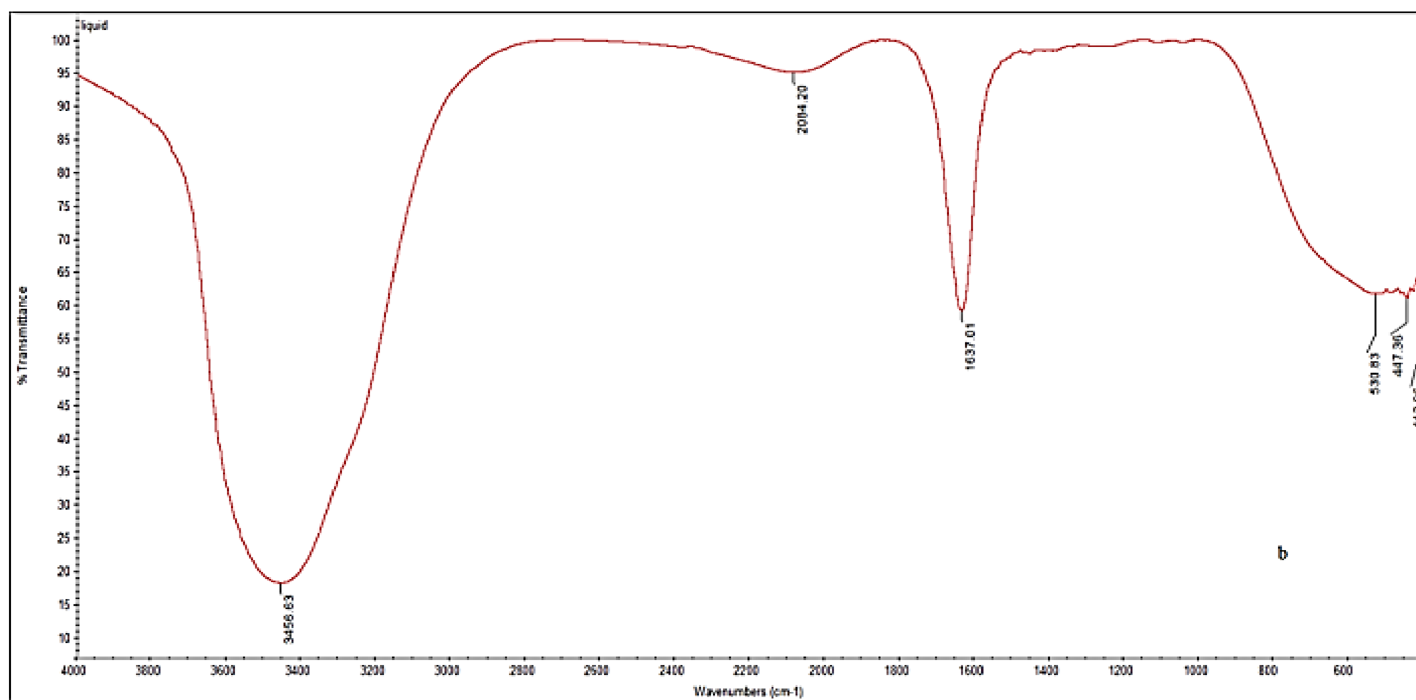

Figure (7): Infrared spectra (FTIR) spectra of the purified bioactive fraction of the biosurfactant produced from *Bacillus altitudinis*.

B. Gas chromatography – Mass spectroscopy

The mass spectra from the target sample were compared to the library mass spectra in scan mode to determine the components of fatty acids. The molecule generated by *Bacillus altitudinis* was identified by GC-MS analysis as a derivative of lipopeptide with hydrophobic moiety found to be 9-Octadecenoic acid (E) (13.75%) as the predominant major compound. The individual compounds peaks are illustrated in **figure (8)** showing **t**otal ion chromatogram formed by *Bacillus altitudinis* biosurfactant.

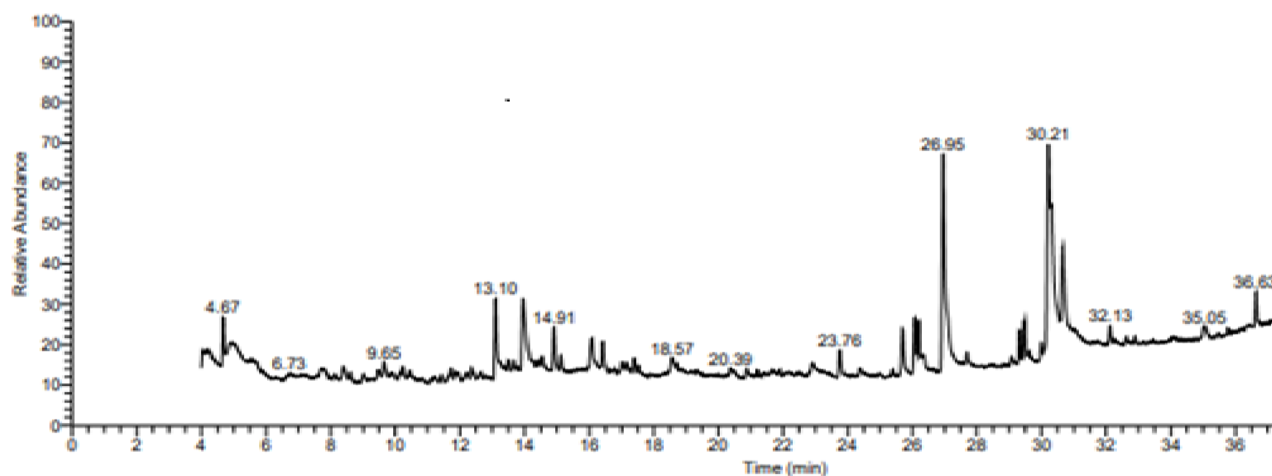

Figure (8): GC-MS of biosurfactant produced by *Bacillus altitudinis* AHMNAZ2.

C. Mass spectrometry

Figure (9) displayed the results of mass spectral studies of the *Bacillus altitudinis* purified biosurfactant fractions. They displayed a well-defined peak cluster that was consistent with substances from the family of iturin. The weights of molecules of 1028, 1042, and 1056, 1072, and 1083 Da in the (M-H) negative ionization mode and 1066, 1074, 1080, 1096, and 1106 in the (M+H) positive ionization mode are corresponding to the iturin family.

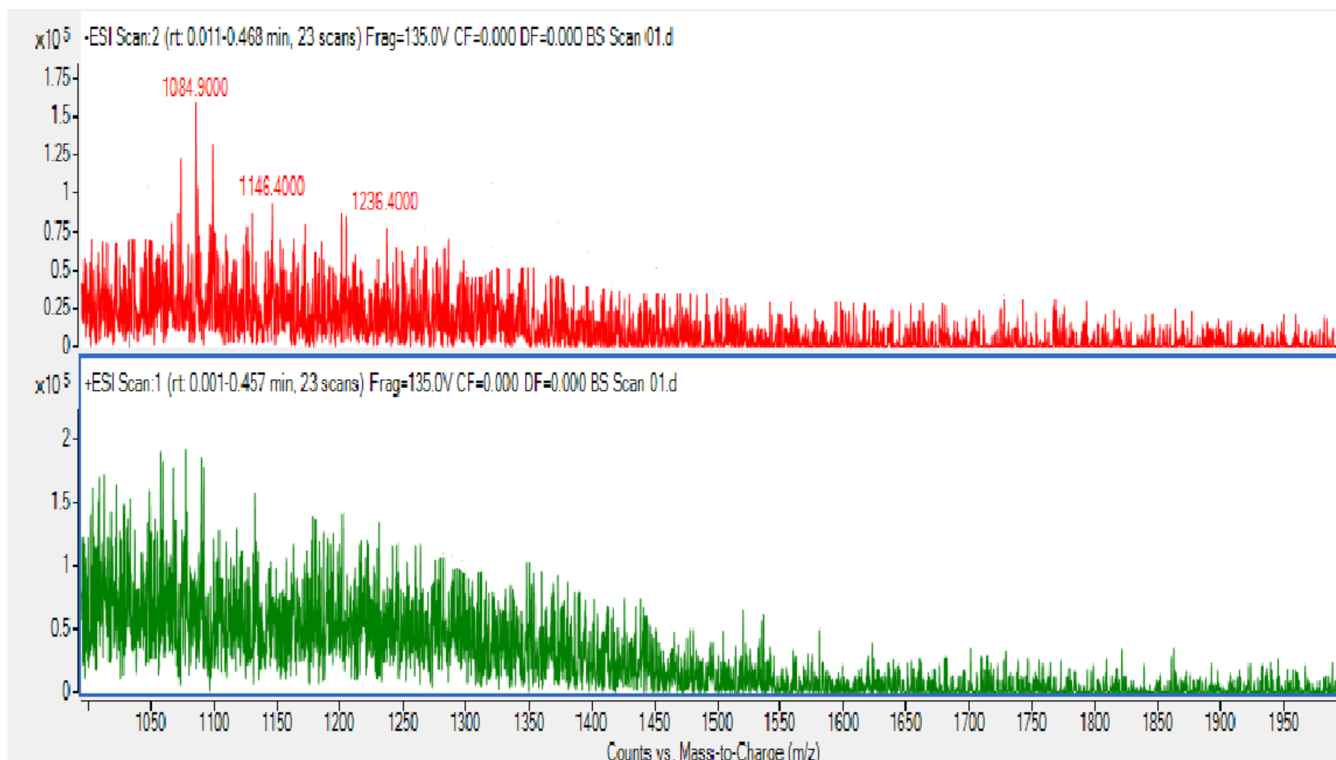

Figure (9): Electrospray ionization mass spectroscopy (ESI–MS) spectra of purified biosurfactant of *Bacillus altitudinis*

6. Applications of lipopeptide iturin

A. Antimicrobial activity of iturin lipopeptide

Iturin was evaluated against various pathogenic microorganisms at a concentration of 10 mg/ml. Its effect on the tested organisms with measured inhibition zone is shown in **table** (4). On the other hand, iturin has not any inhibitory activity to *Bacillus cereus* RCMB 027 (1), *Aspergillus fumigatus* (RCMB 002008) and *Methicillin-Resistant Staphylococcus aureus* (MRSA 2658RCMB) as shown in the table.

Table (4): Biological potency of iturin produced by *Bacillus altitudinis* against some pathogenic microorganisms.

Sample Name Tested microorganisms	Iturin	Control (Standard antimicrobial) (mm)
FUNGI		***Ketoconazole***
***Aspergillus fumigatus* (RCMB *002008*)**	NA	17
***Candida albicans* (RCMB 05031)**	9	20
Gram Positive Bacteria:		***Gentamicin***
***Staphylococcus aureus* ATCC 25923**	23	24
***Bacillus subtilis* NRRL B-543**	21	26
***Bacillus cereus* RCMB 027 (1)**	NA	25
***Staphylococcus epidermidis* RCMB 009 (2)**	12	28
***Enterococcus faecalis* (ATCC 29212)**	11	26
Methicillin-Resistant Staphylococcus aureus (MRSA 2658RCMB)	NA	20
Gram Negative Bacteria:		*Gentamicin*
***Escherichia coli* ATCC 25922**	15	30
***Proteus vulgaris* ATCC 13315**	12	25

B. Minimum inhibitory concentration (MIC)

MIC of iturin was different between tested microorganisms. On the other hand, *Bacillus cereus* RCMB 027 (1), *Aspergillus fumigatus* (RCMB 002008) and Methicillin-Resistant *Staphylococcus aureus* (MRSA 2658RCMB) were not affected at all with iturin as shown in **table (5).**

Table (5): Minimum inhibitory concentrations (MIC) (µg/ml) of iturin against tested microorganisms

Sample code Tested microorganisms	Iturin conc (µg/ml)
FUNGI	
***Aspergillus fumigatus* (RCMB 002008**	NA
***Candida albicans* (RCMB 05031)**	2500
Gram Positive Bacteria:	
***Staphylococcus aureus* ATCC 25923**	156.25
***Bacillus subtilis* NRRL B-543**	312.5
***Bacillus cereus* RCMB 027 (1)**	NA
***Staphylococcus epidermidis* RCMB 009 (2)**	2500
***Enterococcus faecalis* (ATCC 29212)**	2500
Methicillin-Resistant Staphylococcus aureus (MRSA 2658RCMB)	NA
Gram Negative Bacteria:	
***Escherichia coli* ATCC 25922**	1250
***Proteus vulgaris* ATCC 13315**	2500

C. Evaluation of cytotoxic effects of iturin on mammalian cells African green monkey kidney (VERO) normal cell line:

The iturin's cytotoxicity was detected against normal (VERO) cells with CC_{50} = 388.99±12.75µg/ml, as shown in **figure (10).**

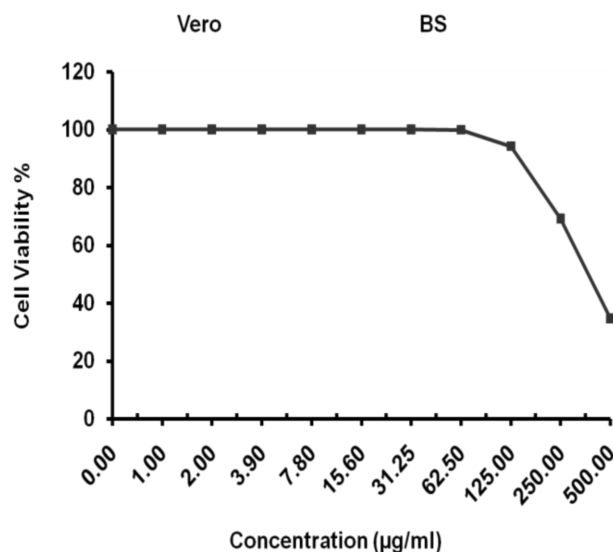

Figure (10): CC_{50} analysis of iturin against (VERO) cell line.

D. Evaluation of antitumor activity of iturin on colon carcinoma (HCT-116) cell line

The present study reveals that IC50 value of 170.51 ± 5.13 µg/ml for iturin against (HCT-116) cells and a maximum inhibitory suppression was observed up to 87.28 % for iturin at 500 µg/ml concentration as shown in **figure (11).** The results showed that the biosurfactant iturin declared important anti-proliferative activity against colon cancer cell line.

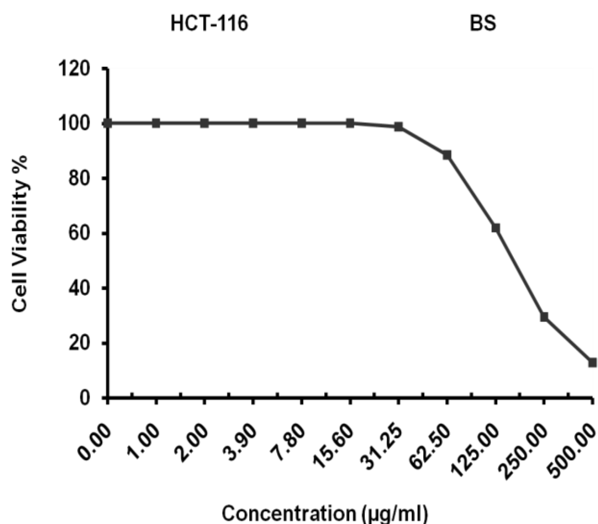

Figure (11): IC_{50} analysis of iturin against colon carcinoma (HCT-116) cell line.

E. Evaluation of antitumor activity of iturin on breast carcinoma (MCV-7) cell line

The present study reveals that IC50 value of 244.03 ± 6.71 µg/ml for iturin against breast carcinoma cells and a maximum inhibitory suppression was observed up to 78.05 % for iturin 500µg/ml concentration as illustrated in **figure (12).** The results declared that iturin showed important anti-proliferative activity against Breast cancer.

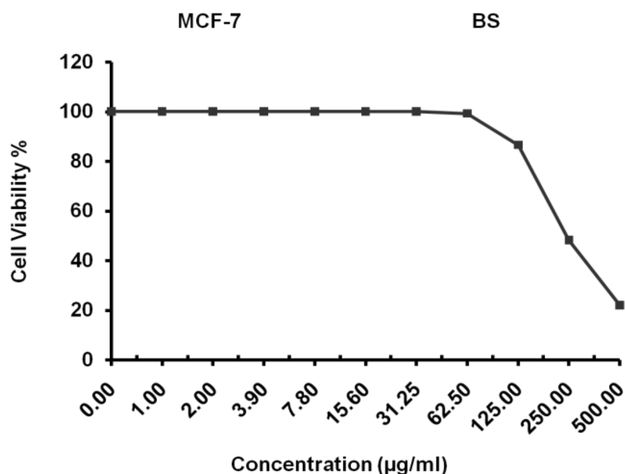

Figure (12): IC_{50} analysis of iturin against Breast Carcinoma (MCV-7) cell line.

DISCUSSION

In the present study, sixty-three (63) isolates (49 bacteria and 14 fungi) were isolated from various types of wastes and were screened for their ability to produce biosurfactant. Concerning the results of other investigators, **Bhuvaneswari and Sivagurunathan** [16] showed that only one of the ten bacteria isolated from the marine sediments of the Cuddalore coastal region in India produced biosurfactants when streaked in **Bushnell and Haas**[6] agar medium supplemented with 1% crude oil for several times. BS bacterial isolate was chosen as the most potent microbial isolate, which showed the highest biosurfactant production in modified BHM with oil spreading zone 9.76 cm, emulsification index 60.92 %, emulsification activity 1.201, as well as 38.1 mN/m reduction of surface tension compared with control used. 16S rRNA was used to identify the BS isolate as *Bacillus altitudinis* strain AHMNAZ2, which has the accession number OP807875.1.

According to **Phulpoto *et al.*** [17]**,** the *Bacillus nealsonii* S2MT bacterial isolate had an oil displacement test that showed a clear zone and an emulsification index for kerosene oil after 24 hours that were 4.2 cm, 55%. According to **Sifour *et al.*** [18]**,** the biosurfactant made from *Pseudomonas aeruginosa* RB28 had an emulsification activity of 0.28 against dodecane. Surface tension was 35.99 mN/m and maximal biosurfactant production of E24 was 68.08 % for *Lysinibacillus fusiformis* after a 120-hour incubation period [19]**.** The greatest amount of biosurfactant occurred only with glycerol with emulsifying activity 55% [20]**.**

According to **Umar *et al.*** [21]**,** *Bacillus subtilis* SNW3 produced crude lipopeptide of 1.17 g/L using optimum medium. A red color spot on a silica gel plate, produced during the extract preliminary characterization using TLC, showed that the biosurfactant from *Bacillus altitudinis* was a lipopeptide with an RF value of 0.69, which is related to the iturin family. When comparing the findings to the standard, **Ramyabharathi *et al.***[22] found that *Bacillus subtilis* Bbv57 synthesized iturin, which was validated on TLC with 0.7 Rf value.

The present study in the case of FTIR was in agreement with the past investigation according to **Umar *et al.*** [21] for lipopeptide biosurfactant produced from a marine *Bacillus circulans* and **John *et al.*** [19] for biosurfactant derived from *Lysinibacillus fusiformis* MK559526. The FTIR spectrum revealed the presence of a peptide moiety and aliphatic groups, a trait that sets the lipopeptide nature of the biosurfactant produced by *Bacillus altitudinis.*

GC/MS declared that 9-Octadecenoic acid, (E) was the biosurfactant's main constituent. This result was in agreement with **John *et al.*** [19] who reported that biosurfactant synthesized from *Lysinibacillus fusiformis*

has 9- Octadecenoic acid (80.80%) as the main component of the biosurfactant.

Bacillus altitudinis' pure biosurfactant fractions were analyzed using mass spectrometry, and the results revealed a well-resolved cluster of peaks that corresponded to molecules from the (Iturin family). When comparing the mass of each group of peaks to the given lipopeptide complexes mass numbers from other *Bacillus* strains, a distinct lipopeptide isoform can be deduced. With varying chain lengths of fatty acids, each group of isoforms may belong to the same family and have the same sequence of amino acids [(23)]. The results of this investigation aligned with those of past study according to **Pyoung *et al.*** [(24)]**.** The molecular weights of these compounds are comparable to those of the iturin A isomers that include acyl chains C14, C15, C16, and C17, according to **Sarwar *et al.*** [(25)]**.** It was determined that these 1,071.7 and 1,085.7 m/z, two lipopeptide precursor ions, were distinct iturin A variants with varying b amino acid chain lengths. The molecular ion of 1,071.7 m/z was identified as iturin A6/A7 and 1,085.7 m/z as iturin A8, according to **Isogai *et al.*** [(26)]**.** The 1,093.7 and 1,107.7 m/z ions were found to be the sodium adducts of the iturin A isomers of the ions of 1,071.7 and 1,085.7 m/z, respectively. Based on literature, it was determined that iturin C with C17 b-amino fatty acid was detected at the molecular ion of 1,086.7 m/z, two iturin C variations (1,100.7 and 1,114.6 m/z), and with C18 and C19 bAA iturin C variants [(27)]. According to investigations on the extracted antibacterial activity of lipopeptide, *K. pneumoniae* (10 mm), *E. coli* (7 mm) and *S. aureus* (6 mm) all responded better to the lipopeptide at a concentration of 5 µg/mL [(28)]**.** A lipopeptide works by attaching to the bilayer surface of bacteria and changing the organizational linkage of local lipid on -ve charged fatty acids, which causes the bilayer of lipid to be restructured and stops functions of the cell [(29)].

Ghribi *et al.* [(30)] have reported that inhibition zone diameter for biosurfactant of 0.5 g/l produced from *B subtilis* SPB1 were 19, 12, 8, 8, 14, 3, 10 and 4 mm for *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Aspergillus oryzae*, *Aspergillus niger* and *Rhizopus oryzae*, respectively. This study's biosurfactant showed a wide range of activities, including antibacterial activity against microbes with profiles of multidrug resistance. The chemical demonstrated greater efficacy against Gram +ve cocci compared to Gram -ve bacilli. Its ability to combat *Enterococcus faecalis* was crucial. These findings are very interesting because, as noted by **Ghribi *et al.*** [(30)]**,** these microorganisms have limited sensitivity to aminoglycosides and penicillin G and natural resistance to chloramphenicol, cephalosporins, co-trimoxazole, aztreonam and clindamycin.

The antimicrobial potency of iturin was also important against *Staphylococcus aureus,* which is known to be resistant to at least two β-lactams [(31)]. Also, it was clear that the activity against Gram -ve bacteria was lesser when compared to Gram +ve bacteria.

Aneurinifactin lipopeptide biosurfactant was found to have MIC against 6 different strains of bacteria, including *Escherichia coli* (8 g mL^{-1}), *Vibrio cholerae* (16 g mL^{-1}), *Pseudomonas aeruginosa* (8 g mL^{-1}), and *Klebsiella pneumoniae* (4 g mL^{-1}) and *Bacillus subtilis* (16 g mL^{-1}) [(32)]**.**

Elkhouly *et al.* [(33)] declared that CC_{50} of rhamnolipid against VERO cell line equal to 44.1 µg/ml. According to **Hajare *et al.*** [(34)]**,** iturin can stop colon adenocarcinoma HCT-15 from growing. According to **Özcan *et al.***[(35)]**,** iturin A blocks the signaling pathway of Akt, which is the main oncogenic protein involved in the formation of cancer, the inhibition of apoptosis, the progression of the cycle of cell**.** Iturin A lowered the cancer of human breast both in vitro and in vivo by interfering with the pathway of Akt [(36)]**.**

CONCLUSION

The current work demonstrated adequate yield of iturin when *B. altitudinis* AHMNAZ2 was grown in a medium containing agro-industrial waste products. The investigation found a cheaper way of creating biosurfactants from industrial wastes, delivering a twofold benefit of lowering pollution of environment and manufacturing useful biotechnological biosurfactant products, in addition to biosurfactant synthesis with high potency. A biosurfactant that can take the place of the conventional chemical surfactants used in the pharmaceutical business was generated by this research. Significant inhibition against many pathogens was demonstrated by the isolated iturin from *B. altitudinis* AHMNAZ2, suggesting that it was a viable biocontrol agent with potential use in the food and medical industries. The biosurfactant showed strong anticancer activity and was non-toxic, indicating that it was safe for use.

Conflicts of interest: There are no conflicts to declare.
Funding: This research received no external funding.

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