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Optimization and improvement of biosurfactant production for *Pseudomonas aeruginosa* 4.2 and *Bacillus cereus* 2.3 strains isolated from oily polluted soil sample

Amr Fouda, Mamdouh Salem El-Gamal, Essam Hussein Abdel-Shakour, and Ahmed Ali Radwan*

Botany and microbiology dept. Faculty of Science (Boys), Al-Azhar University Madent Nasr in Cairo, Egypt.
*Corresponding author: Ahmed Ali Radwan
*Corresponding author: *ahmedradwan56@yahoo.com*

Abstract

Surfactants are amphipathic molecules which reduce surface and interfacial tensions and widely used in pharmaceutical, cosmetic, food and petroleum industries. There are several advantages for biosurfactants in contrast with chemical surfactants, such as lower toxicity; higher biodegradability; better environmental compatibility. The biosurfactant producing strains isolated from oil contaminated soil sample and identified as *Bacillus cereus* 2.3, and *Pseudomonas aeruginosa* 4.2 based on physiological, biochemical tests and 16S rRNA sequence analysis. Maximum biosurfactant yield was obtained in optimized basal mineral medium containing molasses and ammonium nitrate with C/N ratio (12:1) at pH 7-8 incubated at 35-40 ^{o}C under dynamic conditions (150 rpm) for 72 h with 6 % inoculum (0.8 OD_{620}nm) of the isolate at 0.5%,NaCl for *Bacillus cereus* 2.3 while best medium component for *Pseudomonas aeruginosa* were molasses and ammonium nitrate with C: N ratio (15:1) at pH 7-8 incubated at 35-40 ^{o}C under dynamic conditions (150 rpm) for 60 – 72 and 72 h. with 4 % inoculum (0.8 OD_{620}nm) of the isolate at 0.5%.,NaCl.Applying of these standardized conditions improved the biosurfactant productivity by 245% and 210 % for the two bacterial strains respectively.

Keywords: *Optimization, Biosurfactant, Pseudomonas aeruginosa, Bacillus cereus, Oily polluted soil.*

Introduction

Various types of microorganisms produce surface active compounds. The requirementfor industrial production of biosurfactants is very high due to their environmental compatibility compare tochemically synthesized surfactants. The demand for biosurfactant is expected to be around 500 thousand tons by 2018 globally and 21% of total consumption is from developing region such as Asia, Africa and Latin America, Biosurfactants Industry is expected to reach 2,210.5 MillionUSD Globally **(Zainatul, *et al.*, 2014)**

Bacteria are the main group of biosurfactant-producing microorganisms, although it is also produced by some yeasts and filamentous fungi **(Ebrahimi *et al.*, 2011).** The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* **(Nitschke *et al.*, 2005),** while surfactin and iturin produced by *Bacillus subtilis* strains **(Nitschke and Pastore, 2004).**

Some advantages of biosurfactants compared to their chemically synthesized counterpart are their lower toxicity higher biodegradability, greater environmental compatibility, better foaming properties and stability at extremes of pH, salinity and temperature **(Desai and Banat, 1997).** Unlike chemical surfactants, which are mostly derived from petroleum feedstock, biosurfactant molecules can be produced by using cheaper agro based substrates and waste materials **(Banat *et al.*, 2010).**Biosurfactants are also attractive

because they are less damaging to the environment yet are robust enough for industrial use; these properties have led to the increasing interest of biosurfactants for commercial use in Food, Cosmetic, Oil industries and recently in synthesis and stabilization of silver nanoparticles (**Reddy *et al.*, 2009;Kiran *et al.*, 2010).**

The identification and optimization of the cultivation conditions that affect the surfactant production represent key points for the development of a cost-competitive process (**Mukherjee *et al.*, 2006**). There are a number of operating parameters controlling biosurfactant production, which are required to be maintained within a certain range in operating condition whereby the activity of bacteria with the resultant of maximum production of biosurfactant can be optimized. In this regard, temperature, pH of the medium, medium composition and salinity are of prime importance for control and optimization of biosurfactant production. The amount of biosurfactant synthesis depends greatly on the availability of carbon sources and on the balance between carbon and other limiting nutrients (**Abouseoud *et al.*, 2008).**

The aim of this work was to investigate the cultural factors affecting the production of the extracellular biosurfactants by *Bacillus cereus* and *Pseudomonas aeruginosa* isolated from oil-fields and to find the optimal composition of the growth medium for the production in laboratory bench-scale.

Materials and Methods

1.Collection of soil sample

Twelve (12) oil polluted soil samples were collected from different localities (Cairo, Giza, Alexandria and El-Beheraa governorates, Egypt) in sterile polythene bagsat a depth of 0 to 10 cm from the earth surface and stored at room temperature (25 ± 2 ^{o}C), labeled appropriately and transferred to the laboratory for further study.

2. Isolation of biosurfactant producing bacteria

2.1-isolation medium

The collected samples were serially diluted (10^{-1} to 10^{-6}) and plated on basal mineral media (The composition of the mineral medium used was as follows (g/l): NH_4NO_3 (2.0), KCl (0.1), KH_2PO_4 (0.5), K_2HPO_4 (1.0), $CaCl_2$ (0.01), $MgSO_4.7H_2O$ (0.5), $FeSO_4.7H_2O$ (0.01), Yeast extract (0.1) and 10 ml of trace element solution containing (g/l): 0.26 g H_3BO_3, 0.5 g $CuSO_4.5H_2O$, 0.5 g $MnSO_4.H_2O$, 0.06 g $(NH_4)_6Mo_7O_{24}.4\ H_2O$ and 0.7 g $ZnSO_4.7\ H_2O$. The pH of the medium was adjusted to 7.0 ± 0.2) supplemented with 1 % crude oil and using pour plate technique **(Shoeb, 2006; Rashmi *et al.*, 2012)** the plates were then inverted and incubated at 35^{o}C, for 48 h., Colony surrounded by an emulsified halo was considered being positive for biosurfactant production. The purification procedure of each bacterial isolate under investigation was carried out by the agar streak plate method according to **Stephen and Karen (1997).**Slants of each pure culture were prepared and preserved at 4°C for future investigations. Sub-culture was usually done every two months by using the same medium.

2.2- Screening for selection of most potent biosurfactant producing bacteria

Selection of the most potent bacteria isolates depending on results published by **El-Gamal *et al.*, (2015),** select according various test as oil spread technique, blood hemolysis, blue agar plate, drop collapse method, emulsification index (E24) and measurement of surface tension.

2.3- Identification of bacterial isolates.

Identification of the selected most potent bacterial isolate was based mainly on morphological and biochemical test according to **Bergy's manual of systematic bacteria (2005a&b),** and 16S rRNA sequence analysis, the partial 16S rRNA sequence of bacterial isolates was carried out in Sigma Research Company, Cairo, Egypt. DNA was extracted using protocol of GeneJet genomic DNA purification Kit (Fermentas) and amplified using Maxima Hot Start PCR Master Mix (Fermentas), then, the PCR product was purified using GeneJET PCR Purification Kit (fermentas).The forward and reverse primers used for PCR amplification were 27^{f} (5′-AGAGTTTGATCCTGGCTCAG -3′) and 1492^{r} (5′-GGTTACCTTGTTACGACTT-3′) (16SrDNA universal primer). Finally, sequencing of the PCR product was carried out in GATC Company using ABI 3730xl DNA sequencer, dendrogram relationship was applied to confirm similarity of the most potent strains with other bacterial group.

3. Detection and quantification of biosurfactant

One ml of cell free supernatant (CFS) was vigorously shaken for 30 s with 1 ml of 0.003 % methylene blue,

and then an equal amount of chloroform was added to the sample. The mixture was left for 20 min to extract the methylene blue surfactant ion pair into chloroform layer. At this point, it is necessary to note that all blue dye has migrated into the chloroform layer. The tube was centrifuged at 3,000 rpm for 5 min, after the extraction with chloroform, the absorbance of each sample was measured at 625 nm against a reference of pure grade chloroform, compared with standard curve of standard biosurfactant **(Jones and Esposito, 2000).**

4.Factors for improvement of biosurfactant production

Effect of different parameters on biosurfactant productivity was studied as following:

The most potent biosurfactant bacterial producer was allowed to grow on basal mineral medium supplemented with 1% glucose as a sole carbon and energy source and inoculated with 2% (v/v) of previously prepared overnight culture (OD620 = 0.8) containing $3.7X10^5$ bacterial cells/ml for *Bacillus cereus* 2.3 and $4.62X10^5$ for *Pseudomonas aeruginosa* 4.2. for different incubation period ((12, 18, 24, 30, 36, 42, …., 102 h.) under dynamic and static condition respectively; pH values (4, 5, 6, 7, 8, 9, and 10) by using different buffer system; incubation temperature (20, 25, 30, 35, 40, 45, 50, and 60^{o}C respectively); inoculum sizes were applied viz. 0.5, 1, 2, 4, 6, 8, and 10 % (v/v); different nitrogen sources in organic forms urea, peptone, asparagine, cysteine, alanine and inorganic form as NH_4NO_3, NH_4Cl_2, $NaNO_3$, & $NaNO_2$. at 2g/l; equal amount of each carbon source(1%)Glucose, fructose, manitol, sucrose, lactose, maltose, starch, cellulose, glycerol, and sorbitol; different cheap substrate (1%) viz. molasses, corn step,cheese whey, rice straw,bagasse, and Sluggers' west; different oil-sources(1%) viz. olive, sunflower, corn, petroleum, and soybean**.(Moussa *et al.*, 2006; Abouseoud *et al.*, 2008; Kiran *et al.*, 2009)**and different C:N ratio; and finally, different NaCl concentration viz. 0.5, 1, 2, 3, 4, 5, 6, 8, and 10% (w/v).At the end of incubation period the cell free supernatant was obtained by centrifugation at 5000 rpm for 15 min, then the productivity of biosurfactant was detected as previously illustrated.

Results and Discussion

1.Screening for potential biosurfactant producing bacteria

During this study, sixty four (64) purified bacterial isolates obtained from polluted soil samples. According to data reported by **El-Gamal *et al.*, (2015).**Select isolates *Bacillus cereus* 2.3 and *pseudomonas aeruginosa*4.2 as the most potent biosurfactant bacterial isolates, due to each of them represent different bacterial group (Gram positive and negative bacteria) and they have high biosurfactant activities, which will undergo further studying to ensure their identification and optimized physical and nutritional factors which greatly controlled the biosurfactant productivities by bacteria

2.Identification of the most potent bacterial isolates

2.1- Morphological and biochemical test.

The selected isolates were subjected to identification by different physiological and biochemical tests by **El-Gamal *et al.*, (2015),** which recorded that; isolate 2.3 appeared as Gram positive spore forming long rods that present in chains, motile, aerobic, it was able to utilize citrate and hydrolyze each of starch, casein, lipid and urea but failed to grow on the MacConky agar medium, also, this isolate was able to produce H_2S but failed to produce indole and mixed acids.

While, Isolate 2.4 appeared as Gram negative motile thin rods, aerobic, it was able to utilize citrate, grow on MacConky agar and hydrolyze lipid and casein but it was not hydrolyze both of starch and urea, the most important characteristic of this isolate, their ability to produce distinguish green diffusible pigment in the growth media.

2.2- Molecular characterization (16S Ribosomal RNA gene).

The genomic DNA of bacterial isolates 2.3and 4.2 were extracted and the 16S rRNA gene of the selected isolates were amplified by polymerase chain reaction (PCR) using the universal primers. The partial 16S rRNA gene of the isolates under study were generated and detected as sharp bands at (1500bp) by agarose gel electrophoresis as compared with DNA ladder (marker) as showed in **Figure (1).**

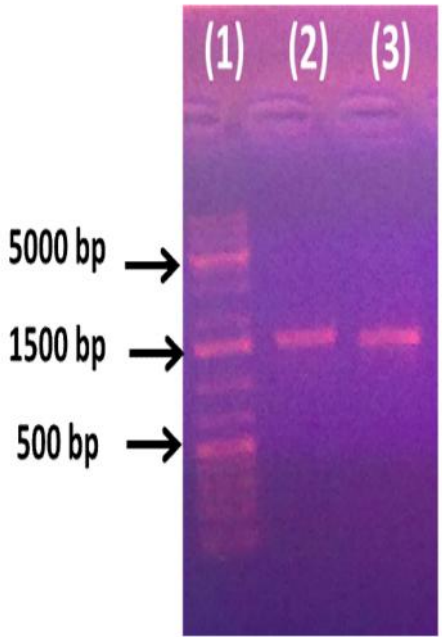

Figure 1: PCR product of 16S rRNA gene of the tested isolates,
Lane (1) DNA ladder (marker), Lane (2) Isolate 2.3, Lane (3) Isolates 4.2.

The alignment showed that isolate 2.3 is similar to ***Bacillus cereus,*** Rock 4-18 with Accession No. (NZ CM000735.1), with Identity 98 %, while isolate 4.2 is similar to ***Pseudomonas aeruginosa***, PAO1, with Accession No. (NC 002515.2), with Identity 99 %, phylogenetic tree of each isolate explain separately as shown in **Figure (2 and 3).**

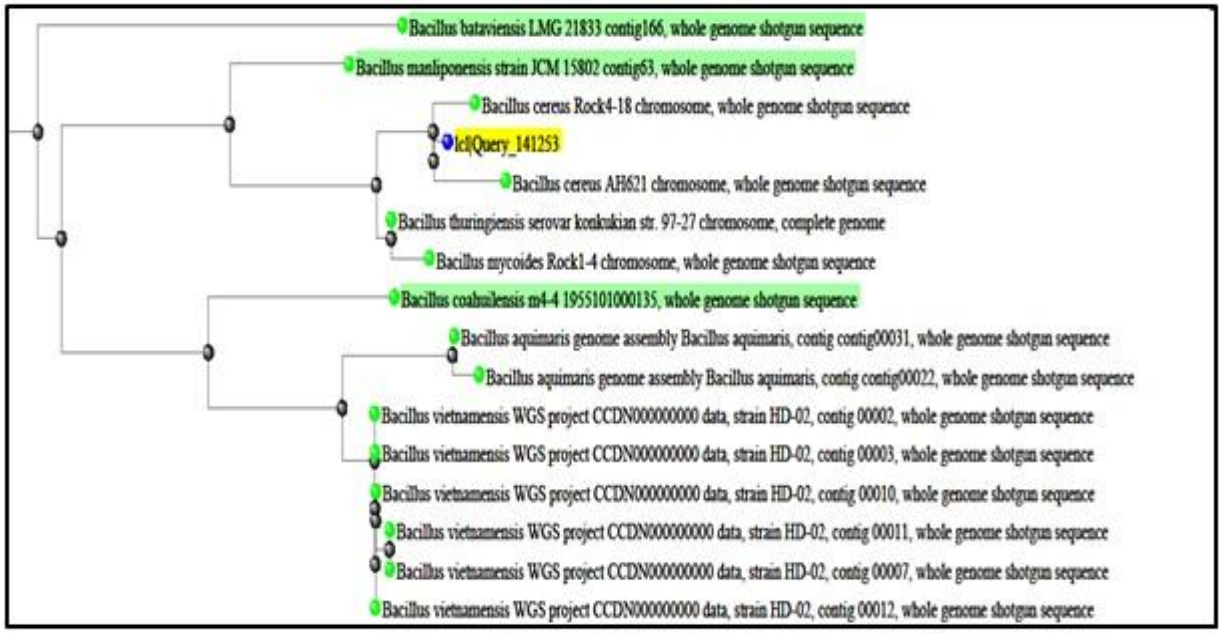

Figure2: Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the position of isolate ***Bacillus cereus 2.3*** and phylogenetically related member of this genus**.**

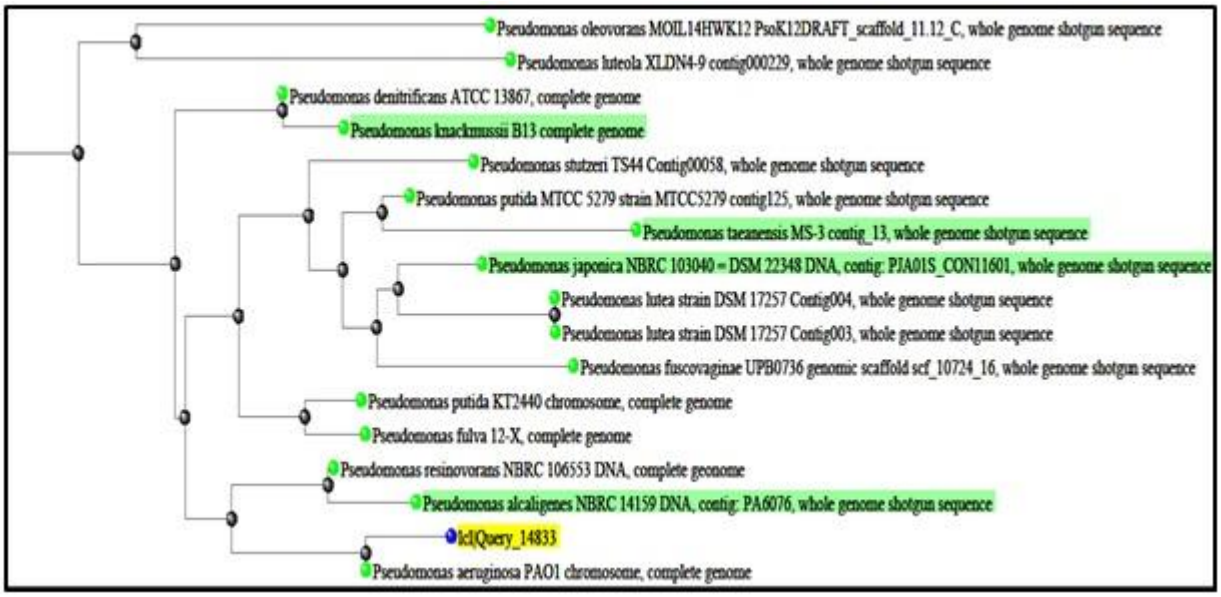

Figure 3: Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the position of isolate *Pseudomonas aeruginosa* 4.2 and phylogenetically related member of this genus**.**

3. Effect of environmental and nutritional factors on biosurfactant productivities by *Bacillus cereus 2.3 and Pseudomonas aeruginosa* 4-2

3.1- Effect of incubation periods and condition.

In general shaking condition was better than static through different incubation time (12 to 102 h.) on the productivity of biosurfactant by the tested organisms as shown in **Figure (4 and 5),** There is a gradual significantly increasing of biosurfactant productivity with the incubation period until it reached to its optimum (60 – 72 h.); (48 – 72 h.) for *B. cereus* 2.3 and *P. aeruginosa* 4-2.respectively, Which indicate that production of biosurfactant occur during the stationary phase.

This result greatly matching with the result that reported previously by **(Saikia *et al*., 2012a&b).** The production of rhamnolipids is typical of a secondary metabolite and increased considerably in the stationary phase in case of *Pseudomonas aeruginosa* RS29 strain, *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* 181 produced highest biosurfactant after 48 h, 132 h and 120 h of incubation respectively, also, agreement with a previous report that *Pseudomonas aeruginosa* WJ-1 exhibited maximum biosurfactant production after 90h **(Xia *et al*., 2012),** while **Ray, (2012)** reported that The maximum biosurfactant production was achieved at 7 days of fermentation for *Bacillus sp.*m28.

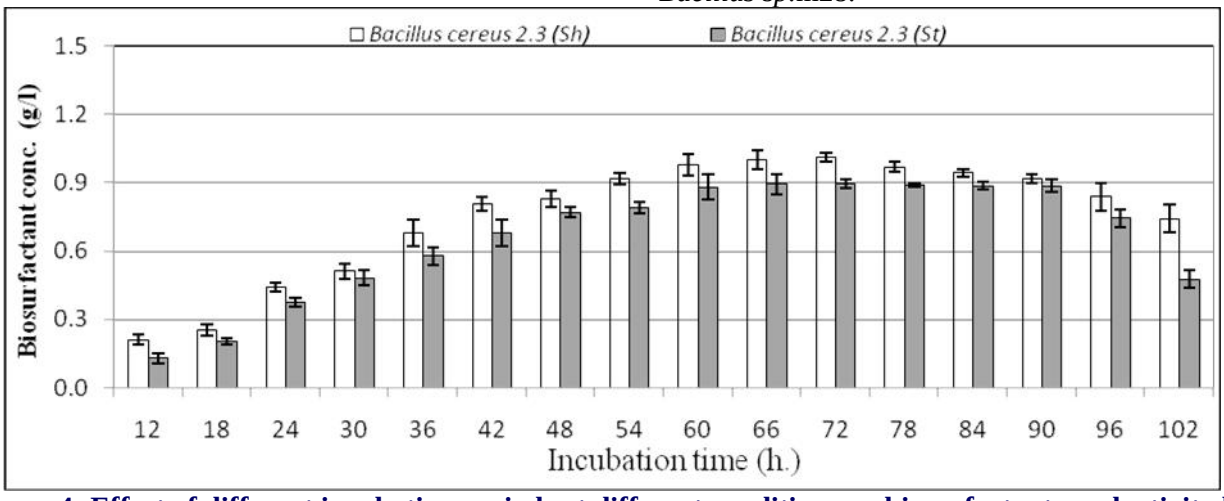

Figure 4: Effect of different incubation periods at different conditions on biosurfactant productivity by *Bacillus cereus* 2.3

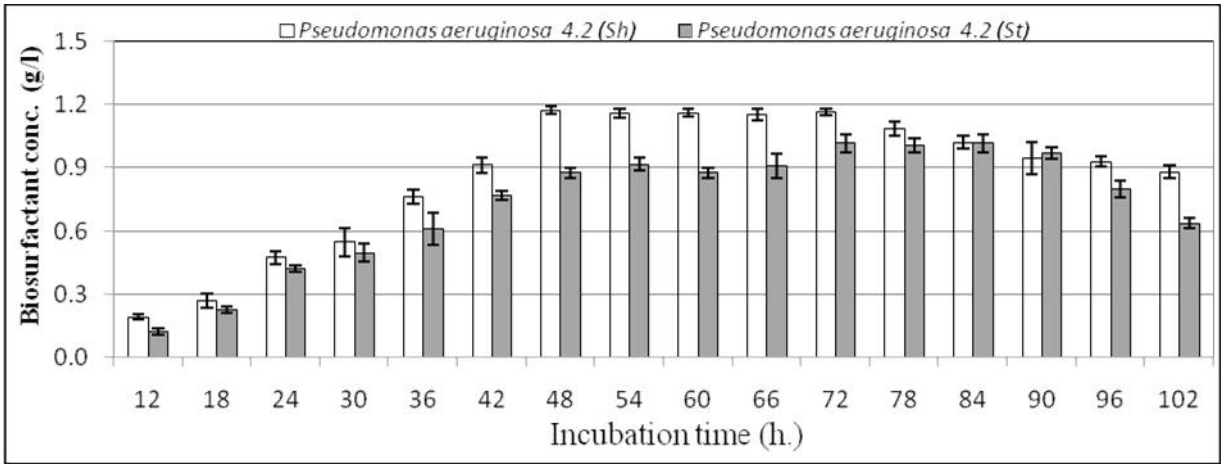

Figure 5: Effect of different incubation periods at different conditions on biosurfactant productivity by *Pseudomonas aeruginosa* 4.2

3.2- Effect of different pH values.

Each isolate was tested for the best pH value that may enhance the production of biosurfactant recorded in **Figure (6)**. Exhibited increasing in pH value from 4-7 was accompanied by enhancing in biosurfactant productivity for both bacterial tested strains, while subsequent increase in pH from 8-10 followed by decreasing in biosurfactant productivity. the highest production rate was recorded at pH 7 and 8 with non-significant difference for the favor of pH value 7.

Similar results were obtained for *Pseudomonas aeruginosa* RS29 and *Pseudomonas aeruginosa* WJ-1 which produced highest biosurfactant at pH range of 7–8 and 6.0–8.0 respectively **(Saikia *et al*., 2012b; Xia *et al*., 2012).**And agreement with the results that recorded for *Bacillus sp*m28by**Ray, (2012).**However, rhamnolipids production in certain *Pseudomonas* spp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 **(Kannahi and Sherley, 2012; Rashedi *et al*., 2005).**

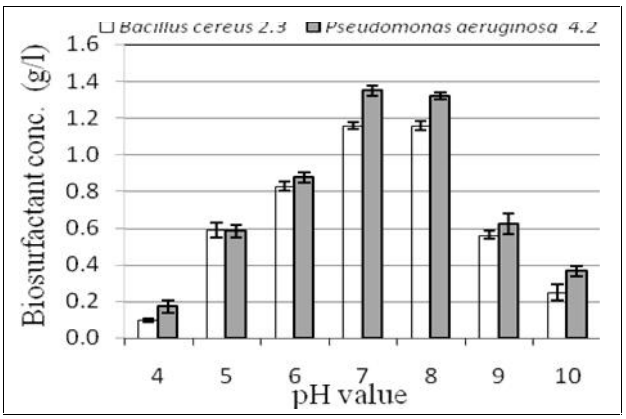

Figure 6: Effect of different pH value

3.3- Effect of different temperature.

The result reported in **Figure (7)** showed highly significant variations among different incubation temperature at significant level (P <0.05) on the productivity, the highest values were recorded at 35 and 40 ^{o}C for both bacterial tested strains while at 20 and 60 ^{o}C have markedly decrease, these indicate that these bacterial strains belonging to mesophilic group.

As well as most of the strains like *Pseudomonas putida***(Kannahi and Sherley, 2012),** *Pseudomonas aeruginosa* RS29 **(Saikia*et al*., 2012b),** *Pseudomonas aeruginosa* WJ-1 **(Xia *et al*., 2012)** and *Bacillus subtilis* MTCC441 **(Chander*etal*., 2012)** produce the maximum biosurfactant at 37°C. In contrast **Swapnil *et al.* (2014)** reported thatoptimum yield of biosurfactant *Pseudomonas aeruginosa* strain F23 demonstrated at 30^{o}C and **Ray, (2012)** reported the same result for *Bacillus sp*m28. In case of *Pseudomonas*, a change in temperature has caused alteration in the composition of biosurfactant **(Youssef *et al*., 2004).**

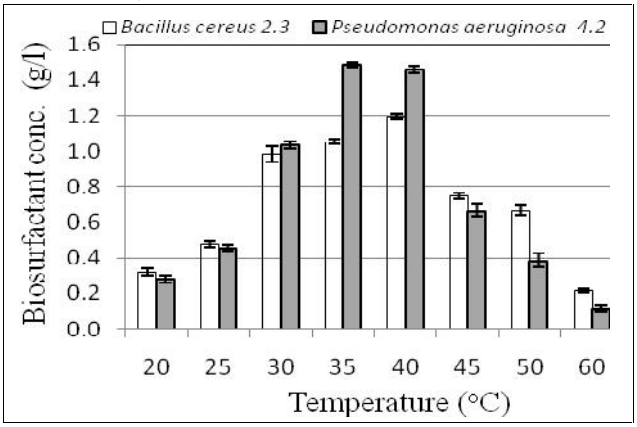

Figure 7:Effect of different temperature

3.4-Effect of different inoculum sizes.

It was observed that 4 and 6 % showed highly significant differences at significance level 0.05, which record maximum production rates; also up to a concentration of 4 – 6 % biosurfactant production was directly proportional to Inoculum size as shown in **Figure (8)**above this concentration it start to decrease.

This result was completely similar with that observed by **(Joshi *et al.*,2008;Swapnil *et al.*,2014).**In another study by **Chander *et al*,.(2012)** the production medium was seeded with 3% inoculum of *B. subtilis* MTCC441 led to good biosurfactant production while **Ray,(2012)** reported that, 1% was the optimum volume of the inoculum size for *Bacillus sp*(m28).

3.5.- Effect of different nitrogen sources.

Nitrogen plays an important role in the production of surface-active compounds by microorganisms **(Mercade*et al.*, 1996).** In the present study, several different organic and inorganic nitrogen sources (2 g/l) were tested of which most inorganic nitrogen sources showed similar growth and productivity by both *B. cereus* 2.3 and *P. aeruginosa* 4.2, except $NaNO_2$,but ammonium nitrate and urea more effective followed by NH_4Cl_2, peptone and $NaNo_3$ as represented in **Figure (9).**

In addition to that NH_4NO_3 and urea are a cost effective alternative for peptone. Similar results were obtained by **Karkera *et al.*, (2012)** for *Pseudomonas aeruginosa* R2 and optimum Nitrogen source was found out to be Ammonium nitrate. The nitrate (NH_4NO_3 or $NaNO_3$) form was found to be the best source of nitrogen for biosurfactant production by different *Pseudomonas* strains **(Tayebe *et al.*, 2009;Chi *et al.*, 2008;Kannahi and Sherley, 2012; Santa-Anna *et al.*, 2002; Xia *et al.*, 2012).**Nitrogen limitation has been reported to increase the rhamnolipids production.**(Joice and Parthasarath, 2014).**

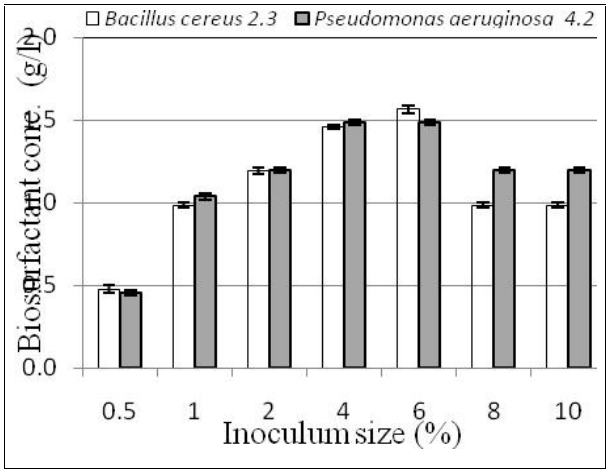

Figure 8: Effect of different inoculum size

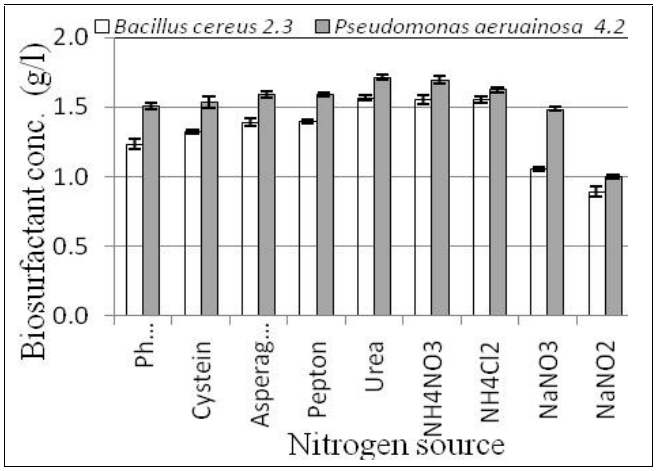

Figure 9: Effect of different N. source

3.6-Effect of different carbon source.

Each one of *B. cereus* 2.3 and *P. aeruginosa* 4.2was able to utilize glycerol as a sole carbon source and produced higher amount of biosurfactant similar to glucose **(Figure 10)** The lowest production was observed with the starch and cellulose (0.894 and 0.884 g/l respectively).

Glycerol is a simple fatty acid precursor with high solubility in medium, so it is easily utilized by bacteria for their carbon and energy source as well as glucose. Which greatly similar to the results that reported by **Joice and Parthasarath (2014)**for *Pseudomonas aeruginosa*PBSC1and partially agreement with the result that reported previously by **Ray (2012),** for *Bacillus sp*.m28 which recorded that, the best carbon source for biosurfactant production was glucose (2%).

3.3- Effect of different cheap and oily substrates.

Due to the importance of cost effective in the biosurfactant production, an alternative cheap and oily substrate were tested as a sole carbon and energy source, The results represented graphically in **Figure (11)** showed that the best cheap substrate was molasses which showed highly significant at 0.05 level which record highly production rates 1.796 ± 0.025 and 1.861 ± 0.015 for the two most potent bacterial strains, *Bacillus cereus 2.3*, and *Pseudomonas aeruginosa* 4.2 respectively .which agreements with the results that reported by **Sanket *et al.* (2008),**Maximum biosurfactant production was achieved with molasses at 5.0–7.0% (w/v) by *Bacillus licheniformis* K51, *B. subtilis* 20B, *B. subtilis R1* and *Bacillus* strain HS3.

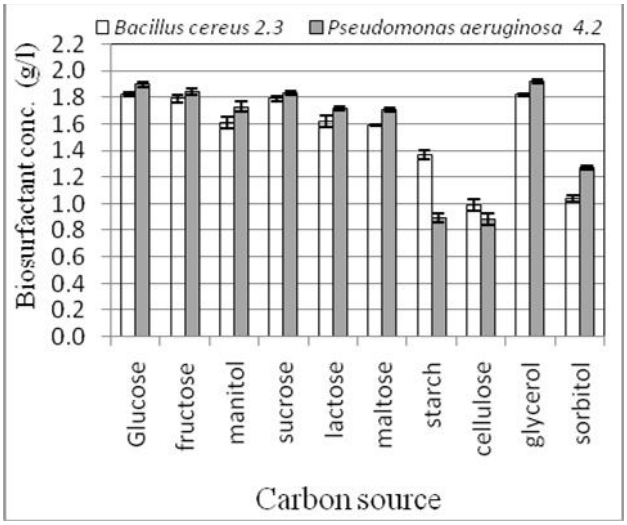

Figure 10: Effect of different C. source

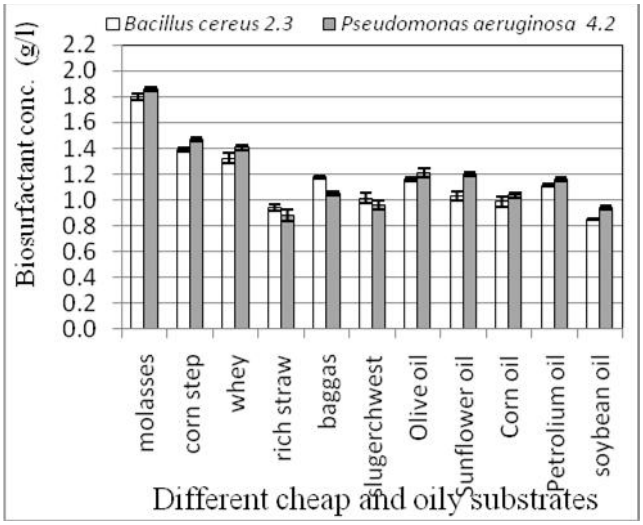

Figure 11: Effect of different substrate

3.8-Effect of C/N ratio.

Optimized C/N (molasses/NH_4NO_3) ratio for biosurfactant production by*B. cereus* 2.3*P.aeruginosa 4.2* was found to be12:1 and 15:1for give (2.48 and 2.47 g/l) respectively as showed in **Figure (12)**. The maximum biosurfactant production by *Pseudomonas nitroreducens* was at C/N (glucose/sodium nitrate) of 22 **(Onwosi and Odibo, 2013)**. However results were obtained for *P. aeruginosa* by using C/N ratio of 7:1 by **Swapnil *et al.* (2014)**; 9:1 by **Santos *et al.* (2002)** and 10:1 **(Abouseoud *et al.*, 2007).Amezcua-Vega *et al.* (2007)** reported that C/N affects biosurfactant production when N is limiting.

3.9- Effect of different NaCl concentrations.

It was observed that all pairwise showed highly significant differences at 0.05 level of significance, and the productivity directly decreased parallel to NaCl concentration until (8%), above this concentration no growth was recorded as represented in **Figure (13).**

Both of bacterial isolates under study found to be moderately halotolerant in nature; maximum biosurfactant production were 2.39 and 2.45 g/lfor *B. cereus* 2.3 and *P. aeruginosa* 4.2 in presence of 0.5% (w/v) of NaCl respectively, and maximum tolerance to NaCl (8%) gives productivity 0.23 and 0.11 g/l respectively, **Saikia *et al* (2012b)** reported that, *Pseudomonas aeruginosa* RS29 strain produced highest biosurfactant after 48 h at salinity <0.8% (w/v).

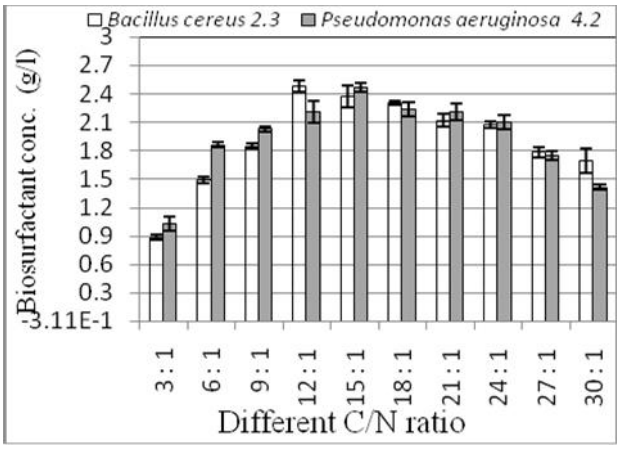

Figure 12: Effect of different C/N ratio

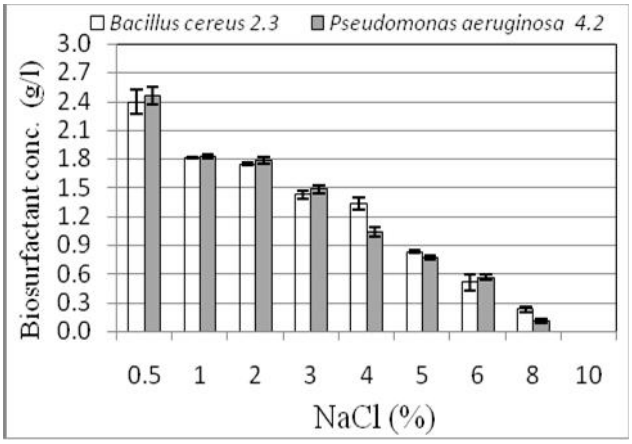

Figure 13: Effect of different NaCl concentration

The final optimal nutritional and environmental factors controlling the productivity of the biosurfactant by *Bacillus cereus* 2.3 and *Pseudomonas aeruginosa* 4.2were summarized in **Table (1).** The applying of this standardized condition improved the biosurfactant productivity by from 1.014 to 2.48 g/l by 245% for *B. cereus* 2.3 and from 1.175 to 2.46 g/l by 210 % for *P. aeruginosa* 4.2 as represented in **Figure (14).**

Table 1: Summary of the optimal nutritional and environmental factors controlling the productivity of the biosurfactant by *Bacillus cereus* 2.3 and *Pseudomonas aeruginosa* 4.2

Factor	*Bacillus cereus* 2.3	*Pseudomonas aeruginosa* 4.2
Dynamic conditions	Shaking	Shaking
Incubation period (h.)	60-72	48-72
pH value	7 - 8	7 - 8
Temperature (^{o}C)	35 - 40	35 - 40
Inoculum size (%)	6	4
Nitrogen source	Urea or NH_4NO_3	Urea or NH_4NO_3
Carbon source	Glucose or Glycerol	Glucose or Glycerol
Cheap & oily substrate	Molasses	Molasses
C/N ratio	12 : 1	15 : 1
NaCl (%)	0.5	0.5

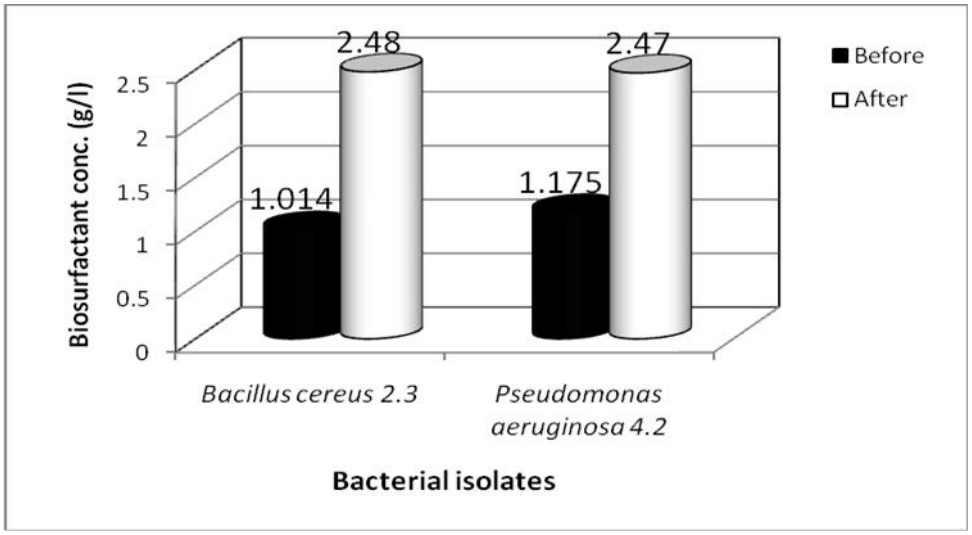

Figure 14: Biosurfactant concentration before and after optimization

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Conclusion:

Different bacterial isolates in contaminated soil have the ability to produce biosurfactant but required optimization for improvement biosurfactant production. Gram positive isolate identified as *Bacillus cereus* 2.3&Gram negative bacterial isolate identified as *Pseudomonas aeruginosa* 4.2. Nutritional factors specially limitation in nitrogen source and high sugar concentration, also environmental factors as pH and Temperature factors have significant effects on the productivity of the biosurfactant and improved the productivity up to210 to 245% for the two isolate respectively.

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