Sustainable Low-Cost Surfactin Production And Optimization By *Bacillus Subtilis* SNW3, Product Characterization, And Its Suitability For Plant Growth Promotion And Bioremediation of Crude Oil

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Abstract

At present time, every nation is absolutely concern about increase agricultural production and bioremediation of petroleum contaminated soil. Hence, with this intention in current study potent natural surfactant (surfactin) was evaluated for low-cost production by *Bacillus subtilis* SNW3, previously isolated from Fimkessar oil field, Chakwal Pakistan. The best results were obtained using substrates in combination (white beans powder (6% w/v) plus waste frying oil (1.5% w/v) and (0.1% w/v) urea) with surfactin production of about 1.17 g/L contributing 99% reduction in cost required for medium preparation. To the best of our knowledge, no single report is present describing surfactin production by *Bacillus subtilis* using white beans powder as a culture medium. Surfactin was confirmed as the principal product characterized by thin-layer chromatography (TLC) and Fourier-transform infrared spectroscopy (FTIR). Additionally, produced surfactin display great physicochemical properties of surface tension reduction value (SFT=28.8 mN/m), significant oil displacement activity (ODA=4.9 cm), excessive emulsification ability (E24=69.8 %), and attains critical micelle concentration (CMC) value at 0.58 mg/mL. Furthermore, surfactin exhibits excellent stability over an extensive range of pH (1-11), salinity (1-8%), temperature (20-121°C) and even after autoclaving. Subsequently, surfactin produced proved suitable for bioremediation of crude oil (86%) and as potent plant growth-promoting agent that significantly ($P<0.05$) increase seed germination and plant growth promotion of chili pepper, lettuce, tomato and pea maximum at concentration of (0.7 g/100 mL), proved as potential agent for agriculture and bioremediation processes by lowering economic and environmental stress.

Introduction

Environmental pollution due to petroleum products such as crude oil, diesel, and gasoline is of major ecological concern nowadays (Jimoh and Lin 2019). Major health problems in humans and animals occurred due to the release of petroleum and its by-products in a terrestrial and aquatic ecosystem because of having mutagenic, carcinogenic, and teratogenic effects (Yadav et al. 2016). Petroleum-derived pollutants result in the limitation of phosphorus, iron, and nitrogen availability in agricultural soil (Nogueira et al. 2011). In today's challenging world enhanced agricultural productivity is the need of the hour to encounter human food demands. However, equally alarming is the damage of agricultural land by pollutants that needs bioremediation strategies. Hence, it is necessary for researchers to focus on remediation of all these issues. Biosurfactants are amphiphilic secondary metabolites that exhibit surface-active properties produced by bacteria, fungi, and yeast (Santos et al. 2016). Biosurfactant-producing microorganisms enhance plant growth with improvement in plant immunity against organic contaminants in the environment, furthermore, they are also efficient in alleviating stress responses in plants along with strengthening plant growth and development (Almansoory et al. 2019). Biosurfactants (lipopeptides) not only help in the detoxification of contaminated soil but also provoke induced systemic resistance (ISR) to provide defence responses for eradicating plant pathogens (Anjum et al. 2016). One of the positive influences of the use of lipopeptides in agriculture is its biocompatibility with living organisms (Ławniczak et al. 2013). Hence, to minimize the initial dose of fertilizers by seed stimulation

strategies and its equal distribution in the soil is made possible by biosurfactants (Krawczyńska et al. 2012). Many researchers verified that plant growth-promoting rhizobacteria (PGPR) positively enhance plant development after association with hydrocarbon-degrading bacteria in contaminated soil (Pawlik et al. 2017). Different plant growth-promoting traits include phosphate solubilization, siderophore production, hydrogen cyanide (HCN) production, indole acetic acid (IAA) production and systemic resistance induction (Benaissa 2019). Hence, for employing biosurfactants in agricultural, bioremediation and its application in other fields the reduction in cost needed for production are of absolute concern. Increase in awareness among public about the use of environment-friendly and sustainable green products demand new strategies development to cut down the production cost for replacement of toxic synthetic surfactants with biosurfactants (Shaban and Abd-Elaal 2017). Biosurfactants with numerous useful applications provide growing interest in diverse industrial sectors including food, medicine, cosmetics, and agriculture (Patil et al. 2014). However, the production cost is still high that depends on the availability of raw materials and downstream processing for scaleup at the industrial level (Akbari et al. 2018). Raw materials used for biosurfactant production accounts for about 50% of the final production cost. Better choice of raw material is a way to cut down the budget and make the process economically feasible. Unlike synthetic surfactants that produced from petroleum feed stock, biosurfactants could be produced using waste materials like agriculture waste (wheat bran), brewery waste and food waste by-products (potato peels and waste frying oil) that not only reduce cost but also helps in waste disposal in environment-friendly manner (Moshtagh et al. 2018; Vea et al. 2018). In the present study, we used potato peels powder, waste frying oil, molasses, and white beans powder as a low-cost substrate for production. Hence, with all the above intentions the current study was conducted to produce stable potent biosurfactants employing various cost-effective renewable resources and to evaluate the potential of produced surfactin for detoxification and management of crude oil contaminated soil and to promote plant growth and development.

Materials And Methods

Microorganism and culture conditions

In the current study, *Bacillus subtilis* SNW3, obtained from Microbiology Research Lab, Quaid-i-Azam University, Islamabad, was previously identified and isolated from contaminated soil of Fimkessar oil field, Chakwal, Pakistan (Malik and Ahmed 2012). The bacterial sample was cultured on nutrient agar plates (Yeast extract 2.0; Beef extract 1.0; Peptone 5.0; Sodium chloride 5.0; Agar 15 g/L) incubated for 24 h at 30 °C to obtain separate pure colonies, stored for regular use at 4 °C and sub-cultured before use. The strain was preserved at -80 °C in nutrient broth (Peptone, 5; Meat extract, 1; Yeast extract, 2.0 and sodium chloride g/L) supplemented with 30% glycerol.

Cost-effective substrates for biosurfactant production.

For low cost biosurfactant production various cost-effective substrates were evaluated that includes: potato peels powder (total carbohydrate 68.7%; starch 25%; protein 18%; non-starch polysaccharide 30%;

acid-soluble and acid-insoluble lignin 20% and nitrogen 1.3%) (Liang et al. 2014), molasses (total sugars 62.3%, sucrose 48.8%, starch 0.33% and ash 13.1%) (Palmonari et al. 2020), white beans powder (protein 15.62%; carbohydrates 60.47%; lipids 2.13%; crude fibre 14.15%) (Alayande et al. 2012), waste frying oil (palmitic acid 15.86%; oleic acid 29.83%; stearic acid 4.87% and linoleic acid 28.85%) (Banani et al. 2015) and nitrogen sources : sodium nitrite, urea and ammonium nitrate while, conventional media yeast extract (protein 62.5%; sugar 2.90%; fat 0.10%; ash 9.50%) was used as control. Each carbon source listed above was designed to use individually, then selected substrates were used in different combinations to achieve an optimized medium composition. Molasses used in current study was obtained from Chashma Sugar Mills Limited in Dera Ismail Khan (Pakistan). Potato peels and waste frying oil were obtained from café located at Quaid-i-Azam University Islamabad (Pakistan). Whereas white beans were obtained from National Agricultural Research Council (NARC) Islamabad Pakistan.

Inoculum

Bacillus subtilis SNW3, streaked and stored on nutrient agar plates at 4 °C was used for inoculum preparation. A loop full of culture from a single isolated colony on plate added in 100 mL nutrient broth (Peptone, 5; Meat extract, 1; Yeast extract, 2.0 and sodium chloride, 5 g/L) incubated at 30 °C for 48 h then seed culture from the nutrient broth was used as inoculum for all experiments.

Production optimization, extraction, and partial purification of biosurfactant

The strain *Bacillus subtilis* SNW3 was grown on conventional yeast extract media (2% w/v) and mineral salt medium (MSM) as described by Abouseoud et al. (2008) of given composition (g/L: KH_2PO_4, 2.0; K_2HPO_4, 4.0; $FeSO_4.7H_2O$, 0.025; $MgSO_4.7H_2O$, 1.0; KCl, 0.2; NaCl, 5.0; $CaCl_2$. $2H_2O$, 0.02; and trace elements solution with composition of $MnSO_4.4H_2O$, 1.78; $ZnSO_4.7H_2O$, 2.32; $CuSO_4.5H_2O$, 1.0; H_3BO_3, 0.56; KI, 0.66 and $NH_4MoO_4.2H_2O$, 0.39) for evaluation of different environmental process parameters significant for biosurfactant production at various range of temperature (15, 30, 37 and 50˚C), pH (2, 4, 6, 8, 10, 12), agitation speeds (0, 150 and 250 rpm) and inoculum size (0.5, 1, 1.5, 2 and 2.5). After that carbon and nitrogen sources used to be screened out were used separately and in different combinations with MSM while yeast extract was used as control media. All designed experiments for substrate evaluation were run with 100 mL media in 250 mL Erlenmeyer flask with pH adjusted to 7.0 ± 0.2 and kept in a shaker for 96 h of incubation at 30 ºC and 150 rpm. The cell-free supernatant obtained after centrifugation at 12,000 rpm was acidified up to pH 2.0 with 1M hydrochloric acid (HCL) and kept overnight at 4 °C. For crude surfactin, pelleted precipitates were extracted with chloroform/methanol (2:1) and concentrated by rotary evaporation (Marchut-Mikolajczyk et al. 2018).

Assessment of biosurfactant production

For estimation of biosurfactant production in cell-free supernatant, various methods like oil displacement activity (ODA) was performed according to the method of Morikawa et al. (2000), emulsification index (E24%) was performed through a protocol of Cooper and Goldenberg (1987) and surface tension (SFT) was measured in mN/m by using KRUSS K20 digital Tensiometer (Kruss GmbH, Hamburg, Germany),

performed at room temperature while using a platinum plate by Wilhelmy plate method according to the protocol given by manufacturer.

Structural characterization of surfactin produced.

For thin-layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR) analysis, extracted form of crude biosurfactant was used while surfactin (from sigma) was taken as standard. Crude biosurfactant components were separated on Silica coated aluminium plates, silica gel 60 F254, MERCK Germany using chloroform: methanol: acetic acid (85:10:5, v/v) visualized under the wavelength of 254 and 365 nm to find retention factor (Rf) as described by Joy et al. (2017). For determination of surfactin functional groups 10 mg crude biosurfactant was loaded and the spectrum was observed at the range of 4500–450 cm^{-1} using Tensor 27 (Bruker) FTIR spectrophotometer, equipped with ZnSe ATR (Marchut-Mikołajczyk et al. 2019).

Functional characterization by Antibiogram of surfactin produced.

For antibiogram analysis, the extracted surfactin (10 mg/mL) dissolved in demineralized water tested with two different antibiotics namely ciprofloxacin and clarithromycin (1 mg/mL) separately and in combination (surfactin:antibiotic 5:0.5 mg/mL) against *Escherichia coli* ATC 25922, poured at a concentration of 100 µL and kept at 37°C for 24 h of incubation, finally examined by diameter (mm) of the clear zone.

Determination of critical micelles concentration (CMC) and critical micelle dilution (CMD)

The CMC of the produced biosurfactant was determined by a change in surface tension reduction with biosurfactant solutions from 0.06 to 1.24 mg/mL prepared in demineralized water (Datta et al. 2018). For critical micelle dilution cell, free supernatant was diluted10-folds up to three levels (i.e. 10x, 100x, and 1000x) named as CMD^{-1}, $CMD^{-2,}$ and CMD^{-3}, respectively, and was analyzed by surface tension reduction values (Joshi et al. 2008a).

Surfactin stability studies

To elucidate the thermal stability of surfactin, the standard solutions were prepared at a concentration of 600 mg/L and incubated at different temperatures (20-121 °C) for 1 h then surface tension of test solutions was measured by the Wilhelmy plate method after cooling at room temperature. Furthermore, a stability test of produced surfactin at saline conditions was performed at different concentrations of sodium chloride NaCl (1-10%) and incubated at 30 °C for 1 hour followed by the analysis of surface tension reduction. To determine pH effect on surfactin activity different buffer solutions were added to biosurfactant standard solution, adjusted to pH 1–5 using citrate-phosphate buffer, pH 7 using phosphate buffer, and pH 9–11 using carbonate-bicarbonate buffer solutions, and check for surface tension reduction after incubation at room temperature for 30 minutes (Goswami and Deka 2019).

Exploration of surfactin for seeds germination and plant growth

The seeds of tomato (*Solanum Lycopersicum),* pea *(Pisum sativum),* chili pepper *(Capsicum annuum),* and lettuce *(Lactuca sativa)* collected from NARC Islamabad, Pakistan were surface sterilized with 10% Na–hypochlorite for 20 minutes and then washed with sterile distilled water before use. The first seed germination experiment was conducted in petri plate containing 40 seeds positioned in filter paper and cotton soaked with four different concentrations (0.1, 0.3, 0.5, and 0.7 g/100 mL) of crude surfactin solution in distilled water while 100% v/v distilled water was used as a control. These plates were kept in yellow light at 25ºC for 7 days after that relative seed germination (G, %): (No. of seeds germinated (treatment) / No. of seeds germinated (control)×100) was calculated. After the germination test seeds treated with surfactin were transferred in pots (seeds without pre-treatment with biosurfactant were used as control) and kept in a greenhouse with temperature maintained between 20 ºC to 22 ºC. Furthermore, for plant growth stimulation crude surfactin solution was added in pots at a concentration (0.1, 0.3, 0.5, and 0.7 g/100 mL) dissolved in distilled water thrice with 10 days interval while in control pots pure water was added. The emergence of plant seedlings was tested and checked for the morphological characteristic of plants like shoot length (mm), root length (mm), and dry weight (g) of plants after 40 days (Huang et al. 2017).

Bioremediation of crude oil through various design treatments

Biodegradation efficiency of crude oil by *Bacillus subtilis* SNW3 was analyzed as illustrated by Rahman et al. (2002) with minor changes. An aliquot of 2 mL pre cultured *Bacillus subtilis* SNW3 was transferred into 250 mL of Erlenmeyer flask containing 100 mL mineral salt media with different concentrations of filter sterilized crude oil of 0.5, 1, 1.5, and 2% (v/v) as sole source of carbon and energy. For monitoring of abiotic loss of the crude oil an uninoculated media was used as control. All these flaks were incubated for 21days at 200 rpm and 35 ˚C. For monitoring the bacterial growth in crude oil, the absorbance rate was detected at (OD600 nm) by using spectrophotometer while for biosurfactant production analysis surface tension reduction was examined by tensiometer. To estimate residual crude oil in media solvent extraction method by hexane was used after that left for evaporation in a pre-weight clean beaker. For quantification of remaining crude oil degradation gravimetric analysis was performed at different time intervals by following formula proposed by Patowary et al. (2017).

Hydrocarbon degradation % = Amount of crude oil degraded /Amount of crude oil added in the media x 100

Surfactin suitability for removing hydrophobic pollutants from soil was analyzed by collecting 5-10 cm deep topsoil while following the protocol of Okop et al. (2012) and transported in a clean container to Microbiology laboratory of Quaid-i-Azam University Islamabad Pakistan. Crude oil used was collected from Pakistan petroleum limited. The soil collected was airdried and sieved with a 2 mm sieve after that 5% of crude oil was sprayed on the soil to pollute soil homogenically. The polluted soil was left undisturbed for 5 days and then divided into 200 g of equal parts and dispensed in pots. These pots were left undisturbed in the open air for a week. Then for conducting bioremediation experiments various designed treatments were established added twice throughout the remediation period: (T0) addition of

distilled water as control, (T1) addition of cell-free supernatant containing surfactin, (T2) addition of cultured *Bacillus subtilis* SNW3 (T3) combination of cell free broth and cultured *Bacillus subtilis* SNW3, (T4) addition of tween 80 (T5) addition of fertilizer (NPK; 20-10-10) shown in (Table 1). The soil content of each pot was tilled twice a week for aeration with moisture maintenance at 60% and temperature of 28-30 ºC, providing all those conditions that are appropriate for crude oil-degrading microbes present in the soil. After that soil samples of 10 g were collected from different areas of the plastic pots at the 30,60, 90th day and were gravimetrically determined using formula given by Ganesh and Lin (2009).

Table 1 Design treatments for removal of crude oil from contaminated soil by surfactin and *Bacillus subtilis* SNW3

Treatments	Soil	Biological treatment	Chemical compounds	Crude oil concentration
Control (T0)	200 g			5%
Treatment 1 (T1)	200 g	200 mL cell free surfactin broth		5%
Treatment 2 (T2)	200 g	2% cultured broth 100 mL		5%
Treatment 3 (T3)	200 g	2% cultured broth 100 mL + 100 mL cell free surfactin		5%
Treatment 4 (T4)	200 g		10 mg/kg of tween 80	5%
Treatment 5 (T5)	200 g		0.8 g/kg of fertilizer	5%

Statistical Analysis

The obtained results were analyzed statistically with the use of STATISTICA software, one-way ANOVA (version 8.1). The difference between obtained results was analyzed by using the Tukeys test to find individual and control mean ± standard deviation. Significance value was set at $p = 0.05$ and p-values ≤ 0.05 were considered significant.

Results

Substrate screening and optimization studies

Biosurfactant production using *Bacillus subtilis* SNW3 was carried out using different carbon sources. Potato peels powder, molasses, white beans powder, and waste frying oil were evaluated as cheap media for surfactin production. The processing of potatoes produces starch-rich waste in form of potato peels, starch-rich wastewater, and unconsumable potato parts that could be used as a substrate for microbial

production (Fox and Bala 2000). According to results obtained for optimization of culture conditions with 2% yeast extract media 30 ˚C temperature was considered as optimum for maximum surfactin production with an ODA value of 1.26 cm. While other optimized cultural conditions were with 1% inoculum size, 150 rpm, and pH of 6 (Fig. 1). In the present study, no significant surfactin was produced at static condition that might be due to lack of oxygenation. At the end of the fermentation process, the obtained ODA values were 1.3, 2.4, 0.9, and 1.8 cm for potato peels powder, white beans powder, sugar cane molasses, and waste frying oil media respectively with 2% w/v concentration, while the surface tension reduction values of all four biosurfactant solutions reduced from 72 mN/m to 41.3, 33.6, 41 and 38.2 mN/m respectively. Though good emulsification values of all these biosurfactant solutions were obtained to about 55 to 57% (Fig. 2a). In the current study among nitrogen sources tested preferably urea act as a good nitrogen source shows surface tension reduction of 31.4 mN/m and ODA value of 2 cm (Fig. 2b). It has been reported that supplementation of peptone, urea, sodium nitrate, ammonium nitrate (Thanomsub et al. 2004), and meat extract (Bednarski et al. 2004) increased biosurfactant production. It was observed that white beans powder and waste frying oil resulted in significant oil displacement value. The white beans powder as a carbon source is more nutritious than other sources used due to high levels of suitable elements, especially proteins. Therefore, it is expected that white beans powder can be a suitable substrate instead of a conventional medium and induce microbial cells to regulate synthesis of enzymes synthases and other peptide moieties for more biosurfactant synthesis. Also, it has been found that combination of carbon sources is more nutritious that enhances biosurfactant synthesis. The final optimized combination media was (white beans powder 6%+waste frying oil 1.5 mL + urea 0.1 g) with significant surfactin yield indicated ODA of 4.9 cm, emulsification index of 69.8% and surface tension reduction value up to 28.8 mN/m (Fig. 2c). Trace elements added in media improve biosurfactant synthesis by acting as co-factor for enzymes.

As in this portion of the study, our focus was on the use of alternative non-conventional media for fermentation of biosurfactant production. It was observed that the concentration of surfactin produced was significantly high while using substrates in combination found to be maximum of 1.17 g/L of surfactin with optimized media that was almost double while making a comparison with 0.56 g/L produced with yeast extract control media (Fig. 2d). On average 1 kilogram of white beans powder with 240 mL of waste frying oil while 640 g of yeast extract media would be enough for preparing 16 liters of fermentation media that gave 1.17 g/L of surfactin production. The cost required for the preparation of one liter of optimized low-cost media in the current study is 0.078 EUR, which is just 0.8% of one-liter synthetic yeast extract media cost 10.5 EUR. Hence utilizing these cost-effective nonconventional media instead of synthetic yeast extract contribute to a 99% reduction in cost required for medium preparation.

Structural characterization of biosurfactant produced.

Characterization of crude biosurfactant produced with final optimized media by *Bacillus subtilis* SNW3 was carried out by thin-layer chromatography (TLC) and Fourier transform infrared spectroscopy FTIR. Results obtained by TLC indicate the lipopeptide (surfactin) nature of the product with a band observed

against standard surfactin having a retention factor (Rf) value of 0.68 as illustrated in (Fig. 3c). The FTIR spectra represent the presence of carboxylic functional groups and aliphatic amines the characteristic of the lipopeptide nature of biosurfactant produced. The FTIR spectra show a sharp peak at 1023 cm^{-1} and 972 cm^{-1} that corresponds to the presence of C-N aliphatic amines in standard and crude biosurfactant (Fig. 3a, b). The peaks in FTIR spectra at 1450 and 1130 suggest the presence of stretching bands between carbon atoms and hydroxyl groups. The absorbance appears at 1762 cm^{-1} and 1757 cm^{-1} attributed to the vibrations due to the ester carbonyl group of peptide components. The peaks observed in FTIR spectra at 2942 and 2926 corresponds to the presence of C–H bands (CH2–CH3 stretching). Another peak ranging from 3500–3200 cm^{-1} indicated presence of alcohols and phenols O–H stretch, H–bond. The spectra presented in the current study in comparison to standard surfactin from sigma suggested the presence of peptide moiety and aliphatic groups, a distinctive feature of lipopeptides.

Functional characterization of surfactin by antibiogram activity

In this study, we observed that surfactin produced by *Bacillus subtilis* SNW3 has an improved antimicrobial effect against *Escherichia coli* as compared to commercially available antibiotics. The maximum inhibitory zone was observed with the combined synergistic effect of surfactin with antibiotics. However, individual use of surfactin also displays a better antimicrobial effect as compared to antibiotics used. We observed that surfactin (27 mm), ciprofloxacin (18 mm), clarithromycin (20 mm) and surfactin in combination with antibiotics displayed (30 mm) inhibitory zone (Fig. 4). Therefore, obtained results demonstrated that surfactin has better antimicrobial properties than commercially available antibiotics and is significantly increased while surfactin and antibiotics are mainly used in combination. Biosurfactants in recent years provide a group of novel antimicrobial compounds. These natural compounds could be applied as safe and effective alternatives to conventional antibiotics. The antimicrobial effect of biosurfactants is due to their potential to form pores inside cell membrane (Gudiña et al. 2010). The results indicated that produced surfactin shows potential antimicrobial effect at a minimum concentration of 10 mg/mL. Hence, these results demonstrate that produced surfactin not only provides new antimicrobial agents against resistant pathogenic strains but also needed to be used at lower concentrations than reported earlier that makes it economically feasible.

Critical micelle concentration (CMC) and critical micelle dilution (CMD) determination

The crude biosurfactant from *Bacillus subtilis* SNW3 dissolved in distilled water at different concentrations showed a reduction in surface tension of water from 72 to 36 mN/m with an increase in surfactin concentration. At the start decrease in surface tension was observed while surface tension set into constant value after concentration of 0.58 mg/mL, indicated that the CMC had been obtained

(Fig. 5a). For estimation of surfactin concentration produced in medium surfactin produced seems to be more competent that remains stable with surface tension reduction values from 29 mN/m to 32 mN/m after making 3-fold dilutions shown in (Fig. 5b). Interestingly surfactin produced during current study is seems to be more potent than reported earlier because only minimum concentration of 0.58 mg/mL was required to attain CMC value.

Stability Studies

The applicability of surfactin produced depends on behaviour it shows at different conditions of temperature, pH, and salinity. The biosurfactant produced during the current study was found to be more stable after exposure to various temperatures ranges since no significant difference was detected for surface tension reduction values from 20 to 121°C. The favourable surface tension reduction values were observed over a pH range of 1 to 11, although in between pH 5 to 7 surfactin produced was found to be more stable (Fig. 6). While with pH reduction at pH 1, surface tension value raised slightly up to 35 mN/m which means that surfactin produced shows stability at acidic conditions but more effectively stable at alkaline ones. The decrease in stability of biosurfactants at acidic conditions might be due to the protonation of negative polar ends of surfactin molecules. Besides this, it was observed that produced surfactin was stable over a wide range of salt concentrations 1 to 8% appeared to be an increase in surface tension reduction values at high concentrations of salinity i.e 10% NaCl. It is assumed that reasons for decreased stability with more NaCl concentration could be due to ion-dipole interactions between salt and water that avoid solute molecules from reaching the interface for the reduction in surface tension.

Effect of surfactin on seed germination

In this study, *Solanum lycopersicum* (tomato), *Pisum sativum* (pea), *Capsicum annuum* (chili pepper), and *Lactuca sativa* (lettuce) were examined to demonstrate the effects of biosurfactant on seed germination. The current study depicts those seeds treated with different surfactin concentrations exhibit significantly ($P < 0.05$) better effects on germination as compared to control water. This increase in germination might be due to the reason that biosurfactant increases the permeability of seed coat to water that indirectly makes quicker the metabolic processes inside seeds. The best results for germination were obtained at higher concentrations of surfactin tested. Among all seeds tested significant ($P < 0.05$) stimulation was observed for chili pepper seeds, which shows almost double 51.7% germination at a concentration of 0.5 g/100 mL in comparison to control 21.6% with MilliQ water. Similarly, tomato seeds show 68.75% germination at 0.7 g/100 mL in comparison to control water (56.25%) shown in (Fig. 7a). The germination of pea and lettuce seeds affected to some extent with not great difference observed as compared to control. According to da Silva et al. (2015) for facilitation of the germination process permeability of embryonic tissues is needed that makes easier the water entrance,

which helps in the activation of metabolism. For increased germination of seeds, the released nutrients must diffuse at suitable rates from liquid-filled intercellular spaces into the seed coat.

Effect of surfactin on the plant dry biomass

The applied biosurfactant treatments also augmented the dry biomass of plants. The plants that arose after treating with different concentrations of surfactin displays higher biomass in comparison to control. This increase in biomass might be due to the enhanced production of phytohormones in plants and improved mineral solubilization in soil (Das and Kumar 2016). During the current study significant ($P < 0.05$) increase in weight was observed for chili pepper and lettuce that exhibit dry biomass of 0.21 g and 0.25 g at 0.7 g/100 mL of surfactin used that is four times increase in relative to control 0.06 g of the seedling. Although for pea and tomato similarly a positive effect was noted with the addition of surfactin that significantly increase ($P < 0.05$) dry biomass at 0.7 g/100 mL almost double in relative to control (Fig. 7b). Interestingly, in the present study positive effect of surfactin was observed for all seeds but maximum for chili pepper and lettuce seeds while analyzing germination and dry biomass.

Effect of surfactin on root length

Almost all surfactin concentrations tested showed an immense effect on root elongation. The plants treated with a higher concentration of 0.7 g/100 mL of surfactin enhance root growth at maximum. The current study depicts that surfactin treatment exhibits significantly ($P < 0.05$) better elongation of seedling roots in lettuce, pea, and chili pepper almost two times greater than control. The tomato seedlings treated with surfactin also show an increase in root development (Fig. 7c). The increase in root elongation might be due to a decrease in the strength of wrapping tissues and seed coating resistance against the extension of the root axis that favours root development (da Silva et al. 2015). Another reason for the increase in root development by applying biosurfactants could be due to minimizing anaerobiosis conditions in the soil, the main cause of root stress (Shukry et al. 2013). Possible reasons that are hypothesized for increase in seed germination and plant growth after treating with surfactin are explained in a schematic way shown in (Fig. 8).

Effect of surfactin on plant growth promotion

All surfactin concentration tested shows significant effect on plant growth parameters. The chili pepper plants show a significant ($P < 0.05$) difference in height 8.06 mm after treatment with 0.7 g/100 mL of surfactin almost double as compared to control (Fig. 7d). Whereas lettuce plants show a gradual increase in height with an increase in surfactin concentration that might be due to a slow reduction in resistance for the leaf axis region of the seed coat. Following our knowledge research conducted for the effect of biosurfactant on plant growth is not more and these results would provide a positive step in the future for growing such species without using toxic agrochemicals. The better plant development with the use of

biological surfactants is due to increase nutrients bioavailability and emulsifying hydrophobic compounds in the soil a beneficial approach for plant growth-promoting microbes living in the rhizosphere (Marchut-Mikolajczyk et al. 2018). All four plant types of tested species with untreated control and treated with surfactin in comparison after 40 days of incubation are shown in (Fig. 9).

Bioremediation of crude oil through surfactin

Biosurfactants are used to emulsify hydrocarbons with the reduction in surface tension, enhancement of water solubility, and increasing oil displacement from soil particles (Andrade Silva et al. 2014; Geetha et al. 2018). Results obtained by the current study revealed that by use of biosurfactants oil-contaminated soil sediments could be remediated in an eco-friendly manner. The current study revealed that crude biosurfactants produced by *Bacillus subtilis* SNW3 effectively remove crude oil from the water and soil. In the present study, the pattern for *Bacillus subtilis* SNW3 growth on crude oil and MSM revealed that there was an increase in microbial growth trend up to 13 days of incubation whereas after that decline in growth was recorded while considering concentration maximum growth was observed for 1 and 1.5% of crude oil used. In the flasks amended with crude oil with increase in microbial growth the SFT value of the culture medium reduced from 72 to 29 mN/m which indicates the surfactin production (Fig. 10a). The simultaneous microbial growth and crude oil biodegradation with surfactin production in culture broth media indicates that various components of crude oil are utilized as substrates for surfactin production. This crude oil utilization by microbes and emulsification by produced biosurfactant boost up the biodegradation process (Antoniou et al. 2015). Mostly biosurfactants are considered as secondary metabolites but, some of biosurfactant molecules helps in microbial survival by aiding transport of nutrients towards microbial cells (Rodrigues et al. 2006a). However, for degradation of oil in MSM degradation percent was increased after every successive week till 21 days and significant values were observed for 1% of crude oil as (81.3%) and (86%) for 1.5% of crude oil (Fig. 10b, 11). After applying different strategies needed for bioremediation the residual crude oil content of each treatment revealed that biodegradation occurred at different extents. It was revealed that the combined strategy of bioaugmentation and biostimulation occurred in T3 shows more potential towards remediation of oil from contaminated soil. The highest reduction was observed with increase in time in all treatments after 90 days and maximum degradation percent observed for T3 treated with *Bacillus subtilis* SNW3 cultured microorganisms and surfactin (80.2%), shows a significant difference from T0 control (11.6%) with distilled water (Fig. 10c). The better bioremediation results (73.2%) were obtained in T1 by stimulation of indigenous microbes with the addition of surfactin than those obtained in T2 by adding surfactin producing strain *Bacillus subtilis* SNW3 (63.8%) that delay in T2 might be due to contaminant stress and time taken by microbial isolates to adopt new environment, while for T1 system facilitate degradation by emulsifying hydrocarbons for indigenous microorganisms who takes time only to produce enzymes needed for oil degradation. Tween 80 is considered to be more suitable for remediation of contaminated soil because of its low cost as compared to other non-ionic surfactants (Bautista et al. 2009). The Tween 80 improved soil washing and extraction of hydrocarbons from the soil, most successfully reported for polycyclic aromatic hydrocarbons PAHs (Gong et al. 2015). In the current study while making a

comparison for bioremediation with chemical compounds it was observed that in T4 addition of Tween 80 shows 65.4% lower than treatments with biosurfactants that might be due to acidic conditions of soil generated by Tween 80 that is unsuitable for microbial growth (Liu et al. 2010). The oil reduction results obtained for T5 in which polluted soil treated with fertilizers showed (32.6%) were lower than those obtained by other treatments (Fig. 10c). Less degradation of oil contaminated soil with addition of fertilizers might be due to absence of crude oil emulsification and only providing suitable nutritional components for oil degrader indigenous heterotrophic bacteria while in other case surfactin not only have ability for hydrocarbons emulsification but also possess good nutrients level. It was observed for all tested treatments that with passage of time after 60 days oil removal percent decreases that might be due to increase in toxic degradation by-products and decrease in the feeding material availability for microorganisms. In recent years, use of biosurfactants for the treatment of oil-contaminated soil is increased. Indigenous microbes that are normally present in oil-contaminated soil are mainly involved in the biodegradation of oil pollutants. Crude oil is a complex mixture of aliphatic and aromatic hydrocarbons that inhibits the uptake of carbon sources required for metabolism and growth. These oil-contaminated environments are treated with biosurfactants for enhancing hydrocarbons bioavailability to microbes present in the environment and to increase the fertility of agricultural soil shown in form of schematic presentation in (Fig. 12). In current study surfactin in combination with *Bacillus subtilis* SNW3 exhibits 80.2% removal of crude oil from contaminated soil higher than other chemical compounds tested for remediation.

Discussion

Biosurfactant production by using cost effective substrates produced by *Bacillus subtilis* SNW3 was previously studied by many researchers. An easy way to achieve cost-effective bioprocesses for surfactin production is by using a low-cost substrate. The use of waste frying oil as a sole source of carbon and energy for lipopeptide production by two *Bacillus* strains was previously reported by Md Badrul Hisham et al. (2019) that gave surface tension reduction values up to 36 mN/m these results are consistent with our study while *Bacillus* strain SNW3 growing on 2% waste frying oil shows 38 mN/m reduction value. De Lima et al. (2009) reported rhamnose production by *Pseudomonas aeruginosa* PACL strain cultivating on waste frying soybean oils indicated biosurfactant production with 100% emulsification index, surface tension reduction up to 26.0 mN/m, and concentration of 3.3 g/L while in the current study 56.3% emulsification was observed with 2% waste frying oil. Research conducted by Abdel-Mawgoud et al. (2008a) investigates surfactin production in a cost-effective manner with the use of 16% molasses and other trace elements that produce a surfactin yield of 1.12 g/L. However, it is also stated in many studies that the presence of hydrophobic substrate is essential for the production of biosurfactants (Karanth et al. 1999). According to literature different types of oils e.g., vegetable oils, waste cooking oil, glycerol, glucose, and diesel were screened out for biosurfactant production by fungal species *M. circinelloides* that shows 11.7 cm ODA with the use of waste cooking oil as carbon source. In another study conducted by Hasanizadeh et al. (2017) for biosurfactant production showed maximum biosurfactant production with the use of 8% (v/v) waste cooking oil as carbon source. However, in these reported cases, these

substrates were used separately while on the contrary in current study substrates were used in combination to increase production and reducing price of culture media. Likewise, some authors like Ohno et al. (1995) reported the use of Okara obtained after processing of ground soybeans as a substrate for lipopeptide iturin and surfactin production by *Bacillus subtilis* NB22. While Zhu et al. (2013) also investigated the use of soybean flour as a substrate for surfactin production by *Bacillus amyloliquefaciens* XZ-173. To the best of our knowledge, for economical biosurfactant production, only a few studies are conducted by using soybean, but no single study is present that shows the use of white beans powder as a substrate for low-cost production. The concentration of surfactin produced (about 1.17 g/L) was close to other reported values for biosurfactant production using cost effective substrates. Also, it was reported by Najafi et al. (2010) that 30°C is the optimum temperature for biosurfactant production that is in correspondence with results obtained in the current study. In the present study, no significant surfactin was produced at static condition that might be due to lack of oxygenation that is also reported in study conducted by Santos et al. (2014) for biosurfactant production by *Candida lipolytica*. In a previous study conducted by Hemlata et al. (2015) for biosurfactant production by *Stenotrophomonas maltophilia* NBS-11 shows maximum production at pH 7. Urea and ammonium nitrate have been already used and reported in literature as very cost-effective nitrogen source to produce biosurfactant by *Artherobacter paraffineus* and various other bacterial species (Karanth et al. 1999). Study conducted by Medeot et al. (2017) showed high yield of biosurfactant (1.7 mg/mL) while using NH_4NO_3 and glucose as substrate for production by *Bacillus amyloliquefaciens* MEP218. In the same way, combination of sucrose and NH_4NO_3 were used by Fernandes et al. (2016) and they reported high yield of biosurfactant (0. 2 g/L) by *Bacillus subtilis* RI4914. Likewise, study conducted for surfactin production by Abdel-Mawgoud et al. (2008b) reported use of different carbon nitrogen sources and ultimate results showed maximum biosurfactant production by *Bacillus subtilis* BS5 while using $NaNO_3$ and NH_4NO_3 as source of nitrogen. In our case, source of nitrogen and carbon plays an important role for surfactin production but amount of surfactin produced was almost double while using carbon/ nitrogen substrates in combination.

Primary characterization for surfactin was carried out by using TLC while using surfactin from sigma as standard. TLC can evaluate about biosurfactants composition, because of having different affinities of molecules to stationary and mobile phase through capillary action. Here our results for TLC of crude biosurfactant sample indicated presence of surfactin with an Rf value of 0.68. These findings are consistent with other reported studies, where Rf value of 0.76 was observed by Cooper et al. (1981) for surfactin produced by *Bacillus subtilis.* Another report shows similar results for Rf values were observed by Arrebola et al. (2010) using *Bacillus subtilis* UMAF6619, UMAF6614, UMAF8561, UMAF6639 and *Bacillus amyloliquefaciens* PPCB004 for fengycin, iturin and surfactin as 0.9, 0.3 and 0.75 respectively. While FTIR results obtained were in accordance with TLC. The chemical structure of surfactin produced by *Bacillus subtilis* SNW3 was revealed by analyzing the crude extract using fourier transform infrared spectroscopy. FTIR analysis of crude biosurfactant produced by *Bacillus subtilis* SNW3 showed it contains alcohols and carboxylic acids (lipids) and peptide moieties (proteins). A similar pattern of FTIR aliphatic and peptide moieties was reported for the presence of lipopeptides by (Joshi et al. 2008b). The

observed pattern of IR spectrum was very similar to spectrum obtained by de Faria et al. (2011) who reported the appearance of the stretch at 1721 cm^{-1} with FTIR analysis of biosurfactant produced by *Bacillus subtilis* isolate LSFM-05 that indicates the presence of lactone carbonyl group. Similar FTIR absorption spectra were reported in the literature for lipopeptide (Pereira et al. 2013). Based on results obtained for antibiogram in current study it was observed that produced surfactin shows potential antimicrobial effect at a minimum concentration of 10 mg/mL. According to literature lipopeptides are reported for antimicrobial activity but in current study only less concentration of surfactin was required to show its antimicrobial effect. Likewise study conducted by Sambanthamoorthy et al. (2014) revealed antimicrobial activities against *A. baumannii, E. coli*, and *S. aureus* at a concentration of 25–50 mg/mL. In current study results obtained proved the produced surfactin is of high efficiency since it shows ability to reduce surface tension of water from 72 to 36 mN/m. The critical micelle concentration (CMC) is the minimum biosurfactant concentration needed to achieve lowest surface tension value after that point micellar aggregates formation starts (Ron and Rosenberg 2001). CMC is considered as important characteristic of surface-active agents for evaluation of their interfacial activity (Zhou et al. 2019b). As shown through obtained results the minimum SFT values (36 mN/m) was obtained at the surfactin concentration of (0.58 mg/mL) where CMC found to be more significant than previously obtained by Ghasemi et al. (2019) for lipoproteins with SFT reduction up to (39 mN/m) with CMC at concentration of (2.7 mg/mL). These results were also efficient as compared to commonly used synthetic surfactants sodium dodecyl sulfate (SDS) that attains CMC value at 2100 mg/L (Chen et al. 2006). These results suggest that superior results of CMC were obtained at 0.5 mg/mL for current surfactin produced over other reported CMC values of 2.7 mg/mL.

After biosurfactant production purification strategies accounts for near 60% of total cost required for production. While considering economic value of the industry, most of biosurfactants are required either in crude form or in form of broth preparations. Therefore, for surfactin application produced by *Bacillus subtilis* SNW3 in crude extracted form without further purification steps was explored. Surfactin produced by *Bacillus subtilis* SNW3 exhibits excellent stability over an extensive range of pH (1–11), salinity (1–8%), temperature (20–121°C) and even after autoclaving. In a previous study conducted by Purwasena et al. (2019) biosurfactant produced shows good stability regarding emulsification at a high temperature of 120°C, pH of 4–10, and NaCl concentration of 10% (w/v) that are consistent with this study. Similarly, it was reported by Moussa et al. (2013) that while studying the stability of biosurfactant produced by *Bacillus methylothrophicus* and *Rhodococcus equi* strains found to be stable between 20–120°C. The persistence of biosurfactant produced with reduced surface tension reduction values under alkaline conditions has also been reported by researchers in different studies (Rodrigues et al. 2006b; Gudina et al. 2010). Several other studies have been reported about stability of the biosurfactants at high salinity and temperature (Das and Kumar 2018; Hentati et al. 2019; Purwasena et al. 2019). The excellent stability of the produced surfactin at wide range of temperature, pH and salinity widens its applicability in several industrial sectors from food, pharmaceuticals, detergents, agricultural to enhanced bioremediation.

In modern agricultural field use of bacterial biosurfactants plays an important role as they are eco-friendly and affordable. Lipopeptides derived from bacterial strains provide an ideal solution as they are eco-friendly, less toxic, remains active in harsh environments because of its more stability and are highly biodegradable in nature as compared to its synthetic counterparts (Lima et al. 2011). The genera *Bacillus* and *Pseudomonas* have been proven to be the major producers of biosurfactant molecules (Zhou et al. 2019a; Hussain and Khan 2020). For studying this the crude lipopeptide surfactin produced by *Bacillus subtilis* SNW3 at different concentrations were investigated on lettuce, pea, tomato and chilli for seed germination and plant growth parameters. We observed that treatment of plants with surfactin enhance not only seed germination but also faster the other parameters of plant growth (total plant dry weight, plant height, shoot length and root length) over the untreated control. These findings provide suggestions about surfactin that it bring about tolerance in host plant and also involved in increased resistance against pathogenesis in future in these plants, in accordance with results reported by (Raaijmakers et al. 2010; Nadeem et al. 2021). Similar results with an increase in plant biomass were observed by Liu et al. (2014). It was demonstrated by Cawoy et al. (2014) that surfactin produced by *Bacillus* isolates induce systemic resistance (ISR) which increases with an increase in surfactin concentration. Surfactin act as signalling molecule that provoke cannibalism and formation of matrix. These surfactin molecules stimulate outflow of potassium to encourage biofilm development (López et al. 2009). Treatment of seeds and plants with crude surfactin increased germination and plant growth parameters (height of plant, dry weight, root and shoot length) greater over those observed in untreated control. These findings revealed that when surfactin was added in higher concentration all these parameters improved with increasing concentration. Surfactin extract of *Bacillus subtilis* SNW3 could therefore be applicable in formulating biological control agents for diverse pathogens. Several researchers have reported the biosurfactant effect on seed germination, but to our knowledge, this is the first time reported about the use of given vegetable plants. Biopreparations are widely used nowadays for the enhancement of seed quality, to improve plant germination in contaminated soil, and also used as a nutrient for plants (Mukherjee et al. 2006). In this sense, due to the amphiphilic structure of biosurfactants, it acts on the external wrapping of seed tissues increases its permeability and facilitates germination. This property of biosurfactants having the potential to increase seed germination rate and high biocompatibility leads to effective application of surfactin produced by *Bacillus subtilis* SNW3 in agriculture, by reducing use of toxic agrochemicals. The increased plant growth parameters and defence provided by surfactin produced by *Bacillus subtilis* SNW3 may be due to more access to nutrients in soil and inhibition of plant pathogens. These surfactin molecules could be used as an alternative to chemical fertilizers and pesticides as biological stimulants and control agents with more the interesting results of the present study will create low-cost and environment-friendly plant growth promoting biological surfactants in agricultural fields. However, some research gaps are still required to be filled about mechanisms followed by biosurfactants concerning enhanced growth and development of plants.

Many reports are present about efficacy of biosurfactant produced by *Bacillus* species in oil recovery methods (Pereira et al. 2013), for bioremediation processes (Greenwell et al. 2016), in industrial sectors and degradation practices (Ismail et al. 2013). While degradation cationic portion of biosurfactant

attracts bacterial membrane that is negatively charged and in contact with crude oil present in environment (Ferradji et al. 2014). Crude oil is composed of hydrophobic water insoluble compounds having diverse chain lengths of n-alkanes. Microorganisms involved degradation of low molecular weight hydrocarbon chains and synthesis of surface-active agents that makes culture media turbid after degradation of crude oil (Chandankere et al. 2014). By introducing microbial culture to contaminated site results in enhanced bioremediation through emulsification of hydrocarbons, solubilization and mobilization (Nievas et al. 2008). In this study *Bacillus subtilis* SNW3 was identified as efficient for bioremediation of crude oil and shows increased degradation as compared to other reported studies for degradation of crude oil (Sathishkumar et al. 2008). It was reported by Iwai et al. (2011) and Lee et al. (2018) that in addition to biosurfactants some other hydrocarbon-degrading indigenous microbes and their metabolic products are involved in the bioremediation of oil contaminants in the polluted environment. In a study conducted for application of microorganisms for total petroleum hydrocarbons (TPH) degradation by *Rhodococcus* sp. NJ2 and *Pseudomonas* sp. BP10 shows degradation percent of 49.5 and 60.6% respectively after 30 days incubation reported by Kumari et al. (2012) while in current study more significant biodegradation percent of 86% was observed after 21 days. Al-Wasify and Hamed (2014) explained that *P. aeruginosa* reveals about 77.8% of maximum degradation after incubation period of 28 days and temperature of 22°C.

In a crude oil degradation study by Kumari et al., it was reported that two biosurfactant producing strain, namely, *Pseudomonas* sp. BP10 and *Rhodococcus* sp. NJ2 degraded 60.6 and 49.5% of TPH respectively when incubated for 30 days. According to results defined in the literature, *P. aeruginosa* produces biosurfactants that shows an ability to remove 49–54% of crude oil from contaminated sand (Bordoloi and Konwar 2008), while more than 85% of removal rates for diesel oil-contaminated sand samples were observed by Silva et al. (2010), but for petroleum-contaminated soil less than 20% removal was observed. Likewise, Nalini and Parthasarathi (2013) showed 92% removal of used engine oil contaminated sand with biosurfactant produced by *S. rubidae*. Biosurfactant produced by *S. marcescens* UEO15 confirmed recoveries of 59% and 78% of kerosene and crude oil, in comparison of 25% and 10% with distilled water used as control (Elemba et al. 2010). Bacterial strains efficiency regarding degradation depends on its degradative enzymes production. It was reported by Mishra and Singh (2012) that among degradative enzymes alkane hydroxylase results in degradation of n-hexadecane enzyme produced by *Rhodococcus* sp. NJ2 and *P. aeruginosa* PSA5. Genes involved in production of these degradative enzymes are reported in studies conducted by Whyte et al. (2002). Efficiency of bacterial degradative enzyme producing capabilities of the bacterial strain make them an efficient strain among other. Recently Mishra and Singh (2012), have reported that alkane hydroxylase enzyme play an important role in the degradation of n-hexadecane by bacterial strains *P. aeruginosa* PSA5 and *Rhodococcus* sp. NJ2. These enzymes play an important role in the hydrocarbon degradation and the respective genes that encode those enzymes were identified in recent studies (Whyte et al., 2002; Hassanshahian et al., 2012). Interesting point is that surfactin is mostly applied in biomedical field and only few reports are present that shows success story of surfactin in bioremediation of oil polluted environments. Biosurfactants have potential to degrade oil polluted environments in environment friendly manner, but problem is of cost competitiveness with

chemical surfactants hence biosurfactant production on large scale using low-cost substrates is required to minimize production cost. The current study demonstrated the use of effective low-cost media for surfactin production by *Bacillus subtilis* SNW3. The possibility of utilizing waste frying oil in combination with low-cost white beans proved to be efficient to substitute yeast extract media and becomes worthwhile for its industrial-scale production. The surfactin obtained exhibited potential emulsifying and surface tension reducing capabilities with strong stability at a wide range of pH, temperature, and salinity. In addition, surfactin showed higher potential for seed germination and plant growth of *Capsicum annuum, Lactuca sativa, Solanum Lycopersicum, Pisum sativum*, and removal of crude oil from contaminated soil, suggesting its potential application in environmental processes and agriculture.

Abbreviations

Thin-layer chromatography,(TLC); Fourier-transform infrared spectroscopy (FTIR); oil displacement activity (ODA); surface tension reduction value (SFT); excessive emulsification ability (E24); critical micelle concentration (CMC); induced systemic resistance (ISR); plant growth-promoting rhizobacteria (PGPR); hydrogen cyanide (HCN); indole acetic acid (IAA); National Agricultural Research Council (NARC); Mineral salt medium (MSM); retention factor (Rf); critical micelle dilution (CMD).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

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Authors contributions

AU and SA, Conception and study design; AU and AZ, carry out experimental work of study; AU, MPS and HW, AHN, testing and data analysis of study; SA, Supervision; AU, draft the manuscript; AU, MAQ, ZAM and SA, revised the manuscript. All authors read and approved the final manuscript.

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Figures

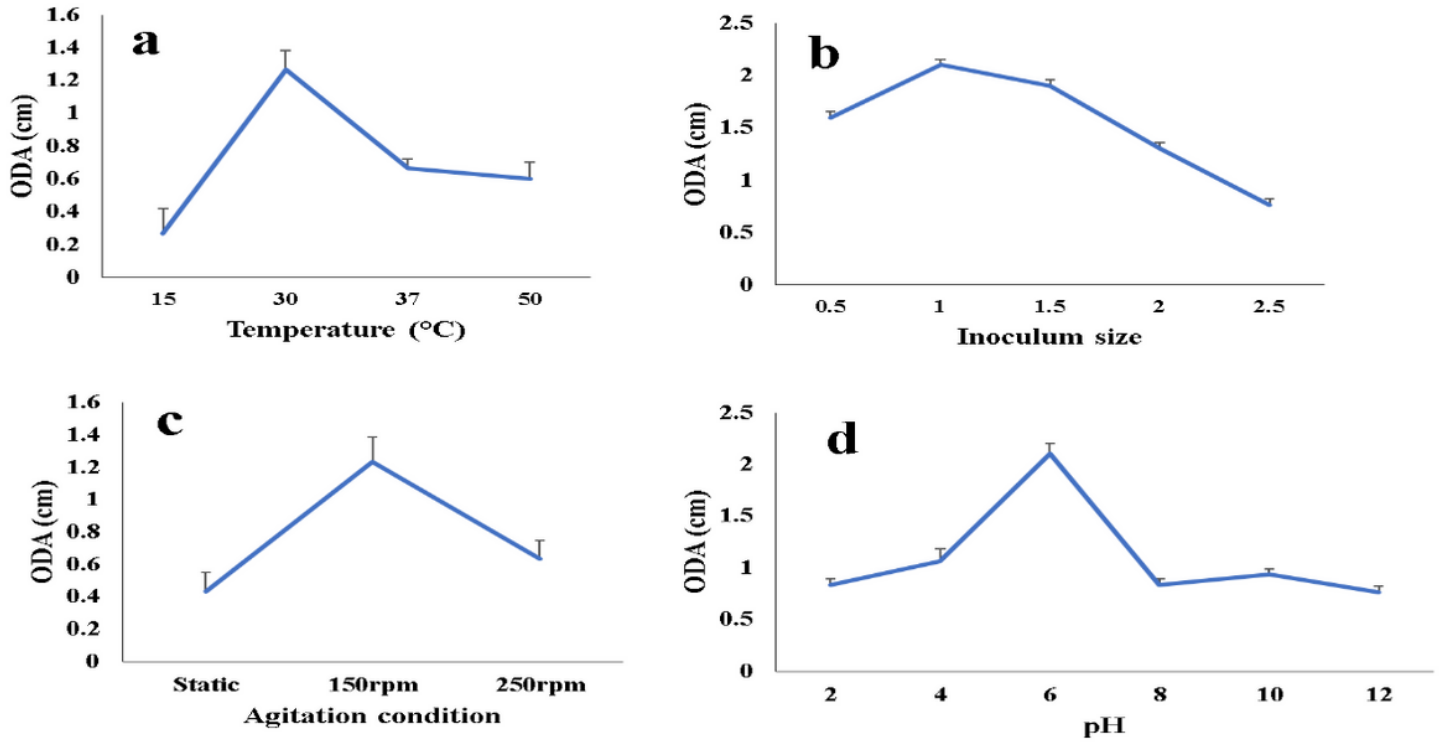

Figure 1

Effect of cultural conditions on ODA of surfactin produced by Bacillus subtilis SNW3 (a) Temperature (b) inoculum (c) agitation and (d) pH error bars represent ± standard deviation that obtained after meaning the value of triplicate experiments.

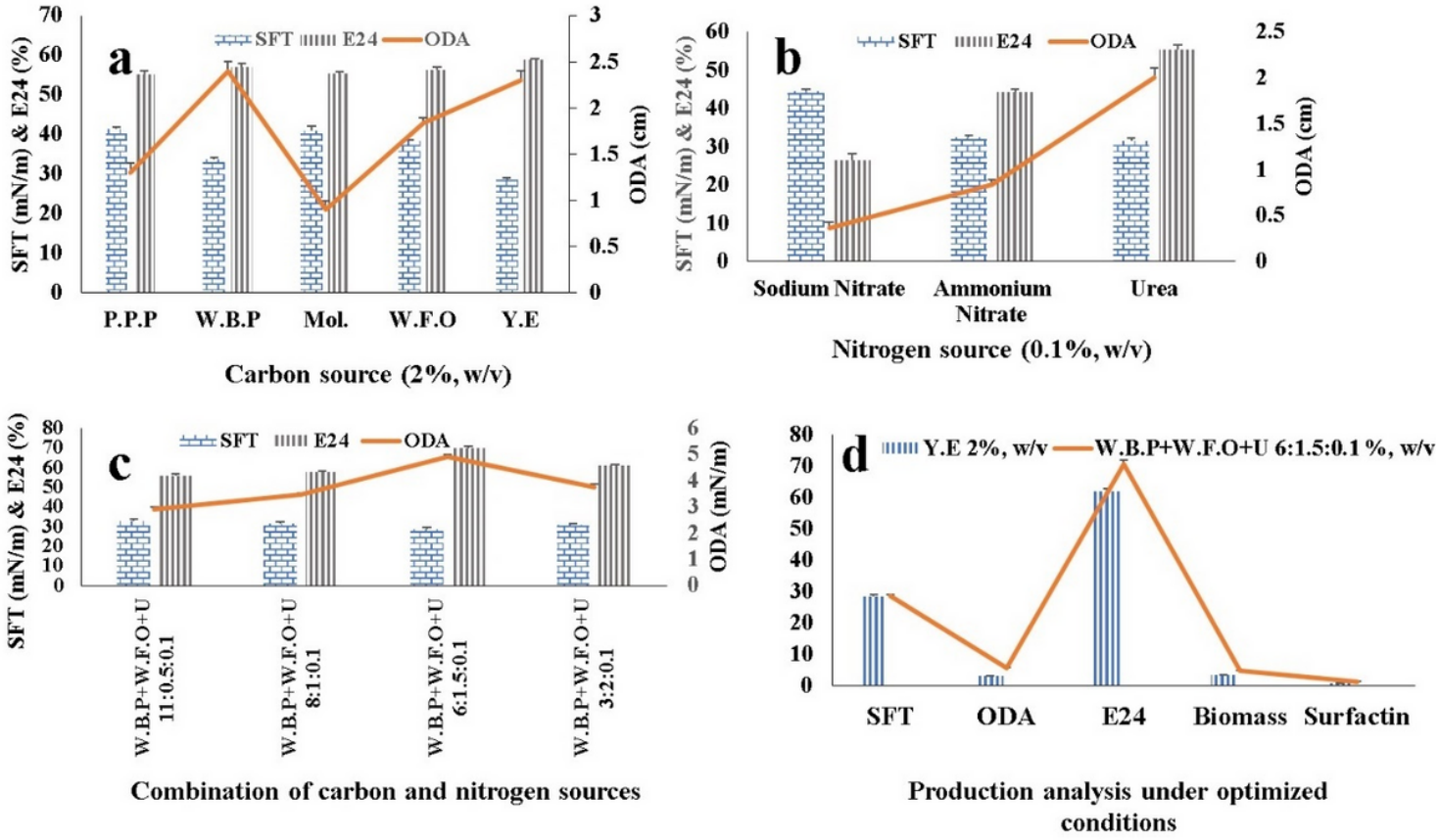

Figure 2

SFT, E24 and ODA values for surfactin production by Bacillus subtilis SNW3 (a) with alternative carbon sources used individually (b) different nitrogen sources (c) with a combination of carbon and nitrogen energy sources and (d) production analysis of surfactin under optimized conditions with yeast extract as a reference, in shake flask fermentation at 30 °C. (Abbreviation: P.P.P Potato peels powder, W.B.P White beans powder, Mol. Molasses, W.F.O Waste frying oil and Y.E Yeast extract).

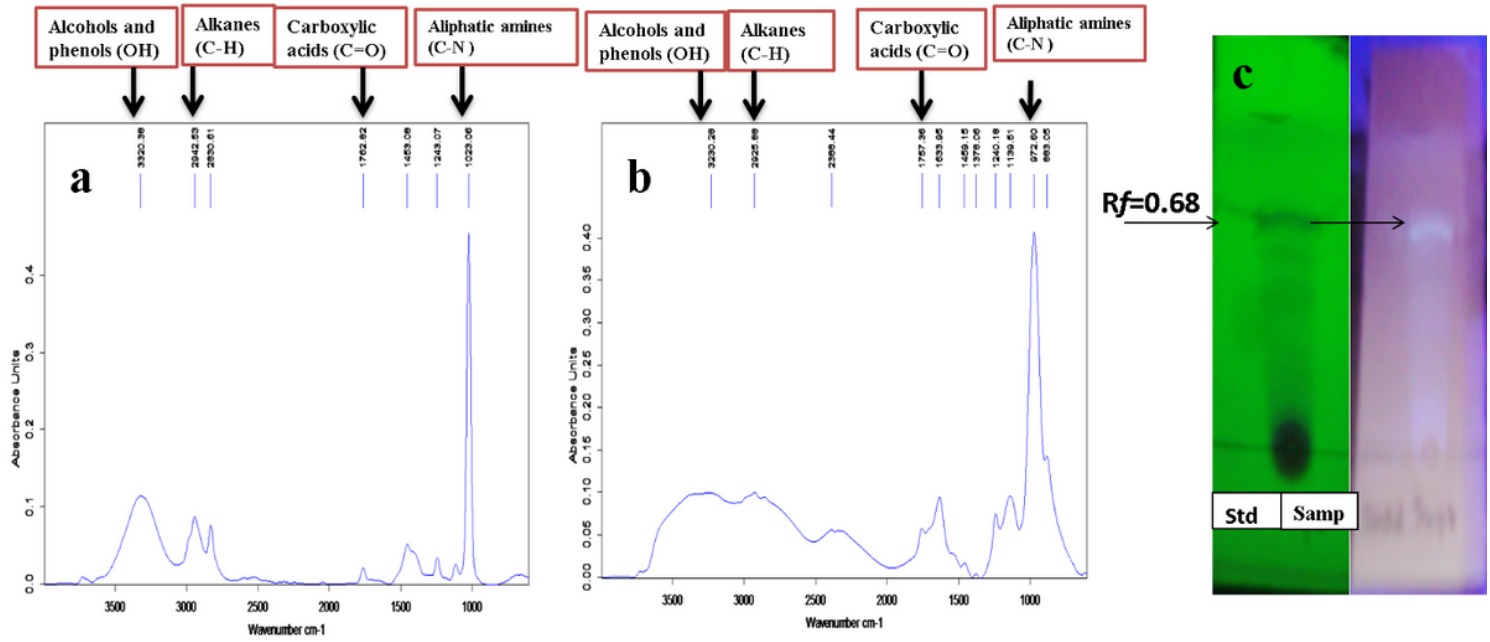

Figure 3

FTIR spectrum and TLC profile of crude surfactin produced by Bacillus subtilis SNW3 in comparison to standard surfactin show as (a) FTIR of standard surfactin (b) FTIR of crude extract (c) TLC profile.

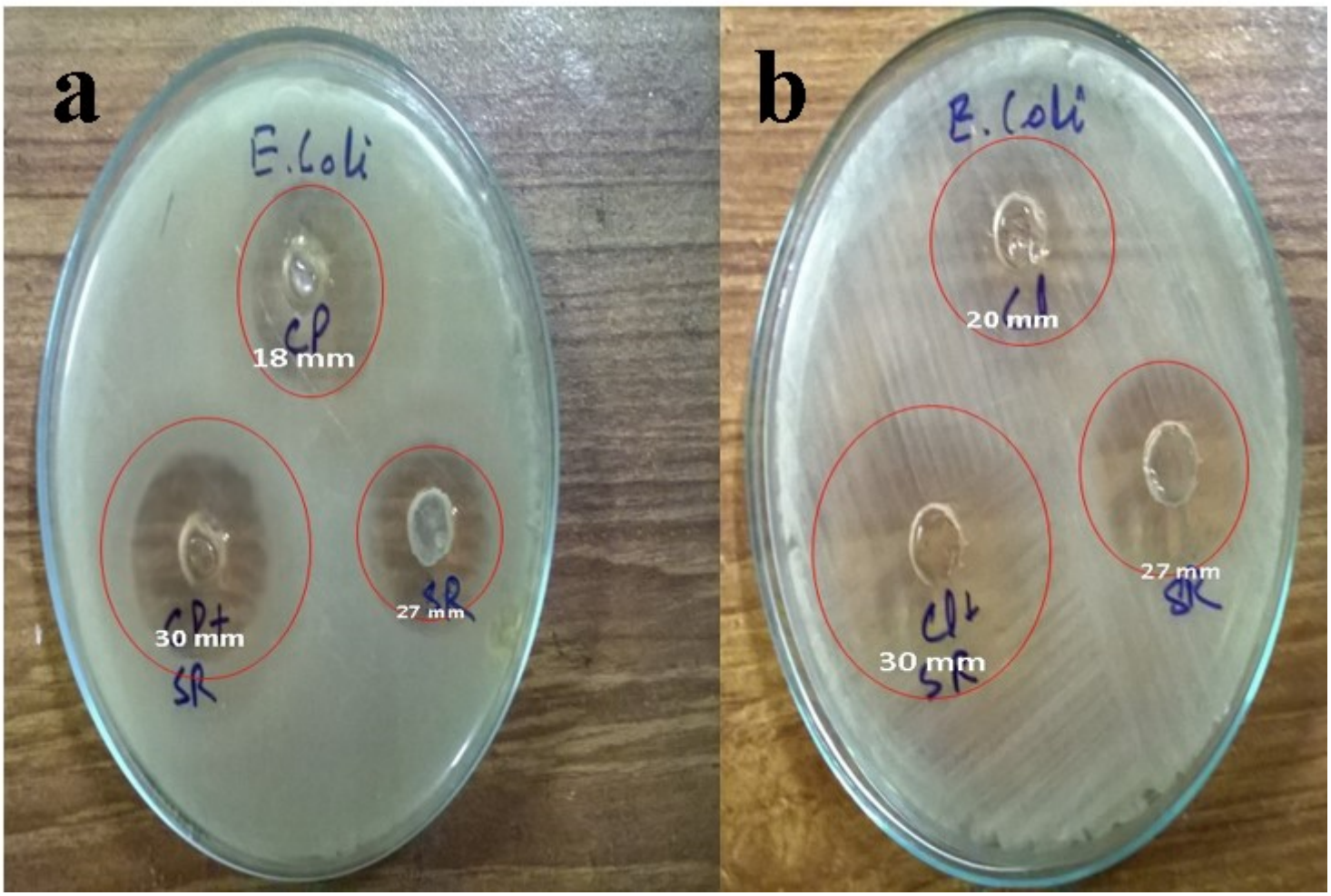

Figure 4

Antibiogram of surfactin produced with antibiotics (a) ciprofloxacin and (b) clarithromycin tested against Escherichia coli.

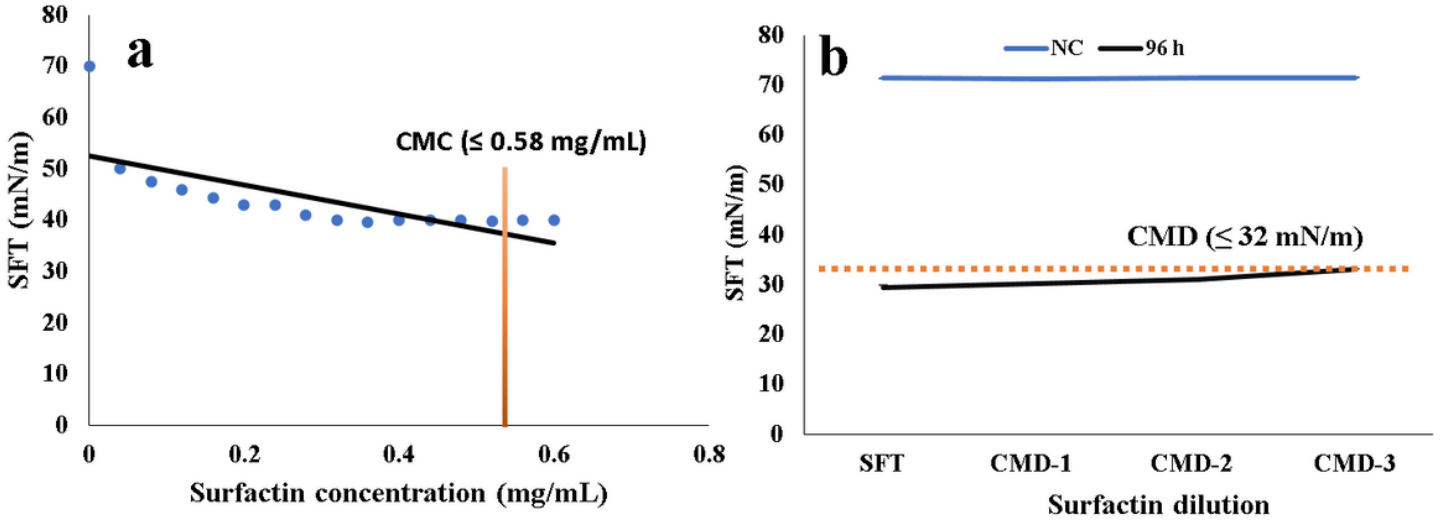

Figure 5

Surfactin analysis by (a) critical micelles concentration (CMC) and (b) critical micelles dilution (CMD); produced by Bacillus subtilis SNW3 about SFT measurement under optimized conditions.

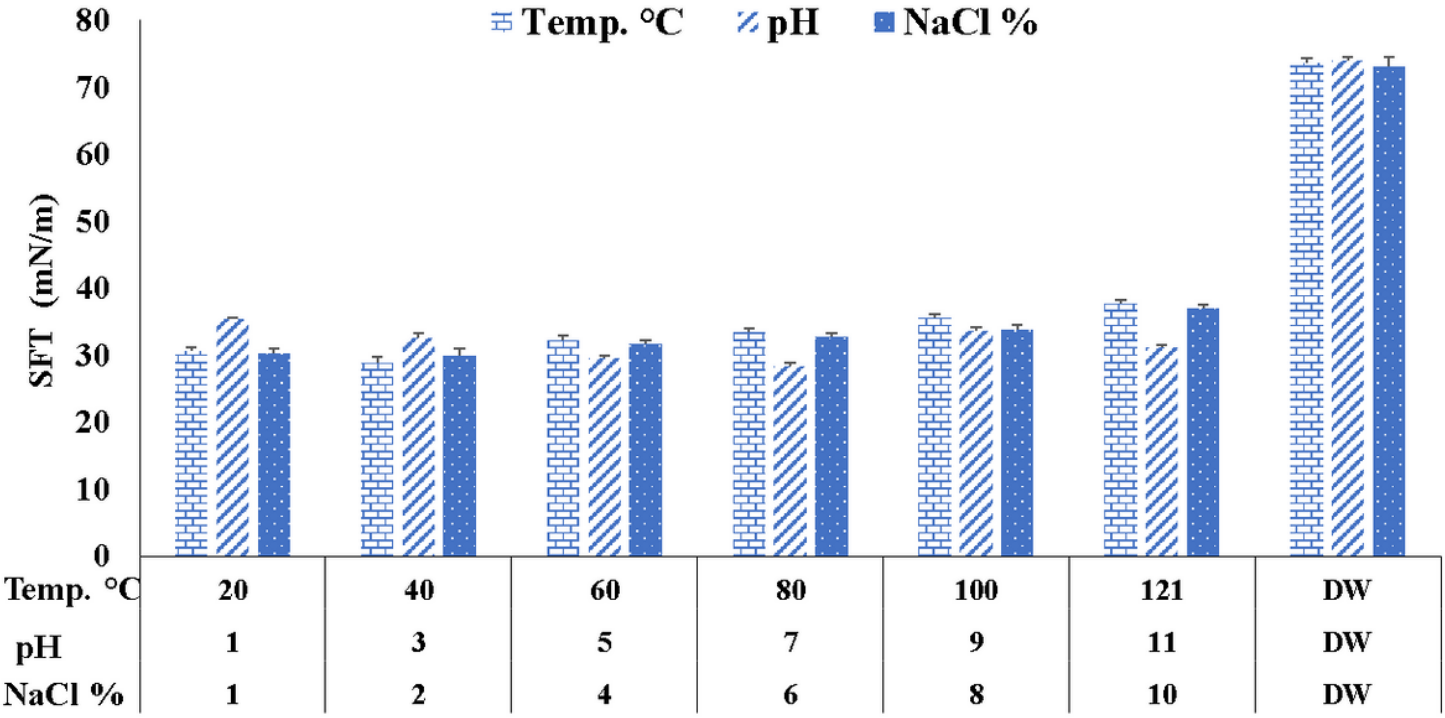

Figure 6

Stability of crude surfactin on various environmental factors like temperature ranges 20–121 °C, NaCl conc. 1–10% (w/v) and pH ranges 1–11 (Abbreviation: DW distilled water, Temp Temperature, NaCl Sodium Chloride).

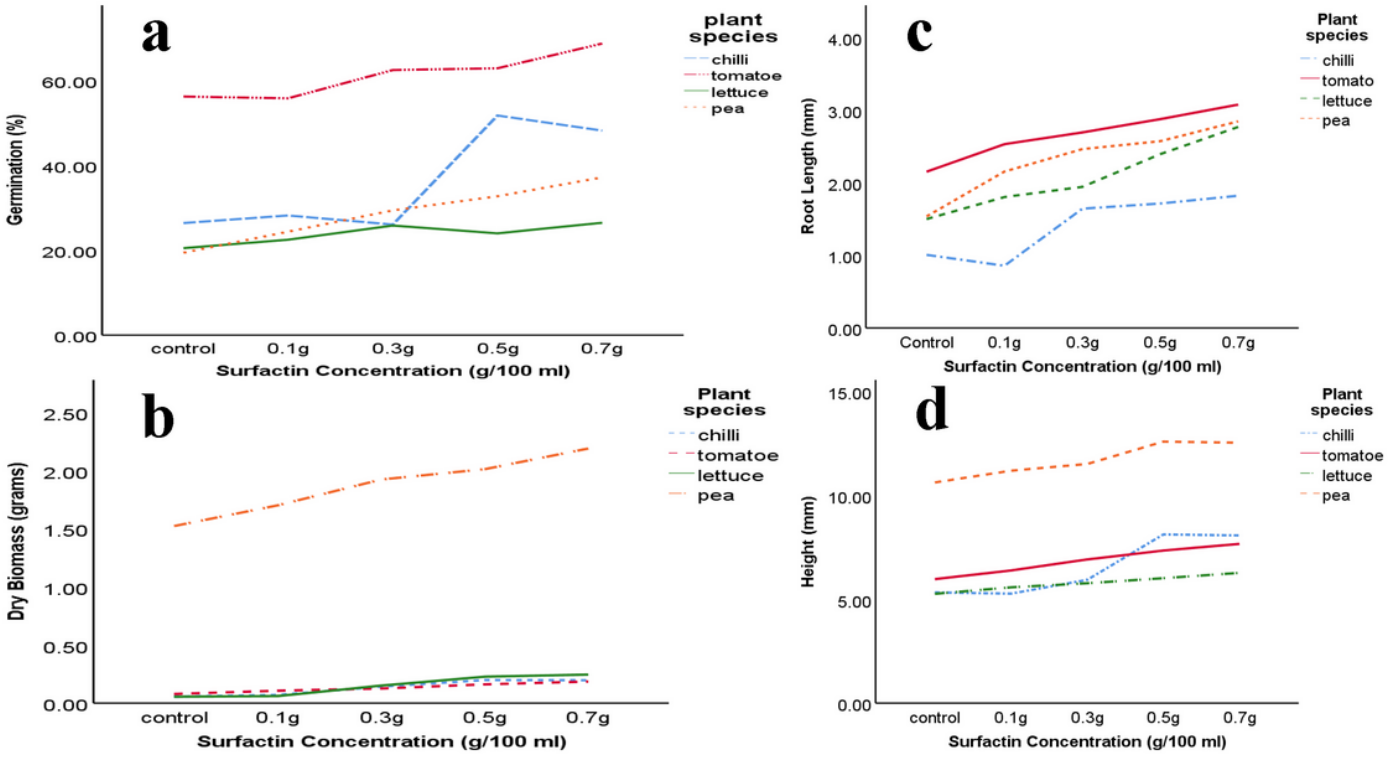

Figure 7

Effect of surfactin obtained from Bacillus subtilis SNW3 cultivated on white beans powder and waste frying oil on (a) germination of seeds (b) dry biomass of plant (c) root length and (d) height of plants.

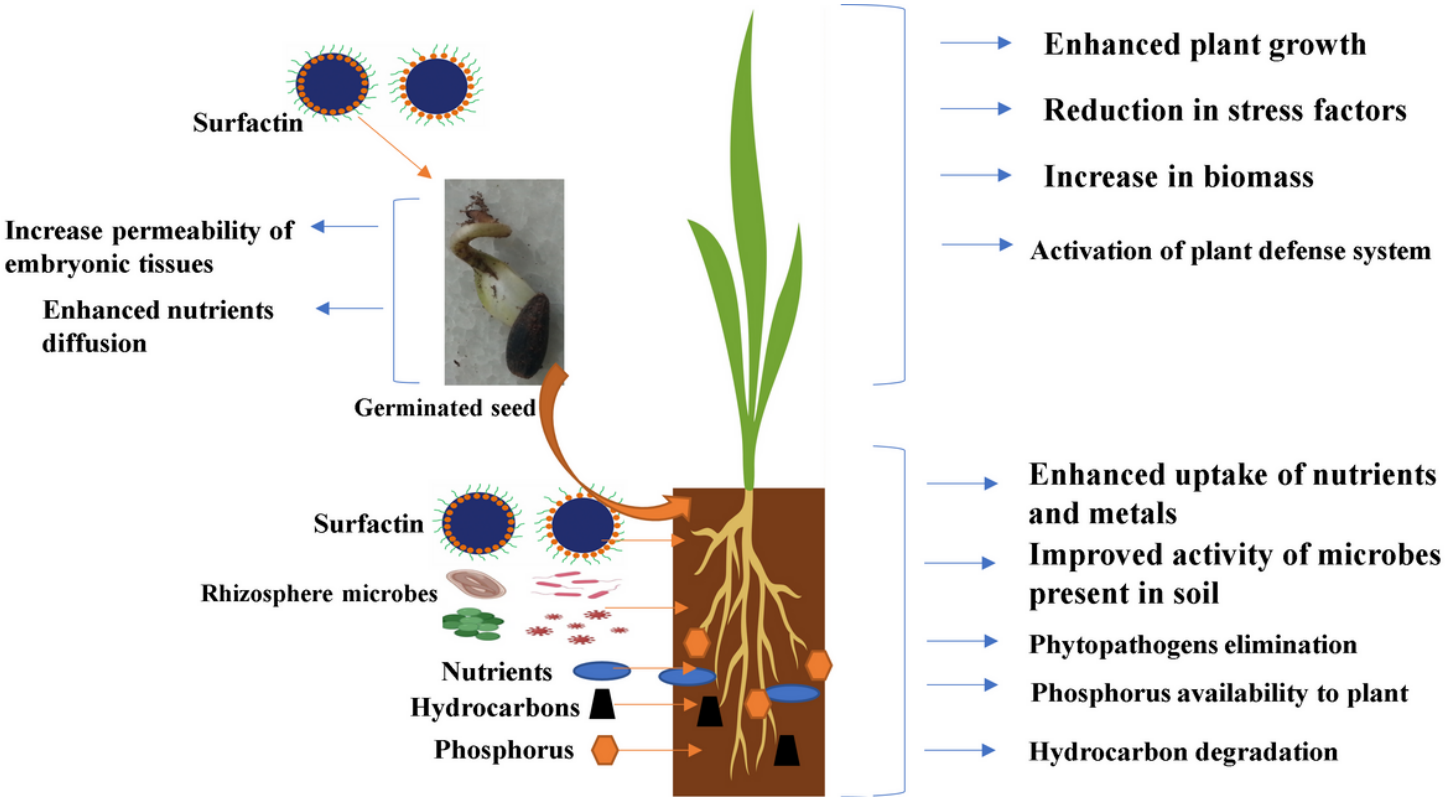

Figure 8

Diagrammatic explanation of possible effects of surfactin on hypothetical base on seed germination and plant growth promotion.

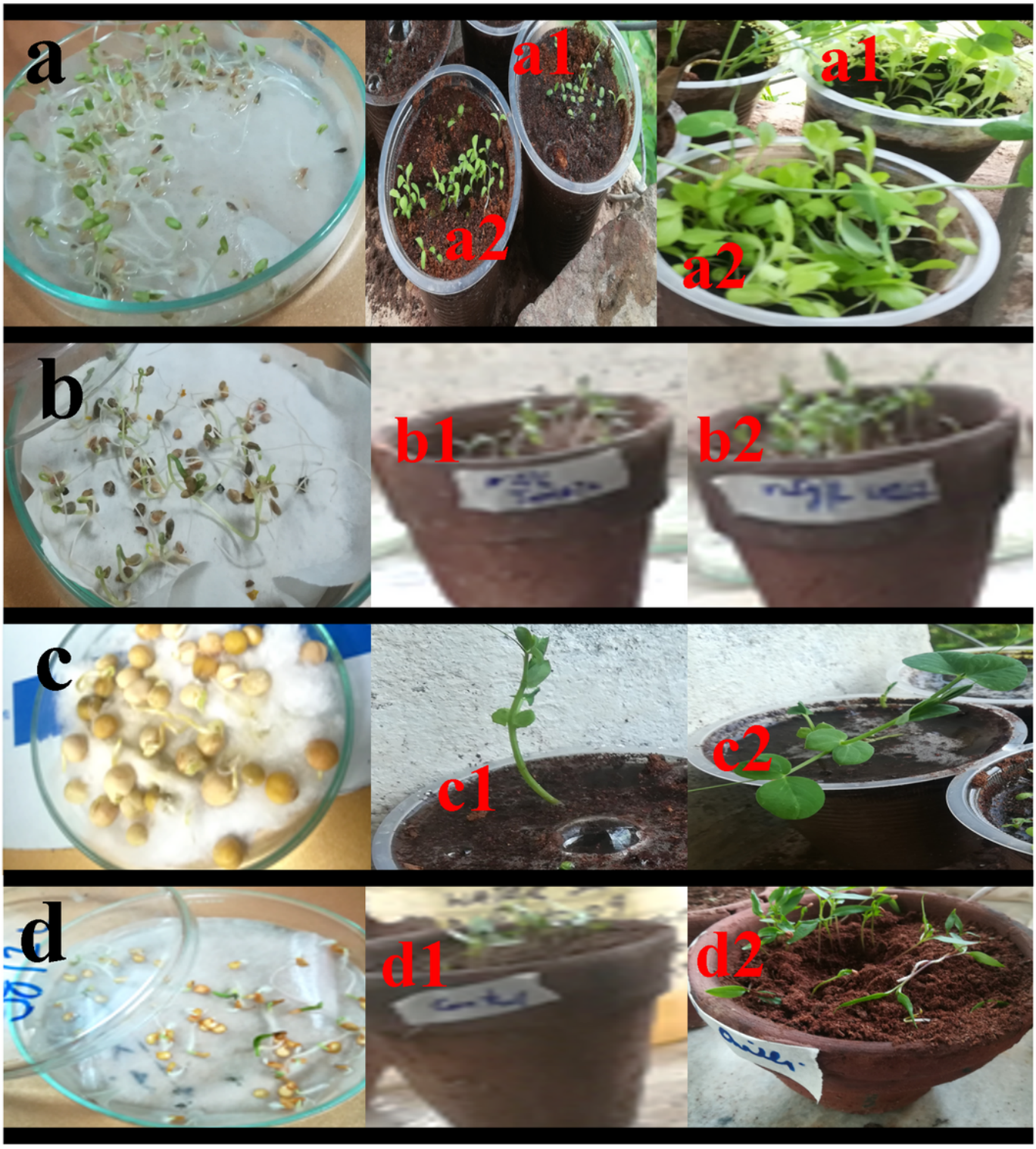

Figure 9

The growth of (a) lettuce, (b) tomato, (c) beans and (d) chilli plants while showing seeds with maximum germination and making comparison (a1,b1,c1,d1) of plants with untreated control and (a2,b2,c2,d2) effect on plants treated with surfactin after 40 days.

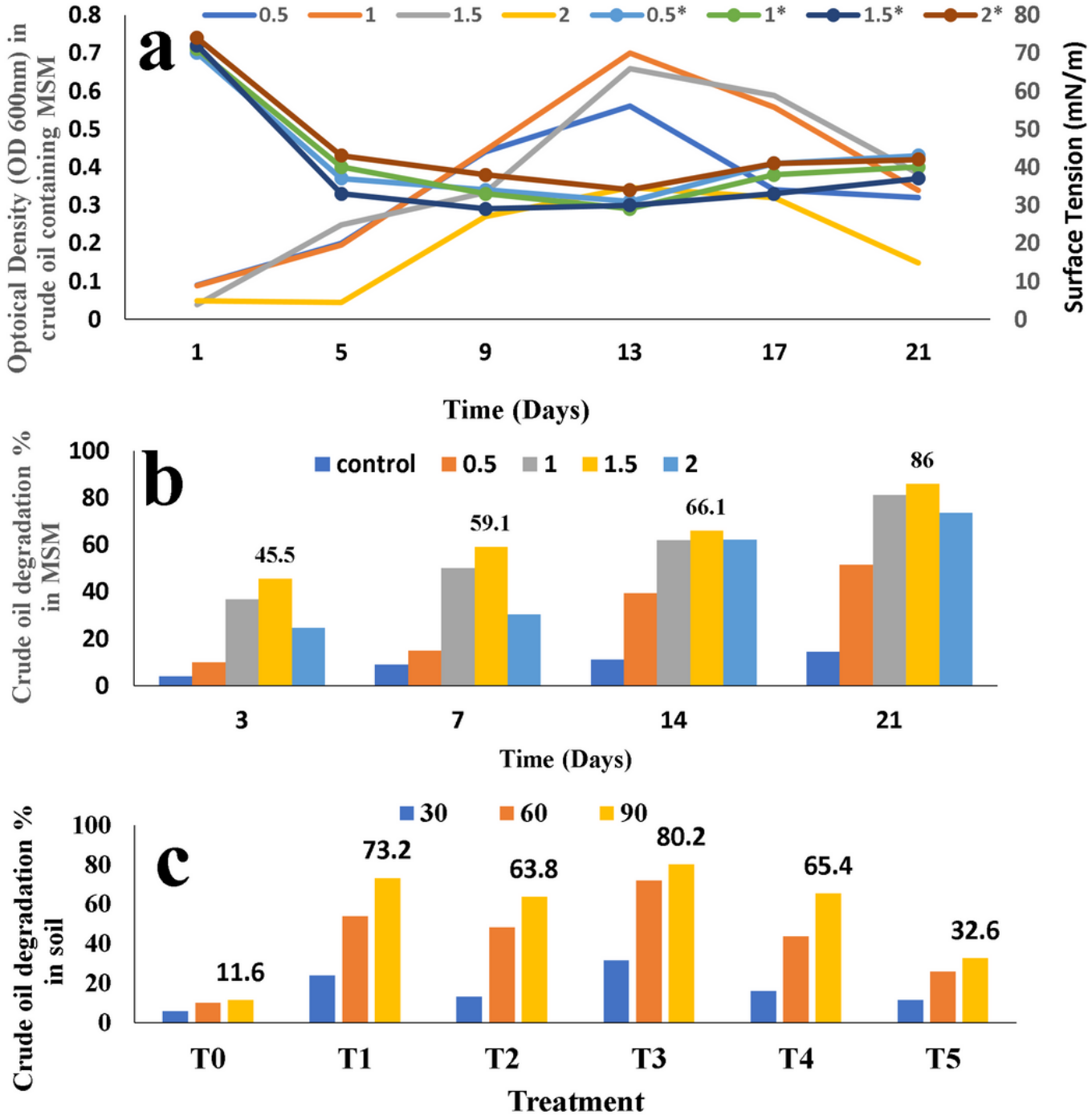

Figure 10

The growth of (a) Bacillus subtilis SNW3 on crude oil and MSM with surface tension reduction values for 21 days and (b) quantity of crude oil degraded (%) by Bacillus subtilis SNW3 for 21 days at different intervals in MSM and (c) (%) degradation for 90 days in crude oil contaminated soil.

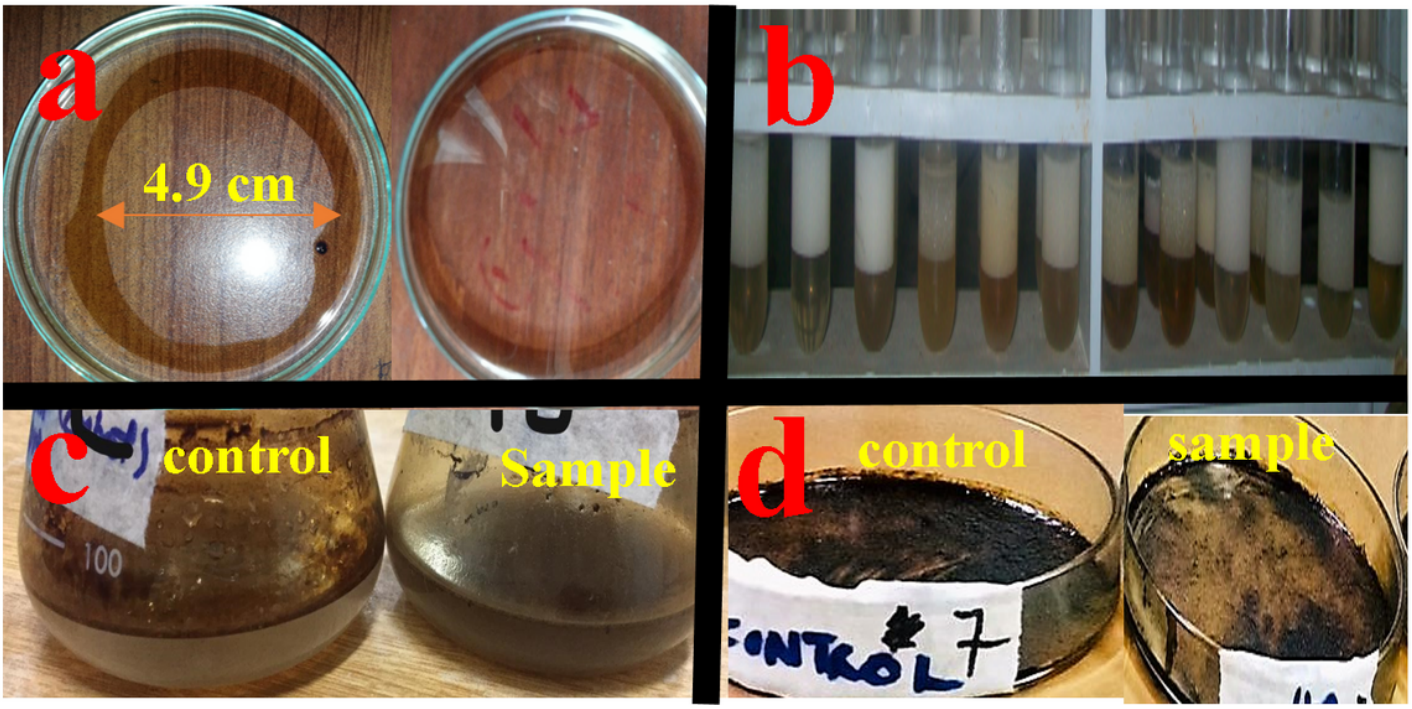

Figure 11

Testing of surfactin for (a) oil displacement activity in crude oil, (b) Emulsification activity (E24 up to 70%) (c) screening of Bacillus subtilis SNW3 for bioremediation of crude oil in uninoculated control and sample with 1% crude oil after 21 days (d) extraction of crude oil with hexane after 21 days from uninoculated control and 1% crude oil sample by gravimetric analysis.

Image not available with this version

Figure 12

Schematic diagram showing bacterial strain activity in degradation of crude oil recalcitrant hydrocarbons with simultaneously surfactin production.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata1.pdf