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Statistical optimization of antifungal iturin A production from *Bacillus amyloliquefaciens* RHNK22 using agro-industrial wastes

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Abstract Biosurfactants are secondary metabolites with surface active properties and have wide application in agriculture, industrial and therapeutic products. The present study was aimed to screen bacteria for the production of biosurfactant, its characterization and development of a cost effective media formulation for iturin A production. A total of 100 bacterial isolates were isolated from different rhizosphere soil samples by enrichment culture method and screened for biosurfactant activity. Twenty isolates were selected for further studies based on their biosurfactant activity [emulsification index (EI%), emulsification assay (EA), surface tension (ST) reduction] and antagonistic activity. Among them one potential isolate *Bacillus* sp. RHNK22 showed good EI% and EA with different hydrocarbons tested in this study. Using biochemical methods and 16S rRNA gene sequence, it was identified as *Bacillus amyloliquefaciens*. Presence of iturin A in RHNK22 was identified by gene specific primers and confirmed as iturin A by FTIR and HPLC. *B. amyloliquefaciens* RHNK22 exhibited good surface active properties and antifungal activity against *Sclerotium rolfsii* and *Macrophomina phaseolina*. For cost-effective production of iturin A, 16 different agro-industrial wastes were screened as substrates, and Sunflower oil cake (SOC) was favouring high iturin A production. Further, using response surface methodology (RSM) model, there was a 3-fold increase in iturin A production (using SOC 4%, inoculum size 1%, at pH 6.0 and 37 °C temperature in 48 h). This is the first report on using SOC as a substrate for iturin A production.

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1. Introduction

Microbial surfactants are a structurally diverse group of surface active molecules produced by a wide variety of microorganisms, including bacteria, fungi and yeasts. These are amphiphilic molecules with both hydrophilic and hydrophobic moieties that confer the ability to accumulate

between fluid phases, thus reducing surface tension at surface and interface respectively (Ongena and Jacques, 2008; Mukherjee et al., 2006). Biosurfactants have advantages over their synthetic counter parts due to their low toxicity, higher biodegradation, better environmental compatibility at extreme temperature, pH, salinity and their ability to be synthesized from renewable feedstock (Romero et al., 2007). Biosurfactants have potential to be applied in pharmaceutical, cosmetics, petroleum, food industries and agriculture sector.

Global demand for microbial biosurfactant is valued at USD 12.7 million in 2012 and is expected to reach USD 17.1 million by 2020, expanding at a Compound annual growth rate (CAGR) of 4% from 2014 to 2020. Of the different biosurfactants, lipopeptides have projected peak annual US revenue of > US $1 billion and are approved in more than 70 countries (Meena and Kanwar, 2015; www.transparencymarketresearch.com). Members of the *Bacillus* genus are considered as efficient microbial factories for large scale production of lipopeptides such as iturin, surfactin and fengycin, inhibiting various fungal pathogens and protecting the crop plants (Singh et al., 2014; Jin et al., 2014). However, a significant obstacle to meet the large scale industrial application of biosurfactants is the high production cost (Makkar and Cameotra, 2002). Hence, optimization of medium composition is most important for the production of microbial metabolites at industrial scale because around 30–40% of production cost is estimated to be the cost of growth medium (Dhanya et al., 2008; Radhika et al., 2014). Agro-industrial wastes contain high amount of carbohydrate, proteins, lipids and are generally used as cattle feed or composted and disposed into land fill. Instead they can be used as substrates for cost effective production of microbial metabolites such as biosurfactants (Yarchoan and Arnold, 2014).

In microbial fermentation, potentially influential variables are numerous and when desired to screen a large number of factors; experimental designs for first-order models, such as the factorial design or Plackett–Burman design, can be used. Plackett–Burman factorial designs are used for reliable short listing of medium components in fermentation for further optimization and allow one to obtain unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations. However, they do not give an optimum value for each variable and further optimization is needed (Ikram and Ali, 2005). Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters (Laxman et al., 2005). RSM, which includes factorial design and regression analysis and can be used to evaluate the effective factor, build models, provide information about the interaction between variables and multiple responses at the same time (Dhouha et al., 2012). The objective of this work is to screen rhizosphere bacteria for biosurfactant production, characterize high biosurfactant producing bacterial isolate and develop a cost effective medium formulation for iturin A production.

2. Material and methods

2.1. Media used for growth of microorganisms

Nutrient broth (NB) and mineral salts medium (MSM) [NaNO$_3$ (0.5), MgSO$_4$·7H$_2$O (0.5), KCl (0.1), FeSO$_4$ (0.01)

K$_2$HPO$_4$ (0.5), KH$_2$PO$_4$ (0.5) g/L], pH 7 and temperature 37 °C were used for bacterial growth. Potato dextrose agar (PDA), glucose casaminoaicds yeast extract medium (GCY) and Kings B (KB) medium were used for fungal growth at 30 °C.

2.2. Isolation of biosurfactant producing bacteria by enrichment culturing

Rhizosphere soil samples of varied crop plants were collected from different areas of Telangana and Andhra Pradesh states of India. Enrichment culturing method was performed by the method described by Dubey and Juwarkar (2001), 10 g of soil sample was added to 90 mL of mineral salts medium (MSM) in 250 mL flasks, amended with kerosene (1 mL or 5 mL or 10 mL) for enrichment and incubated at 37 °C, 180 rpm for 72 h. The enriched soil samples were subjected to serial dilution and appropriate dilutions were spread on nutrient agar plates and incubated at 37 °C for 24–48 h. Colonies of pure cultures were isolated and further characterized by Gram's staining and spore staining. The cultures of selected *Bacillus* spp., were perseved as glycerol stocks (−70 °C) for further studies.

2.3. Preliminary screening for biosurfactant activity

From the above preliminary screening, hundred bacterial isolates were isolated and tested for biosurfactant activity using the following methods like microplate and penetration methods, oil-spread method, blue-agar plate method, blood haemolysis test, lipase assay, emulsification index (EI) and emulsification assays (EA) for preliminary qualitative screening of biosurfactant activity. All the bacterial isolates were inoculated in NB medium and incubated at 37 °C for 24 h. Based on the assay, overnight grown culture or cell free supernatant was used as required.

2.3.1. Microplate method

In this method the culture supernatant (100 μL) of each bacterial isolate was added separately into 96 well microplate placed on a graph paper. Then the plate was observed for curvature of lines on the graph sheet under each well. Curvature of graph lines under the well is a preliminary indication of biosurfactant activity (Vaux and Cottingham, 2001).

2.3.2. Penetration method

All the wells of 96 well microplates were filled with 150 μL hydrophobic paste consisting of oil and silica gel. Then the paste was overlaid with 10 μL of oil. To this, 90 μL of cell free supernatant and 10 μL of staining solution (safranin) were added and biosurfactant activity was identified. Based on the results obtained by microplate and penetration methods, twenty isolates were selected for further studies.

2.3.3. Oil spread method

Oil spread assay described by Plaza et al. (2006) was performed according to which 50 mL of distilled water was taken in a Petridish, and 20 μL of crude oil was overlaid uniformly on water surface. Then, 10 μL of cell free supernatant was added over

the oil surface and biosurfactant activity was measured by observation of clear zone on oil/oil displacement.

2.3.4. Blue agar plate

MSM supplemented with glucose (2%) and cetyl trimethyl ammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (0.2 mg/mL) was prepared. 30 μL of 24 h old culture of each isolate was spot inoculated onto the blue agar plates. Extracellular anionic surfactants form insoluble complex with CTAB and methylene blue, resulting in a blue coloured halo around the colony (Siegmund and Wagner, 1991).

2.3.5. Blood haemolysis test

Haemolysis activity was performed on 5% sheep blood agar plates. 20 μL of 24 h old culture of each isolate was spot inoculated on blood agar medium, incubated at 37 °C for 48 h and observed for clear zone of haemolysis around the bacterial colonies indicating the presence of biosurfactant activity (Anandaraj and Thivakaran, 2010).

2.3.6. Lipase assay (Tributyrin agar plate method)

Lipase assay was performed by the method described by Kokare et al. (2007), where 20 μL of 24 h old cultures were spot-inoculated on tributyrin agar medium [containing (g/L) peptone 5, beef extract 3, tributyrin 10, and agar-agar 20], incubated at 37 °C for 48 h and observed for clear zone of lysis around the colonies.

2.3.7. Emulsification index (EI%)

Twenty bacterial isolates were grown in 100 mL of NB medium in a 500 mL Erlenmeyer flask at 37 °C on a rotary shaker (180 rpm) for 48 h. Supernatants were centrifuged at 10,000 rpm for 15 min at room temperature. Emulsification index (EI%) was measured by adding 2 mL of culture supernatant to 2 mL of each hydrophobic substrates [kerosene, benzene, coconut oil, toluene, diesel, engine oil (red oil), petrol, xylene and sunflower oil], mixed vigorously for 10–15 min and the mixture was left undisturbed for 24 h. EI% was calculated using the formula (1).

$$\text{Emulsfication index}(EI\%) = \frac{\text{Height of the emulsion} \times 100}{\text{Total height}} \quad (1)$$

2.3.8. Emulsification assay

Emulsification assay was performed according to the method described by Satpute et al. (2008) with slight modifications. In this method, 3 mL of culture supernatant was mixed with 0.5 mL of different hydrocarbon oils, vortexed for 2 min and left undisturbed for 1 h at room temperature until a separate aqueous and oil phase was observed. Then the absorbance of aqueous phase was measured at 400 nm.

2.4. Surface tension measurement

Biosurfactant production was monitored by surface tension measurement using a Du Nouy ring type tensiometer (Nitschke and Pastore, 2006). Culture free supernatants of bacterial isolates grown for 24 h and 48 h were tested for reduction in surface tension. The surface tension was measured at room temperature after dipping the platinum ring in solution for a while in order to attain equilibrium condition. For calibration of the instrument, the surface tension of distilled water was first measured and later the supernatant, as prepared above, was measured and uninoculated sterile broth was used as control. The average value of triplicate was used to express the surface tension of the sample (Cooper and Zajic, 1980).

2.5. Anti-fungal activity

Anti fungal activity was detected by the dual culture method. Soil borne plant pathogenic fungi, *Sclerotium rolfsii* and *Macrophomina phaseolina* were grown on PDA, GCY and KBM media. An agar block (five mm dia) was cut from an actively growing (96 h old) fungal culture and placed on the surface of fresh agar medium at the centre of Petri plate. A loopful of 24 h old culture of each bacterium was streaked in a straight line on one edge of a 90 mm dia Petri plate, plates were incubated at 30 ± 2 °C and the inhibition zone between two cultures was measured 5 days after inoculation. Plates inoculated with the same fungus without bacteria were used as control. Three replications were maintained for each and reduction in radial growth was measured and percent inhibition over control was calculated using the formula (2).

$$I\% = \frac{(C - T)}{C} \times 100 \quad (2)$$

where,
   I = Inhibition% of mycelial growth (growth reduction over control).
   C = Radial growth of fungus in the control plate (mm).
   T = Radial growth of fungus on the plate inoculated with bacteria (mm).

2.6. Biochemical identification of the isolate RHNK22

Based on the screening results for biosurfactant and antifungal activity, one potential bacterial isolate RHNK22 was selected for further studies. It was identified morphologically by Gram's staining and biochemical tests like starch hydrolysis, Indole test, Methyl red test, Voges-Proskauer test, Citrate utilization, Catalase test and Glucose fermentation test. All tests were performed according to the Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986).

2.7. Molecular identification of RHNK22 based on 16S rRNA gene sequence

For molecular identification, the isolate was sent to MACRO-GEN (Seoul, Korea) for sequencing using universal 16S rRNA primers. Phylogenetic analysis was done using mega-4 bioinformatics software. 16S rRNA sequence was submitted to EMBL and Nucleotide Sequence Database Accession number is LM651914.

2.8. Detection of iturin A gene in RHNK22 by PCR analysis

Bacterial isolate RHNK22 was inoculated into 5 mL of LB broth and incubated at 37 °C on a rotary shaker at 180 rpm for 18-24 h. DNA extraction was done using DNA isolation

kit by Xcelris labs, India. Iturin A gene specific primers F; TCC AGA CAA TGA CGG ATG GC; R; TTG AAG GAC CAC GAG TTC GG used in the present study were designed by Primer 3 software. Iturin A gene specific primers designed by Ramaratham et al. (2007) F; GATGCGATCTCCTTG-GATGT; R; ATCGTCATGTGCTGCTTGAG were used as positive control. Iturin A gene sequences, (GenBank acc. No. AF534617.1) were searched in the databank through the NCBI Blast search. PCR reaction was performed according to the standard protocols. PCR conditions are 95 °C – 5 min. 30 cycles (94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min) and 72 °C for 5 min and observed for PCR product size 647 bp.

2.9. Production and extraction of biosurfactant

Bacterial isolate RHNK22 was grown in 100 mL of MSM in 500 mL Erlenmeyer flask and incubated at 37 °C, 180 rpm for 48 h and then centrifuged at 10,000 rpm for 15 min. The supernatant was collected and its pH was adjusted to 2.0 using 6 N HCl and kept at 4 °C for overnight to allow for precipitation. The precipitate was centrifuged at 10,000 rpm for 20 min. The biosurfactant was dissolved in methanol and dried in a rotary vacuum evaporator (Kim et al., 2004).

2.10. Antifungal activity of the extracted surfactant obtained from RHNK22

The extracted biosurfactant was dissolved in distilled water and tested for antifungal activity. For testing antifungal activity, an agar plug of actively growing fungal culture was placed in the centre of the plate. Then, wells were made (using sterile agar borer), 2 cm away from the centre where fungus was placed and different concentrations (0.07 mg/mL, 0.15 mg/mL, 0.31 mg/mL, 0.62 mg/mL, 1.25 mg/mL, 2.5 mg/mL and 5 mg/mL) of biosurfactant were added in separate wells and incubated at 30 ± 2 °C for 24 to 96 h. Sterile distilled water was used as control. The inhibition percentage ($I\%$) was calculated using the following formula (3) (Anandaraj and Thivakaran, 2010; Murata et al., 2013).

$$I\% = \frac{(C - T)}{C} \times 100 \tag{3}$$

where,

I = Inhibition% of mycelial growth (growth reduction over control).
C = Radial growth of fungus in the control plate (mm).
T = Radial growth of fungus on the plate inoculated with bacteria (mm).

2.11. HPLC analysis of biosurfactant

HPLC analysis of the extracted biosurfactant was performed by LC-2000 system (Shimadzu) with a C_{18} column (Phenomenex luna C_{18}). Mobile phase components were prepared using 0.1% trifluoroacetic acid (TFA) in methanol. The products were eluted at a flow rate of 1.0 mL/min and elution pattern was monitored by determining absorbance at 210 nm. Pure iturin A (Sigma–Aldrich Co. USA) was used as standard.

2.12. FTIR (Fourier transform infrared spectroscopy) analysis

To understand the overall chemical nature of the extracted biosurfactant, FTIR was employed. The technique helps to explore the functional groups and chemical bonds present in the crude extract. The analysis was done using FTIR spectrophotometer. Samples were prepared by homogeneous dispersal of 1 mg of biosurfactant sample in pellets of potassium bromide. Infra red (IR) absorption spectra were obtained using a built-in plotter. IR spectra were collected over the range of 450–4500 cm^{-1} with a resolution of 4 cm^{-1}. The spectral data given are the average of 10 scans over the entire range covered by the instrument.

2.13. Selection of agro-industrial wastes for iturin A production by unidimensional approach

Initially 16 different agro-industrial wastes i.e., rice bran husk (RBH), Sunflower oil cake (SOC), Coconut oil cake (COC), Cotton seed oil cake (CSOC), Corn cob (CC), Orange peel (OP), Jack fruit peel (JFP), Sugarcane leaf (SCL), Pineapple peel (PP), Banana leaf (BL), Sweet lime peel (SLP), Cheese whey permeate (CWP), Dry yeast cells (DYC), Pongamia seed cake (PSC), Jatropha seed cake (JSC) and Groundnut oil cake (GOC) as substrates were screened for iturin A production. All these substrates, except yeast cells and whey were collected freshly from local markets and farms, cleaned, sliced, dried at 60 °C, blended to fine powder and stored in air tight containers at room temperature. The dry yeast cells used were of commercial, granulated food grade yeast. Whey was collected from a local dairy industry, Hyderabad. All these raw materials were directly used as substrates in the fermentation media without any pre-treatment at 1% level in place of glucose, inoculated with 1% of 24 h culture (O.D-0.6) and incubated at 37 °C at 180 rpm for 48 h.

2.14. Selections of most suitable agro-industrial sources using Plackett–Burman design

Based on the results obtained from preliminary screening of agro-industrial wastes for iturin A production, eight different agro-industrial wastes i.e., SOC, CSOC, COC, POC, JSC, CWP, DYC and GOC were selected and further screened by Plackett–Burman (PB) design. PB design is a two-level factorial design and allows the investigation of n-1 variables in n number of experiments (Plackett and Burman, 1946). In the experimental design, each row represents an experiment and each column represents an independent variable. For screening agro industrial wastes as substrates for iturin A production, a set of 16 experiments were performed at combinations of ' + ' (high-0.2%) and ' − ' (low-0.02%) levels. Plackett–Burman experimental design was based on the first order model:

$$Y = \beta o + \sum \beta i X i$$

where, Y is the response (iturin A productivity), βo is the model intercept, βi is the variable estimates. Plackett–Burman design matrix was developed and the results were analysed using MINITAB-13 statistical software.

2.15. Response surface methodology

After selecting a single best substrate (agro-industrial waste) for iturin A production through preliminary experiments and PB design, response surface methodology (RSM) was employed to determine the optimum conditions for maximum iturin A production. Five variables, i.e., substrate concentration along with pH, temperature, inoculum size and incubation period were selected for optimization. Central composite rotatable design (CCRD) with five coded levels (-2, -1, 0, $+1$, $+2$) was performed in a design of 32 experiments with six replicates at the central point. The following equation was used for coding the actual experimental values of the variables (Neter et al., 1996).

$$Xi = (X_i - X_0)/\Delta X$$

where X_i is dimension less coded level of the variable, X_i is actual value of that variable, X_0 is average of the high and low level values of that variable and ΔX is high value minus low value of that variable. Analysis of variance (ANOVA) for both iturin A production and reduction in surface tension was done by MINITAB-13. The response (iturin A mg/L and reduction in surface tension) was analysed by using a second order polynomial equation and the data were fitted into the equation by multiple regression procedure. The three dimensional graphical presentation of model equation represents the individual and interactive effect of test variables on the response. The optimum levels of variables for high iturin A production as well as reduction in surface tension were obtained by solving the regression equation and analysis of response surface plots. The second-order polynomial equation that defines predicted response (Y) in terms of the independent variables (X_1, X_2, X_3, X_4 and X_5) is given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \\ + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{55} X_5^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 \\ + \beta_{15} X_1 X_5 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{25} X_2 X_5$$

where β_0 is intercept term, β_1, β_2, β_3, β_4, and β_5 linear coefficients, β_{11}, β_{22}, β_{33}, and β_{44} quadratic coefficients and β_{12}, β_{13},..., β_{45} interactive coefficient estimate. The optimum levels of variables (within the experimental range) to obtain maximum iturin A production and reduction in surface tension were determined by running experiment using the optimum values for variables given by response optimization for confirmation of predicted value.

2.16. Statistical analysis

All experiments were performed in triplicates and the results obtained are mean of three independent experiments showing consistent results. ANOVA, Means, CV%, Ranking and standard errors were calculated using the Microsoft Office Excel 2003 (version 7).

3. Results

3.1. Isolation and preliminary characterization of biosurfactant producing Bacillus spp.

Rhizosphere soil samples collected from varied crop plants (chick pea, groundnut, millet, pigeon pea and sorghum) were subjected to enrichment culturing technique to select biosurfactant producers, and a total of 100 morphologically distinct bacterial isolates were obtained. Based on Gram's staining and spore staining, all the isolates were preliminarily identified as *Bacillus* spp. and were selected for further studies. The biosurfactant activity of all the isolates was identified qualitatively by microplate and penetration assays. The microplate assay is performed based on the change in optical irregularity, pure water in a hydrophobic well shows a flat surface whereas the fluid surface containing surfactants is concave and turns into an irregular shape. In penetration assay, it was observed that biosurfactant activity is indicated by a change in colour from clear red to cloudy white within 15 min. Based on results obtained from microplate and penetration assays, twenty isolates were selected for further studies.

Oil spreading assay is a rapid and more sensitive method for detection of biosurfactants. All twenty isolates showed a clear zone on crude oil which varied from 25 to 40 mm in dia with RHNK22 having 40 mm (data not shown). All the twenty isolates showed poor growth on blue agar plates and produced no halos. This indicates that none of the isolates produced glycolipids. In blood haemolysis test, all the twenty isolates showed zone of haemolysis that ranged 4–15 mm in dia with RHNK22 having 15 mm (data not shown). All the twenty isolates showed a clear halo around the colonies on tributyrin agar plates indicating lipase activity and the zone size ranged from 6 to 14 mm in dia (data not shown) and RHNK22 showed maximum zone size of 14 mm dia.

3.2. Emulsification index (EI%) and Emulsification activity (EA) of isolates

In the present study, emulsification index and emulsification assay were carried out with 9 different hydrocarbons like kerosene, benzene, diesel, coconut oil, sunflower oil, toluene, engine oil and petrol. Among twenty isolates, RHNK22 showed highest EI percentage (EI%) 78.1 on kerosene, 62.5 on benzene, 72 on diesel, 53.1 on coconut oil, 62.5 on sunflower, petrol and toluene, 68.7 on engine oil (red oil) and xylene (Table 1). In emulsification assay, the isolate *Bacillus* sp. RHNK22 showed the highest emulsification activity [EA-emulsification units (Eu)/mL] 214.0 on kerosene, 93.1 on benzene, 253.5 on diesel, 226.7 on coconut oil, 291.4 on sun flower oil, 265.1 on toluene, 210 on engine oil, 214.1 on xylene and 252.9 on petrol (Table 2). To our knowledge, this is the first observation of EI and EA with nine different hydrocarbons for screening bacteria for biosurfactant activity.

3.3. Surface tension (ST) measurement

Surface tension measurement was carried out for all the twenty selected isolates and it was observed that RHNK22 was able to show highest reduction in surface tension from 60.50 to 26.12 mN/m in 24 h and 29.04 mN/m in 48 h in comparison with other isolates. Of 20 bacterial isolates, four isolates RHNK 1*, RHNK5*, RHNK22* and RHNK 30* showed significant *p* values less than 0.05. A significant change in surface tension values within 24–48 h was observed for the bacterial isolate RHNK22 whereas the other isolates showed ST values ranging from 60.39 to 27.96 mN/m in 24 h and 50.12–29.86 mN/m in 48 h (Fig. 1).

Table 1 Emulsification index (EI%) of biosurfactant producing bacterial isolates using different hydrocarbons.

S. No.	Isolate label	Kerosene EI%	Benzene EI%	Diesel EI%	Coconut oil EI%	Sun flower EI%	Toluene EI%	Engine oil EI%	Xylene EI%	Petrol EI%	CV %
1	R1	31.2^c(±0.08)	12.5^f(±1.09)	37.5^b(±0.06)	15.6^e(±1.09)	37.5^b(±0.01)	24.9^d(±1.07)	50.0^a(±1.13)	24.9^d(±1.07)	31.2^c(±1.09)	6.08
2	R5	62.5^a(±1.09)	50.0^d(±1.13)	62.5^a(±1.09)	53.1^c(±2.22)	40.6^f(±0.01)	15.6^g(±1.09)	44.6^e(±1.34)	56.2^b(±1.09)	56.2^b(±1.11)	4.62
3	R8	62.4^a(±1.11)	31.2^c(±1.10)	53.1^c(±2.22)	15.6^f(±1.08)	53.1^c(±2.22)	59.3^b(±1.13)	58.1^b(±0.57)	62.5^a(±1.08)	46.8^d(±0.02)	4.78
4	R10	62.5^b(±1.09)	56.2^d(±1.08)	65.6^a(±2.19)	43.7^g(±1.09)	56.2^e(±1.09)	59.3^c(±0.01)	50.7^f(±1.28)	62.5^b(±1.09)	56.2^e(±1.09)	3.91
5	R14	15.4^g(±1.08)	31.2^d(±0.01)	25.2^e(±0.17)	53.1^b(±2.22)	46.8^c(±0.002)	18.7^f(±0.01)	63.3^a(±1.31)	15.6^g(±0.01)	31.2^d(±1.09)	3.94
6	R16	31.2^c(±1.08)	15.6^f(±1.10)	24.9^d(±1.07)	46.8^a(±0.01)	31.2^c(±0.01)	21.8^e(±0.01)	36.2^b(±0.61)	10.1^g(±0.10)	25.2^d(±0.17)	3.46
7	R19	56.2^a(±1.08)	43.7^d(±0.06)	56.2^a(±1.09)	40.6^e(±0.01)	46.8^c(±0.002)	53.1^b(±2.22)	45.0^c(±0.67)	56.2^a(±1.09)	43.7^d(±1.09)	3.30
8	R20	56.2^a(±1.09)	46.8^c(±0.01)	50.0^b(±1.13)	37.5^e(±0.01)	43.7^d(±1.09)	46.8^c(±0.04)	44.2^d(±1.17)	18.7^f(±1.08)	50.0^b(±1.13)	3.42
9	R22	78.1^a(±1.08)	62.5^d(±1.10)	71.8^b(±1.08)	53.1^e(±2.22)	62.5^d(±1.11)	62.5^d(±1.09)	68.9^c(±1.11)	68.7^c(±1.09)	62.5^d(±1.09)	3.72
10	R30	56.2^b(±1.08)	12.5^g(±1.10)	56.2^b(±1.09)	46.8^c(±0.01)	37.5^d(±0.04)	24.9^f(±1.07)	63.3^a(±1.29)	31.2^e(±1.08)	37.5^d(±0.01)	3.70
11	R33	71.8^a(±1.09)	50.0^d(±1.13)	56.2^c(±1.09)	43.7^e(±1.09)	40.6^f(±0.01)	56.2^c(±1.09)	50.4^d(±1.19)	62.5^b(±1.09)	43.7^e(±1.09)	3.29
12	R40	12.5^f(±1.08)	15.6^e(±1.10)	15.6^e(±1.09)	46.8^b(±0.01)	40.6^c(±0.06)	6.23^g(±0.01)	46.8^b(±0.01)	31.2^d(±1.08)	50.0^a(±1.13)	4.17
13	R44	31.2^f(±0.01)	43.7^c(±1.10)	31.2^f(±0.01)	56.2^a(±1.09)	46.8^b(±0.01)	40.6^d(±0.05)	43.7^c(±1.08)	56.2^a(±1.10)	37.5^e(±0.01)	2.32
14	R48	15.6^d(±1.10)	56.2^a(±1.11)	15.6^d(±1.10)	15.6^d(±1.08)	56.2^a(±1.09)	15.7^d(±1.02)	40.6^b(±0.09)	37.5^c(±0.01)	37.5^c(±0.01)	4.52
15	R52	31.2^e(±1.10)	31.2^e(±1.08)	18.7^f(±1.09)	56.2^a(±1.11)	43.7^c(±1.09)	47.4^b(±0.57)	56.2^a(±1.09)	37.5^d(±0.01)	15.6^i(±0.01)	4.25
16	R61	15.6^e(±1.08)	18.7^d(±0.01)	18.7^d(±0.01)	53.1^a(±2.22)	50.0^b(±1.13)	50.0^b(±1.13)	40.6^c(±0.01)	15.6^e(±0.01)	15.6^e(±0.01)	4.07
17	R66	15.6^f(±0.01)	25.2^d(±0.14)	15.6^f(±1.11)	40.6^c(±0.04)	43.7^b(±1.09)	15.6^f(±1.11)	53.1^a(±2.22)	15.6^f(±0.01)	15.6^f(±0.02)	4.77
18	R72	71.8^a(±1.09)	50.0^d(±1.13)	62.5^b(±1.09)	50.0^d(±1.13)	56.2^c(±1.08)	56.3^c(±1.10)	62.5^b(±1.09)	56.2^c(±1.09)	46.8^e(±0.01)	3.45
19	R85	68.7^a(±1.09)	43.7^g(±1.08)	65.6^b(±2.19)	50.0^f(±1.11)	50.0^f(±1.13)	47.6^f(±0.51)	53.4^e(±2.31)	62.5^c(±1.11)	59.3^d(±1.13)	4.66
20	R91	46.8^d(±0.01)	31.2^g(±1.09)	53.1^b(±2.22)	53.1^b(±2.22)	50.0^c(±1.11)	35.4^f(±0.69)	59.3^a(±1.13)	59.3^a(±1.13)	43.7^e(±1.09)	4.96

Values superscribed by a–g are ranking highest to lowest of significant, same alphabet are insignificant according to Fischer's least significance difference test ($p < 0.05$). Values in the brackets are standard error, values in column are mean two independent experiments of 4 replications and values in last column are CV%.

Table 2 Emulsification activity (EA; EU/mL) of biosurfactant producing bacterial isolates using different hydrocarbons.

S. No.	Isolate label	Kerosene EA	Benzene EA	Diesel EA	Coconut oil EA	Sun flower EA	Toluene EA	Engine oil EA	Xylene EA	Petrol EA
1	R1	100.3(±0.01)	75.0(±0.05)	193.1(±0.24)	205.5(±0.03)	215.1(±0.07)	207.4(±0.07)	151.9(±0.99)	186.7(±0.04)	181.3(±0.08)
2	R5	181.8(±0.07)	85.1(±0.07)	226.4(±0.07)	215.0(±0.07)	216.4(±0.10)	223.5(±0.08)	203.4(±0.07)	204.8(±0.09)	190.4(±0.07)
3	R8	192.6(±0.05)	86.7(±0.04)	232.2(±0.07)	218.3(±0.08)	215.1(±0.07)	227.3(±0.07)	211.9(±0.21)	207.7(±0.10)	161.6(±0.07)
4	R10	193.0(±0.14)	90.1(±0.07)	235.7(±0.04)	214.7(±0.07)	210.6(±0.10)	232.0(±0.31)	206.3(±0.67)	203.4(±0.07)	162.4(±0.08)
5	R14	98.9(±0.05)	90.1(±0.03)	200.0(±0.35)	209.5(±0.03)	215.3(±0.08)	232.6(±0.05)	172.5(±0.72)	204.4(±0.08)	132.5(±0.07)
6	R16	166.3(±0.07)	92.5(±0.04)	235.2(±0.07)	224.1(±0.07)	213.4(±0.07)	236.9(±0.07)	144.2(±1.30)	201.6(±0.08)	186.9(±0.08)
7	R19	211.4(±0.12)	90.1(±0.25)	204.0(±0.12)	222.7(±0.07)	219.8(±0.03)	238.8(±0.07)	192.1(±1.92)	201.6(±0.08)	175.7(±0.12)
8	R20	203.1(±0.01)	90.1(±0.07)	241.0(±0.35)	222.2(±0.07)	217.3(±0.08)	242.2(±0.37)	208.4(±1.32)	207.1(±0.08)	231.0(±0.55)
9	R22	214.0(±0.14)	93.1(±0.28)	253.5(±0.07)	226.7(±0.07)	291.4(±0.10)	265.1(±0.20)	210.0(±0.70)	214.1(±0.07)	252.9(±0.07)
10	R30	201.2(±0.07)	83.2(±0.14)	238.5(±0.10)	226.9(±0.08)	216.0(±0.07)	244.0(±0.31)	200.2(±0.04)	204.7(±0.08)	204.1(±0.04)
11	R33	208.6(±0.08)	85.7(±0.08)	247.3(±0.07)	226.2(±0.07)	218.4(±0.10)	251.4(±0.04)	203.9(±0.21)	213.2(±0.07)	221.0(±0.15)
12	R40	158.3(±0.01)	88.4(±0.07)	144.8(±0.09)	219.2(±0.07)	220.4(±0.07)	237.4(±0.21)	136.5(±0.09)	187.9(±1.07)	138.3(±0.06)
13	R44	194.8(±0.11)	90.3(±0.08)	185.4(±0.04)	231.6(±0.10)	218.1(±0.10)	247.0(±0.56)	139.0(±0.21)	171.5(±0.10)	158.9(±0.11)
14	R48	175.5(±0.25)	91.2(±0.02)	216.6(±0.49)	203.7(±0.07)	215.7(±0.07)	253.9(±0.16)	191.4(±0.07)	213.8(±0.07)	219.8(±0.08)
15	R52	148.5(±0.14)	91.0(±0.07)	213.0(±0.49)	189.1(±0.33)	218.2(±0.08)	259.2(±2.85)	196.2(±0.04)	213.7(±2.54)	169.6(±0.08)
16	R61	210.4(±0.12)	84.5(±0.05)	248.6(±0.07)	223.8(±0.07)	214.6(±0.04)	256.3(±0.67)	163.7(±0.07)	209.5(±0.08)	204.9(±0.07)
17	R66	135.2(±0.01)	57.2(±0.05)	172.1(±0.33)	213.2(±0.08)	213.9(±0.07)	251.2(±0.24)	130.1(±0.34)	105.8(±0.05)	172.5(±0.08)
18	R72	179.2(±0.01)	93.6(±0.07)	204.6(±0.03)	227.5(±0.07)	219.8(±0.08)	255.0(±0.29)	118.9(±0.05)	199.8(±0.08)	238.6(±0.07)
19	R85	201.0(±0.10)	89.1(±0.08)	205.8(±0.07)	222.7(±0.11)	216.4(±0.07)	262.3(±0.55)	213.9(±0.42)	209.8(±0.04)	224.3(±0.08)
20	R91	103.6(±0.18)	91.0(±0.22)	249.0(±0.39)	226.2(±0.07)	215.3(±0.07)	263.8(±1.92)	164.9(±0.43)	200.1(±0.08)	114.4(±0.05)

EA: (EU/mL) = 1 emulsification unit = 0.01 O.D multiplied by a dilution factor of absorbance at 400 nm. Values in the brackets are standard error; values in column are mean two independent experiments of 4 replications.
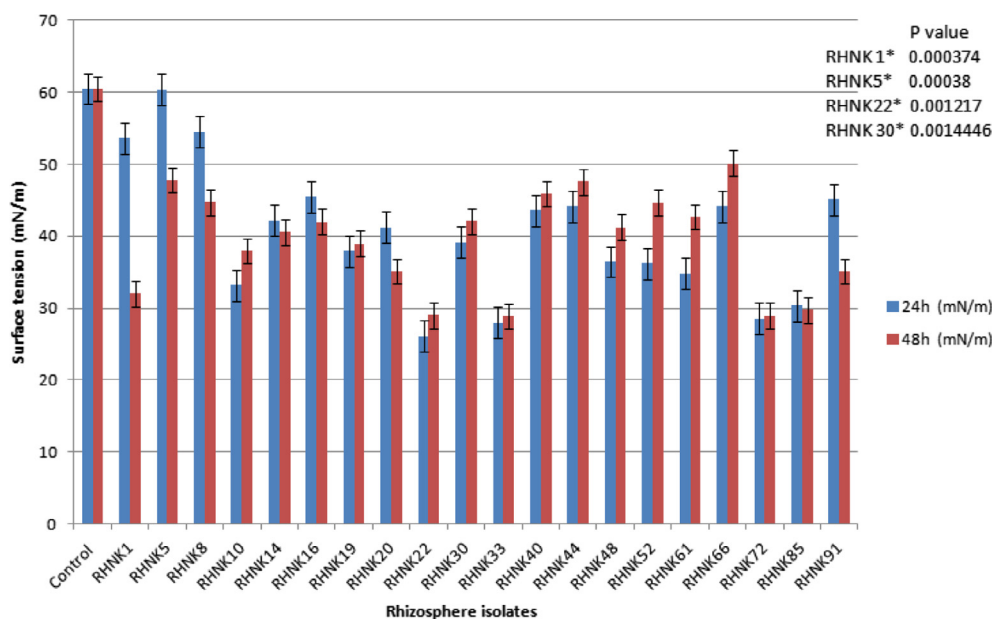
Figure 1 Surface tension measurement of the different biosurfactant producing bacterial isolates.

3.4. Anti-fungal activity by dual culture method

Anti-fungal activity against phytopathogens, *M. phaseolina* and *S. rolfsii* was studied. Among 20 isolates, *Bacillus* sp. RHNK22 showed effective antifungal activity against tested fungi in three different media. The isolate RHNK22 exhibited an inhibition (*I*%) of 76.9 on PDA, 80 on GCY, 72.5 on KB against *M. phaseolina* and 73.3 on PDA, 80 on GCY, 75.5 on KB medium against *S. rolfsii* (Fig. 2).

3.5. Identification of isolate Bacillus spp. RHNK22

Bacterial isolate RHNK22 was identified as Gram positive, rod shaped motile and sporulating bacterium. Biochemical tests revealed that the isolate was positive for indole test, Voges–Proskauer test, citrate utilization test, catalase test, starch hydrolysis and glucose fermentation and negative for methyl red test (Table 3). The 16S rRNA gene sequence results were obtained from a BLAST search of EzTaxon server and RHNK22 was identified as *Bacillus amyloliquefaciens* (Fig. 3).

3.6. Antifungal activity of the extracted iturin A

In this study, the extracted biosurfactant (iturin A) at different concentrations was tested for antifungal activity. It was observed that iturin A produced by strain RHNK22 showed 77.7% inhibition of the phytopathogens tested at 5 mg/mL concentration (Fig. 4).

3.7. PCR, HPLC and FTIR analysis for iturin A

Primers were used for the amplification of gene involved in the antibiotic biosynthesis from *B. amyloliquefaciens* RHNK22 in this study PCR product which was amplified using primers and showed 647 bp of band corresponding to the iturin A gene

(Fig. 5). HPLC analysis of the extracted surfactant of RHNK22 showed a homologue peak with standard iturin A (Fig. 6). FTIR spectra of the extracted surfactant absorption valleys at 1521 and 1558 indicate that the compound contains peptide bonds. A lactone ring is suggested by the absorption at 1732 cm^{-1} and valleys that result from C–H stretching (1174, 1234, 1319, 1338, 1361, 1404 cm^{-1}) indicate the presence of an aliphatic chain. Bands in the range 1570–1515 cm^{-1}, resulting from the deformation mode of the N–H bond combined with C–N stretching mode (amide II band), both indicate the presence of a peptide component. Also C–H stretching was observed at 1730 cm^{-1} (data not shown). *B. amyloliquefaciens* RHNK22 produced iturin A which was confirmed by HPLC & FTIR.

3.8. Uni-dimensional screening of agro-wastes as substrates for iturin A production

Results of unidimensional screening of agro-industrial wastes as substrates are listed in (Table 4). The data on iturin A production using different agro-industrial wastes as substrates, at 48 h of incubation showed a wide variation from 103 to 276 mg/L. The top eight substrates (SOC, COC, CSOC, CWP, DYC, POC, JSC and GOC) which showed maximum iturin A production were selected for second level statistical screening.

3.9. Plackett–Burman design for screening different agro-industrial wastes as substrates for iturin A production

Iturin A production varied from 360 mg/L to 780 mg/L among the sixteen runs of PB design (Table 5). On analysis of regression coefficient of eight variables SOC, CSOC, COC, JSC, CWP and GOC showed positive effects for iturin A production. Out of eight different substrates tested, three substrates (SOC, CWP & DYC) were found to be significantly affecting
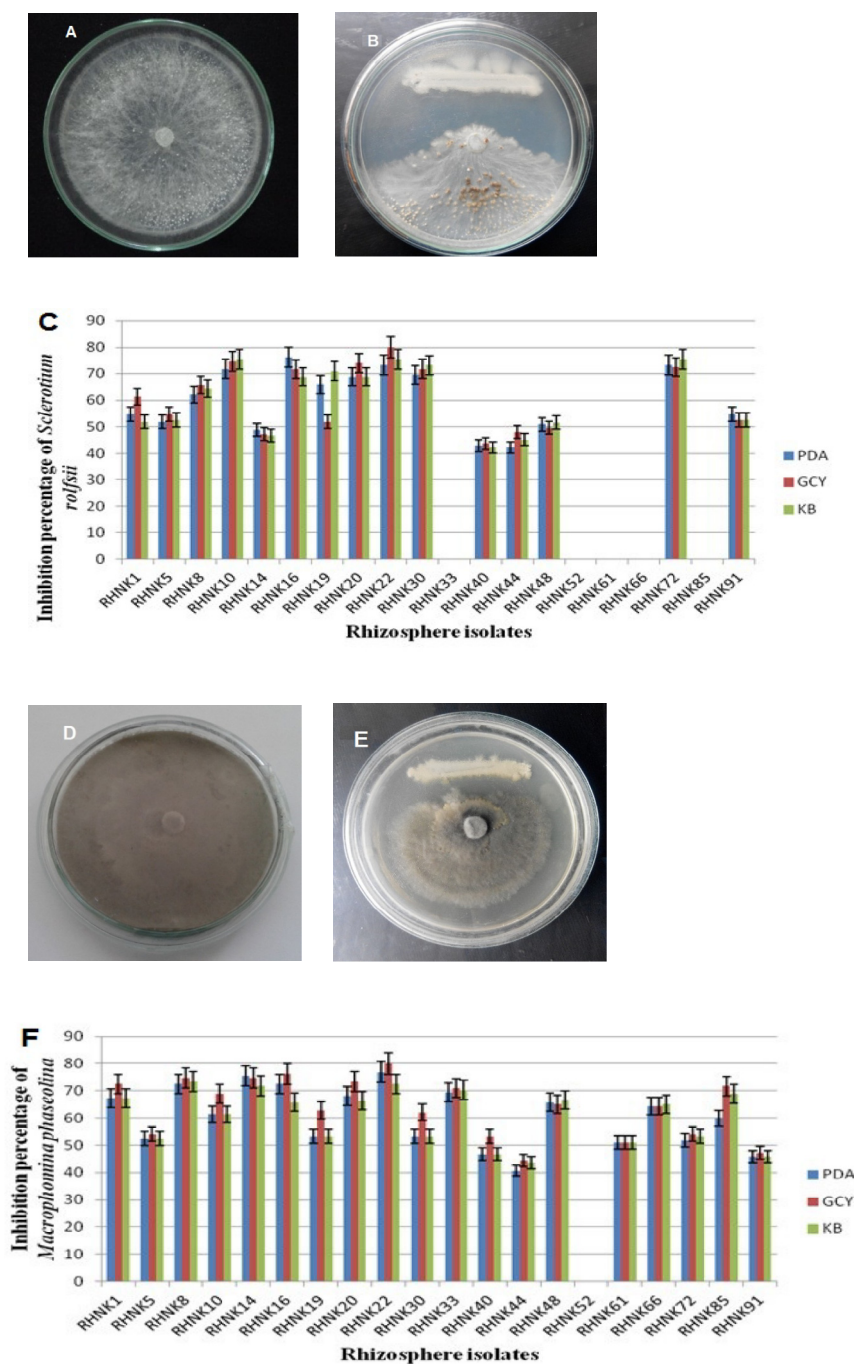
Figure 2   Antifungal activity of biosurfactant producing bacterial isolates. (A) Control (*Sclerotium rolfsii*), (B) antifungal activity of bacterial isolate RHNK22 against *Sclerotium rolfsii* on PDA medium. (C) Antifungal activity of biosurfactant producing bacterial isolates against *Sclerotium rolfsii*. (D) Control (*Macrophomina phaseolina*), (E) antifungal activity of bacterial isolate RHNK22 against *Macrophomina phaseolina* on PDA medium. (F) Antifungal activity of biosurfactant producing bacterial isolates against *Macrophomina phaseolina*.

iturin A production with *P* values less than 0.05, of which SOC with a *P* value of 0.002 was found to be highly significant and was used for further studies (Table 6). The other variables, CSOC, COS, JSW, GOC and POC were found to be insignificant, with *P* values greater than 0.05, that is increase in their concentrations would not have any effect on iturin A production (Table 7).

3.10. Response surface methodology (RSM)

For optimization of iturin A production by *B. amyloliquefaciens* RHNK22 in submerged fermentation using SOC as substrate, RSM was employed. Five variables i.e., substrate (SOC) concentration, along with physical parameters like temperature, pH, inoculum size (IS) and incubation period (IP) were

Table 3 Morphological and biochemical identification of the bacterial isolate RHNK22.

Morphological and biochemical tests	Result
Gram staining	Gram +ve
Motility	+ve
Endo spore	+ve
Starch hydrolysis	+ve
Indole	+ve
Methyl red	−ve
Voges Proskauer	+ve
Citrate utilization	+ve
Catalase	+ve
Glucose fermentation	+ve

studied by central composite rotatable design (CCRD) at five levels and their interactions on iturin A production and reduction in surface tension was determined (Table 8). Thirty two trails or runs were performed to locate the optimum conditions for maximum iturin A production and reduction in surface tension. Both, highest iturin A production and reduction in surface tension were observed at run 6. The student 't' distribution and corresponding values, along with parameter estimate are given in (Tables 9 and 10). The probability (P) values were used as a tool to check the significance of each of the coefficient.

For understanding the simultaneous influence of variables, regression analysis was performed to fit the response function with experimental data. Multiple regression analysis was used to analyse the data and thus a polynomial equation was derived from regression analysis for both iturin A production and reduction in surface tension as follows:

$$Y1 = 643.61 + 14.92X_1 - 29.08X_2 - 23.25X_3 - 47.0X_4$$
$$- 18.33X_5 - 5.24X_1^2 + 5.14X_2^2 - 1.11X_3^2 - 11.11X_4^2$$
$$+ 13.89X_5^2 - 12.63X_1X_2 + 3.63X_1X_3 - 17.0X_1X_4$$
$$- 8.75X_1X_5 - 25.25X_2X_3 + 65.88X_2X_4 - 2.88X_2X_5$$
$$- 11.12\ 5.65X_3X_4 - 4.62X_3X_5 - 13.25X_4X_5$$

Figure 3 Identification of *Bacillus* sp RHNK22 using 16S rRNA sequencing and construction of phylogenetic tree.

$$Y2 = 31.112 - 0.3313X_1 + 0.0286X_2 + 0.9279X_3 - 0.2285X4$$
$$- 0.1104X_5 - 0.0427X_1^2 - 0.2038X_2^2 - 0.6050X_3^2$$
$$- 0.1644X_4^2 + 0.1331X_5^2 + 0.0830X_1X_2 - 0.3340X_1X_3$$
$$- 0.2368X_1X_4 - 0.9519X_1X_5 + 0.4091X_2X_3$$
$$- 0.9706X_2X_4 - 0.1232X_2X_5 + 0.3404X_3X_4$$
$$+ 0.5020X_3X_5 - 0.1422X_4X_5$$

where $Y1$ and $Y2$ are the predicted responses of iturin A yield and the reduction in surface tension and $X1$, $X2$, $X3$, $X4$ and $X5$ were the coded values of the test variables SOC concentration, temperature, pH, inoculum size and incubation period respectively. The significance of each coefficient determined by Student's t-test and P-values is given in (Tables 9 and 10). Larger t-value and smaller P-value indicate a higher level of significance for the corresponding coefficient. In ANOVA for iturin A production, the P-value of each model term, the constants, $X1$; X_2; X_3; $X4$; X_5; X_4^2; X_5^2; $X1X2$, $X1X4$, X_2X3, $X2X4$, $X4X5$ were found to be significant ($P < 0.05$) and in the ANOVA for surface tension reduction $X1$, $X3$, $X4$, X_2^2, X_3^2, $X1X3$, $X1X5$, $X2X3$, $X2X4$, $X3X4$, $X3X5$ were found to be significant.

The results of the second-order response surface model fitting in the form of ANOVA are given in (Tables 11 and 12). F-Test for regression was significant at 5% level. The ANOVA indicates that the effect of interactions of variables, quadratic effects and regression between independent variables are quite significant (Table 11 and 12). To test the fit of the model equation, regression-based determination coefficient R^2 was evaluated. The nearer the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values. The model presented a high determination coefficient ($R^2 = 0.97$) explaining 97% of the variability in response and only 3% of total variations could not be explained by the model. The value of adjusted determination coefficient (Adj $R^2 = 0.92$) was also very high to reflect a good fit between the observed and predicated responses. The present analytical results indicated that the response equation provided a suitable model for process optimization of iturin A production.

Response surface plots were generated to show the interaction of each pair of variables on iturin A production and reduction in surface tension (Fig. 7). Each figure presented the interactive effects of two variables on the yield by keeping the other variables at their middle levels, exhibiting a visual interpretation of the location of optimum experimental conditions. An elliptical contour plot indicated a significant interaction between variables. The results show that, among the independent variables, all the variables tested have significant effect on iturin A production, however, substrate concentration, incubation period and pH were found to have significant effect on reduction in surface tension. In square terms, pH–pH and temperature–temperature were significant for iturin A production and IS–IS and IP–IP were significant for reduction in surface tension. Among the interactions, substrate concentration–IS, substrate concentration–pH, IS–IP, IS–pH and pH–temperature were significant for iturin A production (Fig. 7). High iturin A production and reduction in surface tension could be observed with higher concentration of substrate up to a limit
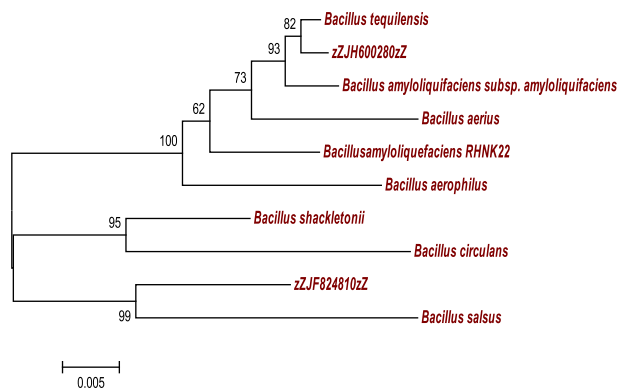
A and B showed inhibition on phytopathogens with different concentration levels of extracted
Iturin A (mg/mL) [A=0.07, B=0.15, C=0.31, D=0.62, E=1.25, F=2.5, G=5.0 and
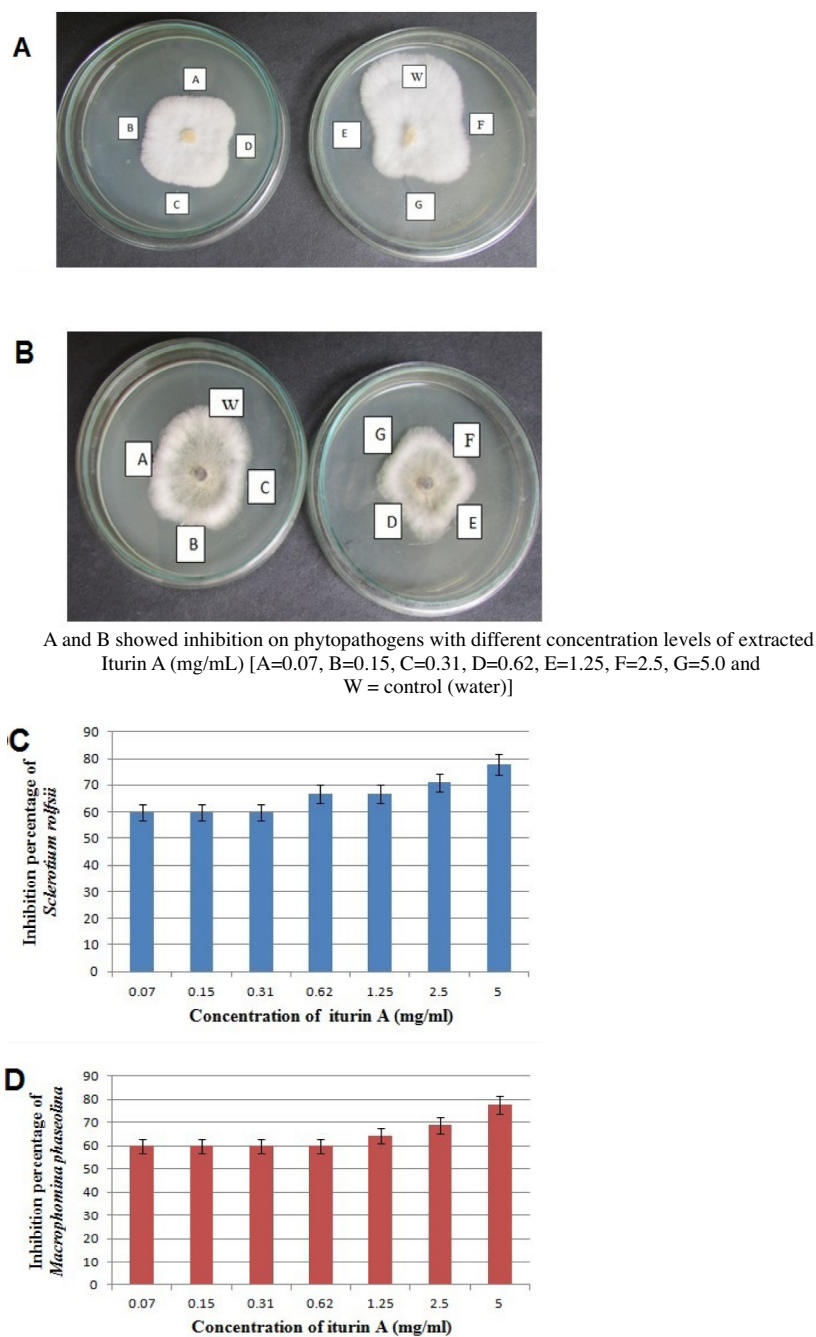W = control (water)]

Figure 4 Antifungal activity of extracted lipopeptide (iturin A). (A) Inhibition of *Sclerotium rolfsii* on PDA medium using different concentrations of Iturin. (B) Inhibition of *Macrophomina phaseolina* on PDA medium using different concentrations of iturin A. (C) Inhibition of *Sclerotium rolfsii* at different concentrations of iturin A produced by *B. amyloliquefaciens* RHNK22. (D) Inhibition of *Macrophomina phaseolina* at different concentrations of iturin A produced by *B. amyloliquefaciens* RHNK22.

(4%) and also with higher incubation period at hold values of other respective parameters. As per Fig. 7, lower IS and higher IP lead to higher iturin A production and reduction in surface tension. Maximum iturin A production and reduction in surface tension could be observed at higher concentration of substrate (4%), 1% IS, 48 h-IP, pH-6.0 and an incubation temperature of 37 ± 2 °C.

4. Discussion

Biosurfactants are a heterogeneous group of secondary metabolites having surface active properties which are known to be produced by a variety of microorganisms. The biosurfactants possess unique properties like higher biodegradability (Lovaglio et al., 2011), lower toxicity
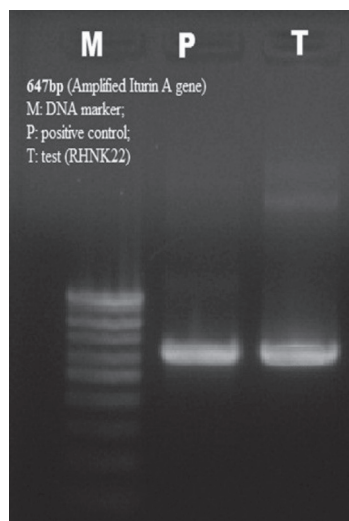
Figure 5 PCR product of amplified iturin A gene from *B. amyloliquefaciens* RHNK22. M: DNA marker. P: iturin A gene specific primers designed by Ramaratham et al. (2007). T: Primers designed in this study.

(Chtioui et al., 2010) and greater stability, which make them use as alternative for chemical surfactants, for the applications in refinery industry, agriculture, pharmaceuticals, cosmetics, plastic, textile, food and machinery fields (Ongena and Jacques, 2008; Mukherjee et al., 2006; Edwards et al., 2003; Liu et al., 2010; Mulligan, 2005). The present study was conducted to screen for biosurfactant producing bacteria with potential antagonistic activity against phytopathogens, characterization of the biosurfactant and development of a cost-effective medium formulation for biosurfactant production.

Of the different rhizosphere bacterial isolates characterized for biosurfactant and antifungal activity, RHNK22 isolated from groundnut rhizosphere was potential. Rhizosphere microorganisms are known to produce biosurfactants which play a key role in plant–microbe interactions (Sachdev and Cameotra, 2013). Due to diverse chemical nature and mode of action of biosurfactants, screening for their activity by a single method is not valid and it is necessary to perform more than one screening method for isolating bacteria with potential biosurfactant activity (Youssef et al., 2004). In this study, nine different screening methods were used for selecting biosurfactant producer from rhizosphere. Plaza et al. (2006) reported that the area of oil spreading zone depends on the concentration of biosurfactant, the isolate RHNK22 showed highest zone (40 mm in dia) [data not shown] compared to the other isolates in this study. Blue agar plate method is a semi-quantitative method to detect anionic surfactants and other extracellular glycolipids (Siegmund and Wagner, 1991). In the present study, as none of the isolates formed blue coloured zones on CTAB agar plates, it was confirmed that the biosurfactant produced by all the twenty bacterial isolates was not glycolipids and of anionic nature.

Camilios-Neto et al. (2011) found an association between haemolytic activity and biosurfactant production and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant production. However, not all bacteria with haemolytic activity are biosurfactant producers, as compounds other than biosurfactants can also cause haemolysis (Youssef et al., 2004). Surfactants with lipase activity have good interfacial activity on water–oil surfaces (bioemulsifiers) (Kokare et al., 2007). In our observation, twenty isolates have good haemolytic and lipase activities. Previous reports of Satpute et al. (2008), Amiriyan et al. (2004) suggest EI and EA methods are essential methods to screen for potential biosurfactant producers and a study on *Bacillus subtilis* MTCC 2422 exhibited an EI% of 68 with kerosene and emulsification assay on petrol 291.4 (EU/mL). In this study, we have tested for EA and EI with nine different hydrocarbon oils and surfactant produced by the isolate RHNK22 showed promising EI and EA, which varied with different hydrocarbons (Table 1 and 2). Das et al. (2009) reported that an efficient biosurfactant can reduce the surface tension of water to < 35 mN/m. In this study, we observed that isolate RHNK22 has shown 26.12 mN/m reduction in surface tension of water within 24 h (Fig. 1).

Soil borne plant pathogens such as *Sclerotium rolfsii* and *Macrophomina phaseolina* cause collar rot and charcoal rot disease respectively in crop plants and are mostly managed by chemical pesticides which may lead to development of resistant pathogens and can also affect symbiotic soil microflora apart from soil fertility. Moreover, such chemicals can enter the food chain and accumulate as undesirable chemical residues (Kim et al., 2004). Lipopeptide antibiotics produced by *Bacillus* spp. are known to inhibit phytopathogenic fungi (Cao et al., 2009; Chen et al., 2009). Singh et al. (2014) have reported that the antifungal activity of iturin A is due to membrane permeabilization properties. Murata et al. (2013) have reported the inhibition of phytopathogenic fungi, *Rhizoctonia solani* with an inhibition of 80% by iturin A produced by *B. amyloliquefaciens* on PDB medium. In the present study, we have tested the antifungal activity of *B. amyloliquefaciens* RHNK22 for both cell-mediated and extracted iturin A against phytopathogens *M. phaseolina* and *S. rolfsii* by dual culture method on 3 different media. RHNK22 showed inhibition of 80% and the extracted iturin A showed 77.7% at 5 mg/mL concentration (Figs. 2 and 4). Bacterial isolate RHNK22 was characterized by the morphological, biochemical characteristics and 16S rRNA gene sequence and identified as *Bacillus amyloliquefaciens* RHNK22 (Table 3, Fig. 3). According to Chun et al. (2007) all the results were obtained from BLAST search EzTaxon server to determine the exact nomenclature of isolate. Presence of iturin A gene in RHNK22 was confirmed by PCR amplification with specific primers and the biosurfactant produced was identified as iturin A (Fig. 5 and 6) by HPLC according to the method reported by Murata et al. (2013). FTIR analysis of the extracted biosurfactant revealed the presence of carboxyl group and peptide component which was in accordance with the results reported by Ramaratham et al. (2007).

The use of cheaper substrates (oil cakes, molasses, distillery wastes, starchy materials, cheese whey, etc) obtained as by-products from various agro industrial sectors have been reported as carbon and nitrogen sources for biosurfactant production (Makkar and Cameotra, 2002). Oil cakes obtained as by-product after oil extraction from seeds are mainly composed of proteins, fibres, crude lipids and minerals, and can
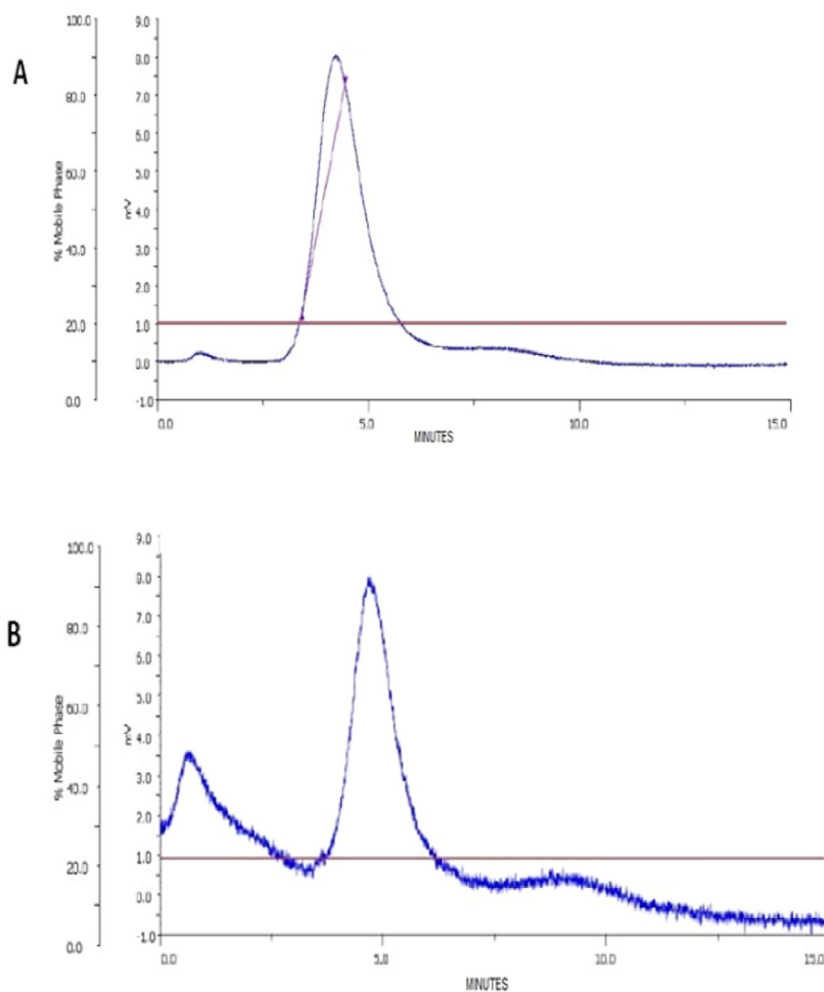
Figure 6 (A) HPLC peak of Standard iturin A (sigma chemical). (B) HPLC peak of iturin A from *B. amyloliquefaciens* RHNK22.

Table 4 Uni-dimensional screening of different agro-industrial wastes as a source for iturin A production by *B. amyloliquefaciens* RHNK22.

Substrate	Iturin A (mg/L)	P value
Rice bran husk	177 ± (1.06)	0.552
Sunflower oil cake*	276 ± (1.08)	0.019
Coconut oil cake*	253 ± (1.70)	0.013
Cotton seed oil cake*	263 ± (1.03)	0.019
Corn cob	135 ± (1.63)	0.105
Orange peel	103 ± (1.03)	0.051
Jack fruit peel	178 ± (1.08)	0.105
Sugarcane leaf	211 ± (0.86)	0.105
Pine apple peel	177 ± (0.64)	0.591
Banana leaf	197 ± (0.89)	0.420
Sweet lime peel	183 ± (1.08)	0.105
Cheese Whey*	268 ± (1.08)	0.029
Dry yeast cells*	253 ± (1.08)	0.029
Pongamia seed cake*	241 ± (1.08)	0.019
Jatropha seed cake*	238 ± (1.02)	0.019
Groundnut oil cake*	258 ± (1.08)	0.019
Glucose with MSM	270 ± (1.06)	0.105

Iturin A production by *Bacillus amyloliquefaciens* RHNK22 (mg/L). Values in the brackets are standard error; values in column are mean values of two independent experiments of 4 replications and * indicating significant (less than 0.05 of the *P* value) for production.

Table 5 The Plackett–Burman experimental design matrix: Screening agro-industrial waste for iturin A production by *B. amyloliquefaciens* RHNK22.

Run	SOC	CSOC	COC	POC	JSC	CWP	DYC	GOC	ITURIN mg/L
1	+	−	−	−	+	−	−	+	640
2	+	+	−	−	−	+	−	−	780
3	+	+	+	−	−	−	+	−	560
4	+	+	+	+	−	−	−	+	720
5	−	+	+	+	+	−	−	−	460
6	+	−	+	+	+	+	−	−	760
7	−	+	−	+	+	+	+	−	560
8	+	−	+	−	+	+	+	+	660
9	+	+	−	+	−	+	+	+	560
10	−	+	+	−	+	−	+	+	480
11	−	−	+	+	−	+	−	+	560
12	+	−	−	+	+	−	+	−	472
13	−	+	−	−	+	+	−	+	680
14	−	−	+	−	−	+	+	−	416
15	−	−	−	+	−	−	+	+	380
16	−	−	−	−	−	−	−	−	360

Number of runs-16, variables-08 and centre points-0.

Table 6 Estimated effects and coefficients for iturin A production (coded units): Plackett–Burman design.

Term	Effect	Coefficient	SE Coefficient	t	P	Significance
Constant		565.50	15.71	35.99	0.000	*
SOC	157.00	78.50	15.71	5.00	0.002	Significant
CSOC	69.00	34.50	15.71	2.20	0.064	*
COC	23.00	11.50	15.71	0.73	0.488	*
POC	−13.00	−6.50	15.71	−0.41	0.691	*
JSC	47.00	23.50	15.71	1.50	0.178	*
CWP	113.00	56.50	15.71	3.60	0.009	Significant
DYC	−109.00	−54.50	15.71	−3.47	0.010	Significant
GOC	39.00	19.50	15.71	1.24	0.255	*

SE, standard error; t, student t value; P, probability, *insignificant.

Table 7 Analysis of variance for iturin A production (coded units): Plackett–Burman design.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main effects	8	233,952	233,952	29,244	7.41	0.008
Residual error	7	27,644	27,644	3949		
Total	15	261,596				

DF, degrees of freedom; Seq SS, sum of squares; Adj SS, adjusted sum of squares; Adj MS, adjusted sum of squares; F, variance ratio; P, probability.

act as a nutrient source for microbial growth and secondary metabolite production. In 2014 the production of SOC was estimated at about 220 million tons (http://www.index-mundi.com). In the present study, an initial screening of different agro-industrial wastes as substrates for biosurfactant production was done to understand the significance of their effect on product formation. A few better ingredients were selected for further level of statistical screening by Plackett–Burman design and the optimum levels of the selected substrates were optimized by RSM.

Previous studies revealed that, using rapeseed meal iturin A production was 600 mg/L in submerged fermentation conditions. However, reports on SOC for biosurfactant production are meagre and study by Mital et al. (2011) revealed that *Enterobacter* sp. showed 1.5 g/L of glycolipid production. In this study, using SOC, iturin A production with RSM showed a 3 fold increase (819 mg/L) when compared with mineral salt medium. Sunflower oil cake consists of 58.25% carbon, 5.88% nitrogen 36.76% protein and crude lipid 1.15% (Lomascolo et al., 2012). The exact reason for enhanced biosurfactant production by SOC cannot be pointed; however, it could be possible because of the high lipid content, free amino acids, and soluble protein present in SOC. The lowest yield of iturin A (103 mg/L) was obtained using orange peel as the substrate which might be attributed to the absence of lipid content, though carbon 41.25% and nitrogen 1.28% are present (http://nutritiondata.self.com; Gnaneshwar Goud et al., 2013).

Table 8 Central composite rotatable design CCRD for optimization of five variables for iturin A production and surface tension reduction by *B. amyloliquefaciens* RHNK22.

Run	SC	IS	IP	pH	Temp. (°C)	Iturin A production (mg/L)		ST (mN/m)	
						Pred. value	Exp. value	Pred. value	Exp. value
1	−1(2%)	−1(1%)	−1(24 h)	−1(6.0)	1(37)	744.2	730.0	30.396	30.466
2	1(4%)	−1(1%)	−1(24 h)	−1(6.0)	−1(30)	821.2	809.0	30.067	30.184
3	−1(2%)	1(2%)	−1(24 h)	−1(6.0)	−1(30)	613.5	621.0	30.446	30.689
4	1(4%)	1(2%)	−1(24 h)	−1(6.0)	1(37)	638.1	636.0	28.569	28.508
5	−1(2%)	−1(1%)	1(48 h)	−1(6.0)	−1(30)	750.1	746.0	27.871	27.841
6	1(4%)	−1(1%)	1(48 h)	−1(6.0)	1(37)	832.8	819.0	26.835	26.192
7	−1(2%)	1(2%)	1(48 h)	−1(6.0)	1(37)	533.0	539.0	33.493	33.285
8	1(4%)	1(2%)	1(48 h)	−1(6.0)	−1(30)	604.0	612.0	33.317	33.156
9	−1(2%)	−1(1%)	−1(24 h)	1(8.0)	−1(30)	578.9	581.0	30.747	31.176
10	1(4%)	−1(1%)	−1(24 h)	1(8.0)	1(37)	544.5	537.0	27.514	27.640
11	−1(2%)	1(2%)	−1(24 h)	1(8.0)	1(37)	691.8	704.0	28.273	28.524
12	1(4%)	1(2%)	−1(24 h)	1(8.0)	−1(30)	714.8	729.0	28.391	28.689
13	−1(2%)	−1(1%)	1(48 h)	1(8.0)	1(37)	513.5	514.0	33.443	33.421
14	1(4%)	−1(1%)	1(48 h)	1(8.0)	−1(30)	608.5	611.0	32.063	32.089
15	−1(2%)	1(2%)	1(48 h)	1(8.0)	−1(30)	616.7	639.0	29.808	29.959
16	1(4%)	1(2%)	1(48 h)	1(8.0)	1(37)	516.4	529.0	29.758	29.606
17	−2(1%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	592.8	581.0	31.603	31.254
18	2(5%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	652.4	656.0	30.278	30.441
19	0(3%)	−2(0.5%)	0(36 h)	0(7.0)	0(35)	722.3	750.0	30.239	30.140
20	0(3%)	2(2.5%)	0(36 h)	0(7.0)	0(35)	605.9	570.0	30.353	30.266
21	0(3%)	0(1.5%)	−2(12 h)	0(7.0)	0(35)	685.6	690.0	29.498	29.165
22	0(3%)	0(1.5%)	2(60 h)	0(7.0)	0(35)	592.6	580.0	30.547	31.004
23	0(3%)	0(1.5%)	0(36 h)	−2(5.0)	0(35)	693.1	710.0	30.911	31.184
24	0(3%)	0(1.5%)	0(36 h)	2(9.0)	0(35)	505.1	480.0	29.997	29.537
25	0(3%)	0(1.5%)	0(36 h)	0(7.0)	−2(25)	735.8	720.0	31.864	31.421
26	0(3%)	0(1.5%)	0(36 h)	0(7.0)	2(40)	662.4	670.0	31.423	31.680
27	0(3%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	643.6	647.0	31.111	31.034
28	0(3%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	643.6	641.0	31.111	31.245
29	0(3%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	643.6	642.0	31.111	31.176
30	0(3%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	643.6	643.0	31.111	31.284
31	0(3%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	643.6	648.0	31.111	31.046
32	0(3%)	0(1.5%)	0(36 h)	0(7.0)	0(35)	643.6	649.0	31.111	31.054

SC-substrate concentration, IS-inoculum size, IP-incubation period, Temp-temperature.

Table 9 Coefficients and *t*-values for iturin A production by *B. amyloliquefaciens* RHNK22 using central composite rotatable design (CCRD).

Term	Coefficient	Standard error Coefficient	*t*-value	Probability
Constant	643.61	8.900	72.320	0.000
Substrate	14.92	4.554	3.275	0.007
IS	−29.08	4.554	−6.386	0.000
IP	−23.25	4.554	−5.105	0.000
pH	−47.00	4.554	−10.320	0.000
Temperature	−18.33	4.554	−4.025	0.002
Substrate * Substrate	−5.24	4.120	−1.272	0.230
IS * IS	5.14	4.120	1.247	0.238
IP * IP	−1.11	4.120	−0.270	0.792
pH * pH	−11.11	4.120	−2.698	0.021
Temperature * Temperature	13.89	4.120	3.371	0.006
Substrate * IS	−12.63	5.578	−2.263	0.045
Substrate * IP	3.63	5.578	0.650	0.529
Substrate * pH	−17.00	5.578	−3.048	0.011
Substrate * Temperature	−8.75	5.578	−1.569	0.145
IS * IP	−25.25	5.578	−4.527	0.001
IS * pH	65.88	5.578	11.810	0.000
IS * Temperature	−2.88	5.578	−0.515	0.616
IP * pH	−11.12	5.578	−1.994	0.071
IP * Temperature	−4.62	5.578	−0.829	0.425
pH * Temperature	−13.25	5.578	−2.375	0.037

$S = 22.31$; R-Sq = 97.4%; R-Sq (adj) = 92.7%.

Table 10 Coefficients and *t*-values for surface tension reduction by *B. amyloliquefaciens* RHNK22 using central composite rotatable design (CCRD).

Term	Coefficient	Standard error coefficient	*t*-Value	Probability
Constant	31.1112	0.17288	179.960	0.000
Substrate	−0.3313	0.08847	−3.744	0.003
IS	0.0286	0.08847	0.323	0.753
IP	0.9279	0.08847	10.488	0.000
pH	−0.2285	0.08847	−2.583	0.025
Temperature	−0.1104	0.08847	−1.248	0.238
Substrate * Substrate	−0.0427	0.08003	−0.533	0.605
IS * IS	−0.2038	0.08003	−2.546	0.027
IP * IP	−0.6050	0.08003	−7.560	0.000
pH * pH	−0.1644	0.08003	−2.054	0.064
Temperature * Temperature	0.1331	0.08003	1.663	0.124
Substrate * IS	0.0830	0.10836	0.766	0.460
Substrate * IP	0.3340	0.10836	3.082	0.010
Substrate * pH	−0.2368	0.10836	−2.185	0.051
Substrate * Temperature	−0.9519	0.10836	−8.785	0.000
IS * IP	0.4091	0.10836	3.776	0.003
IS * pH	−0.9706	0.10836	−8.958	0.000
IS * Temperature	−0.1232	0.10836	−1.137	0.280
IP * pH	0.3404	0.10836	3.141	0.009
IP * Temperature	0.5020	0.10836	4.633	0.001
pH * Temperature	−0.1422	0.10836	−1.313	0.216

S = 0.4334 R-Sq = 97.5% R-Sq (adj) = 92.8%.

Table 11 Analysis of variance for iturin A production by *B. amyloliquefaciens* RHNK22 using CCRD.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	20	205,083	205083.3	10254.2	20.60	0.000
Linear	5	99,697	99696.5	19939.3	40.05	0.000
Square	5	11,880	11880.3	2376.1	4.77	0.015
Interaction	10	93,507	93506.5	9350.7	18.78	0.000
Residual error	11	5476	5476.2	497.8		
Lack-of-Fit	6	5418	5418.2	903.0	77.85	0.000
Pure error	5	58	58.0	11.6		
Total	31	210,559				

DF, degrees of freedom; Seq SS sum of squares; Adj SS, adjusted sum of squares; Adj MS, adjusted sum of squares; *F*, variance ratio; *P*, probability.

Table 12 Analysis of variance for surface tension reduction by *B. amyloliquefaciens* RHNK22 using CCRD.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	20	79.3313	79.3313	3.96657	21.11	0.000
Linear	5	24.8634	24.8634	4.97269	26.47	0.000
Square	5	12.9744	12.9744	2.59488	13.81	0.000
Interaction	10	41.4935	41.4935	4.14935	22.09	0.000
Residual error	11	2.0665	2.0665	0.18786		
Lack-of-Fit	6	2.0028	2.0028	0.33381	26.23	0.001
Pure error	5	0.0636	0.0636	0.01273		
Total	31	81.3978				

DF, degrees of freedom; Seq SS sum of squares; Adj SS, adjusted sum of squares; Adj MS, adjusted sum of squares; *F*, variance ratio; *P*, probability.
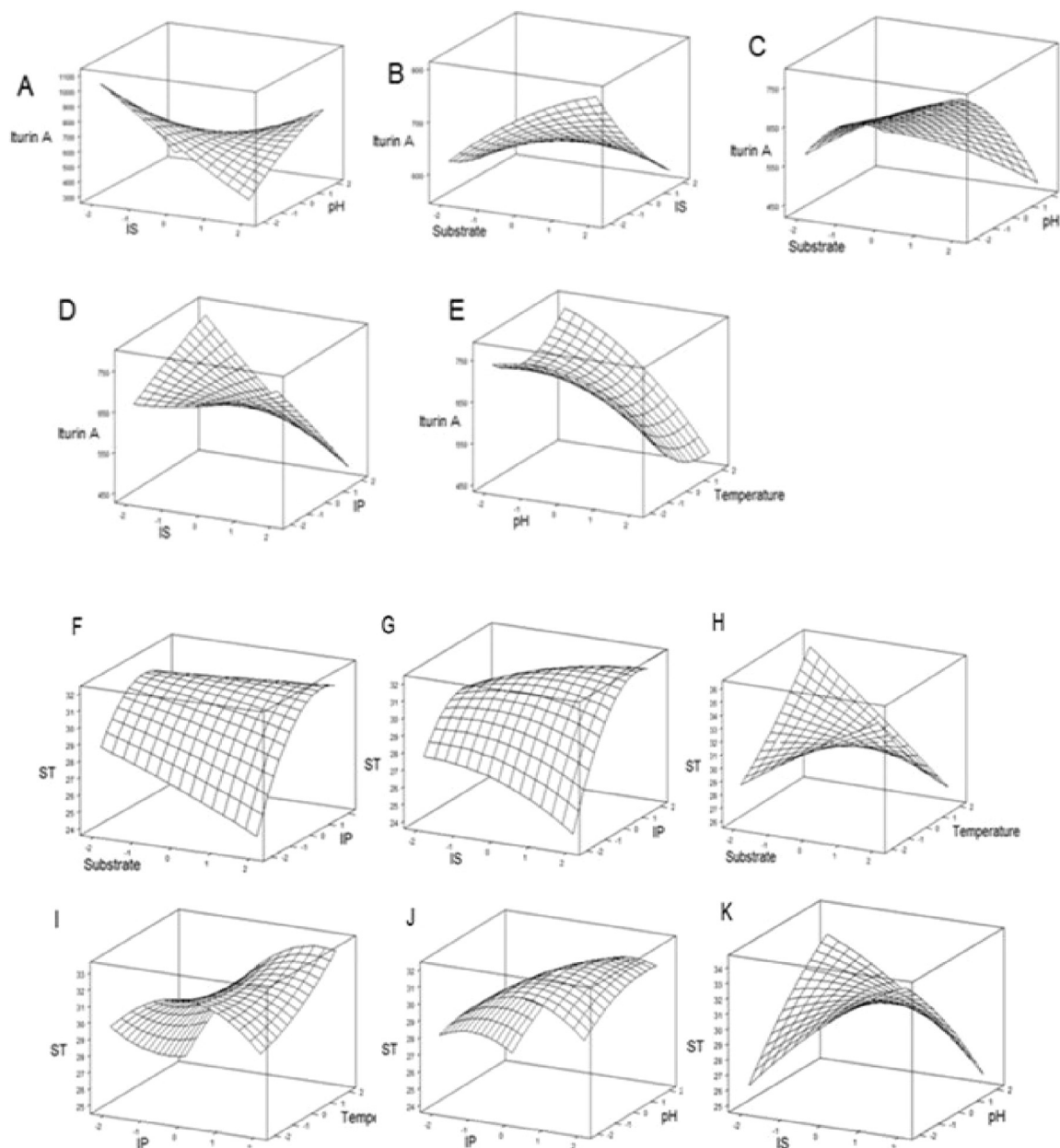
Figure 7 Response surface plots of iturin A production and surface tension (ST) by *B. amyloliquefaciens* RHNK22. (A)–(E) are Response surface plots of iturin A production, in terms of interaction between (A) inoculum size level and pH, (B) substrate concentration and inoculums size, (C) substrate concentration and pH, (D) inoculum size level and incubation period and (E) pH and temperature. (F)–(K) are Response surface plots of reduction of surface tension, in terms of interaction between (F) substrate concentration and incubation period, (G) inoculum size level and incubation period, (H) substrate concentration and temperature, (I) incubation period and temperature, (J) incubation period and pH and (K) inoculums size and pH.

5. Conclusion

In present study, 100 *Bacillus* spp. from rhizosphere soil samples were screened for biosurfactant activity using various basic and high-throughput screening methods. Among them, one bacterial isolate identified as *B. amyloliquefaciens* RHNK22 isolated from groundnut rhizosphere showed significant biosurfactant activity and reduction of surface tension. Biosurfactant, identified as iturin A by FTIR and HPLC analysis and it had potential antifungal activity. Using RSM model, Sunflower oil cake showed 3 fold increases in iturin A production and in view of this it can be recommended for large scale production, to meet the demand of global requirement of biosurfactants.

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