Objective of the Project:

To overcome the disposing problem of animal fat, biosurfactant production is an effective and alternative way. The main objective of this project was the utilization of fish fat as double beneficial approach like overcoming of environmental hazards as well as cost reduction of biosurfactant production.

The basic focus of the project includes

a) To isolate and identify the biosurfactant producing bacteria from oil contaminated soil.
b) To isolate and identify the produced biosurfactant.
c) Process optimization of culture condition for maximizing the biosurfactant production.
d) To examine the pharmacological and antimicrobial effect of produced biosurfactant.

Whether Objectives were achieved   **Annexure II**

Yes

Two effective fat degrading as well as biosurfactant producing bacteria were isolated from oil contaminated fertile soil sample among a number of isolate. The isolated bacteria were primarily identified by biochemical tests according to Bergey's manual of systematic bacteriology and finally confirmed by 16S rRNA gene sequencing analysis. The isolated biosurfactants were structurally identified after purification. Pharmacological activity in terms of wound healing effect and antibacterial activity of the produced biosurfactants were examined.

Achievements from the Project

1. Isolation and identification of two biosurfactant producing bacteria i.e. *Pseudomonas aeruginosa* DSM 50071 (GenBank accession no. NR026078) and *Bacillus stratosphericus* 41KF2a(T) (GeneBank accession no. KU644139).

2. Isolation and identification of the produced biosurfactants from the two isolated bacteria.

3. Bacterial culture condition optimization to achieve highest biosurfactant yield.

4. Evaluation of pharmacological activity in terms of *in vivo* wound healing activity of the produced biosurfactants and examining its antibacterial activity.

Summary of the findings   **Annexure – IV**

Except a study of Deshpande M. and Daniels L. *et al* till date there are no such significant application of fish fat is available, this experiment can play a double beneficial role by conquering the environmental hazard as well as acting as a promising economic substrate for biosurfactant production. Instead of fish fat degradation, the produced biosurfactant showed potential application as a wound healing and antibacterial agent which could make it a promising alternative of traditional antibiotic in near future.

Contribution to the Society   Annexure V

During 2006-07 it was reported that 3,02,750 tonnes of fish waste was generated in India. Among this waste, the largest waste generation was observed from Gujarat (30.51%), followed by Maharashtra (23%) and Kerala (17.5%). Processing of fish leads to enormous amounts of waste. It was estimated that fish processing waste accounts to be 75% of the total fish weight after fillet preparation. About 30% weight of total part remains as net waste in the form of skin and bone during preparation of fillet. Animal fat and plant derived fatty substances may solidify or become viscous between the temperatures of 32 ^{0}F to 150 ^{0}F (0 ^{0}C to 65 ^{0}C), and during their disposal, these are separated from wastewater by gravity (Wright-Pierce, 2006) as the density is lower than water. This leads to blockages in pipes and sewers in cold weathered countries and increases disposal risk. This waste is full of protein, fatty acids, vitamin and minerals. So, these fats can be used for the production of biosurfactant by microbial degradation which is also cheaper than vegetable oils. The utilization of this waste could help to overcome the environmental hazards.

No. of Publications out of the Project (Please Attach Re-Prints)   **Annexure VI**

1. Bhattacharya, M., Biswas, D., Sana, S. and Datta, S., 2014. Utilization of waste engine oil by Ochrobactrum pseudintermedium strain C1 that secretes an exopolysaccharide as a bioemulsifier. Biocatalysis and Agricultural Biotechnology, 3(4), pp.167-176.
2. Bhattacharya, M. and Biswas, D., 2014. Enhancement of waste engine oil biodegradation by optimization of media using factorial design study. Indian J Biotechnol, 13, pp.293-300.
3. Bhattacharya, M., Biswas, D., Sana, S. and Datta, S., 2015. Biodegradation of waste lubricants by a newly isolated Ochrobactrum sp. C1. 3 Biotech, 5(5), pp.807-817.
4. Bhattacharya, M., Guchhait, S., Biswas, D. and Datta, S., 2015. Waste lubricating oil removal in a batch reactor by mixed bacterial consortium: a kinetic study. Bioprocess and biosystems engineering, 38(11), pp.2095-2106.
5. Bhattacharya, M., Guchhait, S., Singh, R., Biswas, D. and Datta, S., 2015. Evaluation of two bioemulsifier producing strains in a microbial consortium for crude oil spill bioremediation and its potential uses in enhanced oil recovery. Journal of Petroleum Science and Engineering (under review).
6. Sana, S., Bhattacharya, M., Datta, S. and Biswas, D., 2015. RSM study for the production of rhamnolipid using Catla catla Fish fat. Int J Curr Microbiol App Sci, 4(1), pp.169-178.
7. Datta, S., Biswas, D. and Auddy, R.G., 2014. Correlation between oil substrates and biosurfactant activity using Acinetobacter junii and Pseudomonas aeruginosa. JOURNAL OF THE INDIAN CHEMICAL SOCIETY, 91(3), pp.497-502.

Book chapter publication during the period of project

1. M. Bhattacharya, I.S. Roy, D. Biswas, S. Datta and S. Sana (2015) Towards efficient PDE waste management by biodegradation of waste transformer oil. In: Adhikary K et al. (Eds) Environment: Pollution and protection, pp. 55-65, Narosa Publishing House Pvt. Ltd. New Delhi, India (ISBN No. 978-81-8687-4105).

Papers presented in the conference during the period of project.

a. Munna Bhattacharya, Sugata Guchhait and Dipa Biswas. Bioemulsifier modified enhanced oil recovery: a study on future potential of biotechnology in the field of petroleum research. Page 153, Proceedings of 4th International conference of World Science Congress, Jadavpur University, Kolkata, India, December 16-18, 2014.
b. Munna Bhattacharya and Dipa Biswas. Enhancement of waste engine oil biodegradation by optimization of media using factorial design study, (Abstract Code MIB-117) Page C163, Proceedings of International Conference on advances in Biotechnology and Bioinformatics

(ICABB), DPU Publications (ISBN: 978-81-926619-1-9), Dr. D.Y. Patil Vidyapith, Pune, India, November 25-27, 2013.

c. Munna Bhattacharya, Sugata Guchhait and Dipa Biswas. Growth kinetic models for crude oil biodegradation by mixed culture in a batch scale bioreactor, Page 15. Proceedins of 10th Annual session of Student's Chemical Engineering Congress (SCHEMCON), Department of Chemical Engineering, Indian Institute of Chemical Engineers, Haldia Regional Centre, India, September 19-20, 2014.

d. M. Bhattacharya, I.S. Roy, D. Biswas, S. Datta and S. Sana. Towards efficient PDE waste management by biodegradation of waste transformer oil. Page 93, Proceedings of National Conference on Environment: Pollution and Protection (EPP), Department of Earth and Environmental Studies, National Institute of Technology, Durgapur, January 30-February 1, 2014.

e. Munna Bhattacharya and Dipa Biswas. Degradation of waste lubricants by bacterial having potential for bioemulsifier production. Page 139, Proceedings of the 100th Session of Indian Science Congress (ISC), Section of Environmental Sciences, University of Calcutta, Kolkata, January 3-7, 2013.

f. Santanu Sana, Munna Bhattacharya, Sriparna Datta & Dipa Biswas. Study on utilization of Fish Fat for Biosurfactant production. Proceedings of 4th International conference of World Science Congress, Jadavpur University, Kolkata, India, December 16-18, 2014.

g. Santanu Sana, & Sriparna Datta. Comparative study of different Fats for Rhamnolipid production. Proceedings of International Conference on advances in Biotechnology and Bioinformatics (ICABB), DPU Publications (ISBN: 978-81-926619-1-9), Dr. D.Y. Patil Vidyapith, Pune, India, November 25-27, 2013.

h. Santanu Sana, & Sriparna Datta. Study on utilization of Fish Fat for Biosurfactant production. Proceedings of the 100th Session of Indian Science Congress (ISC), Section of Environmental Sciences, University of Calcutta, Kolkata, January 3-7, 2013.

i. Santanu Sana, Sriparna Datta & Dipa Biswas. Wound healing activity of a biosurfactant produced by *Bacillus stratosphericus* A15. Proceedings of the 103rd Session of Indian Science Congress (ISC), Section of Medical Science (including Physiology), University of Mysore, Mysore, January 3-7, 2016.

j. Santanu Sana, Sriparna Datta & Dipa Biswas. Isolation and characterization of a lipopeptide type biosurfactant from a *Bacillus stratosphericus* using waste fish fat. Proceedings of National Symposium on Recent Advances in Chemistry and Industry (2016, with special emphasis on Pharmaceutical Industry). University of Calcutta, Kolkata, August 2-3, 2016.

k. Santanu Sana & Sriparna Datta. Utilization of fish fat for production of biosurfactant with multifunctional activities. Proceedings of the International Conference on Advances in Bio-Informatics and Environmental Engineering - ICABEE 2016, Rome, Italy, August 18-19, 2016. (Abstract accepted)

l. Santanu Sana, Sriparna Datta & Dipa Biswas. Antimicrobial activity of rhamnolipid and BS15 against *Escherichia coli* and their synergistic interaction. Proceedings of 11th International Conference on Biotechnology, Bio Informatics, Bio Medical Sciences and Stem Cell Applications (B3SC), London, September 21-22, 2016. (Abstract accepted)

Sriparna Datta
2/8/16

Principal Investigator
(Signature with seal)

PROFESSOR
Dept of Chemical Technology
University of Calcutta
92, A.P.C. Road, Kolkata-9

Registrar
(Signature with seal)

REGISTRAR (Actg).
Calcutta University 08/09/16.

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

Annual/Final Report of the work done on the Major Research Project.

(Report to be submitted within 6 weeks after completion of each year)

1. **Project report No.:** 3rd/Final

2. **UGC Reference No.**:   F/41-501/2012(SR) dated 23.07.2012 and 11/11/2014

3. **Period of report:**   From 01/04/2014 to 30/06/2015

4. **Title of research project:**   Biosurfactant production from waste oils by microbes

5. **(a) Name of the Principal Investigator:**   Dr. (Ms.) Sriparna Datta

   (b) Department: Chemical Technology

   (c) University/College where the work has progressed: University of Calcutta

   92, A.P.C. Road, Kolkata – 700009, India

6. **Effective date of starting of the project:** 1st July, 2012

7. **Grant approved and expenditure incurred during the period of the report:**

   a. **Total amount approved**   Rs. 13, 14,753/-

   b. **Total expenditure**   Rs. 12, 92, 263/-

   c. **Report of the work done: (Please attach a separate sheet)**

      i.   **Brief objective of the project:** Annexure – I

      ii. **Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication**
                          Annexure – II

      iii. **Has the progress been according to original plan of work and towards achieving the objectives, if not state reasons.**
      The work has progressed as per proposed original plan of work to achieve objective.

iv. Please indicate the difficulties, if any, experienced in implementing the project.

The project was implemented on 1st July'2012. After official formalities, the project actually started on 1st week of October'2012. The UGC sent 85% of recurring expenditure including project fellowship and HRA on 2nd installment, which are almost exhausted. Non-availability fund influence the progress of the work.

The main two difficulties are
   a)   Administrative delay
   b)   Non-availability of the funds

v. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet.

The project work was completed on June 2015. Documentation for paper presentation is progressing.

vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission.
<div align="center">Annexure – III</div>

vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any

Ph.D. Enrolled, if yes, details.

1. Munna Bhattacharya done her PhD. registration, entitled "Biodegradation of Waste Mineral Oils for Environmental Production and Production of Value Added Products" (Registration No. 5655 Ph.D. (Tech.) Proceed/12, dated 03-12-2012).
2. Santanu Sana has joined as a Project Fellow on 1st October, 2012. His Ph.D. registration, entitled "Microbial Degradation of Animal Fat for Novel Product Formation" was done (Registration No. 4837Ph.D.(Tech.) Proceed/13, dated 14-08-2013).

UNIVERSITY GRANTS COMMISSION

BAHADUR SHAH ZAFAR MARG

NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE

FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project   Biosurfactant production from waste oils by microbes

2. Name and Address of the Principal Investigator   Dr. (Ms.) Sriparna Datta
                              Department of Chemical Technology, University of Calcutta,

    92, A.P.C. Road, Kolkata, PIN 700009

3. Name and Address of the Institution

    Department of Chemical Technology, University of Calcutta,

    92, A.P.C. Road, Kolkata, PIN 700009

4. UGC Approval No. and Date   F/41-501/2012(SR) dated 23.07.2012

5. Date of Implementation   First July'2012

6. Tenure of the Project   3 (Three) Years

7. Total Grant Allocated   Rs. 13, 14, 753.00

8. Total Grant Received   Rs. 12, 25, 768.00

9. Final Expenditure   Rs. 12, 92, 263.00

10. Title of the Project   **Biosurfactant production from waste oils by microbes**

11. Objective of the Project   **Annexure – I**

    The proposal was processed through

    ➢ Isolation of a number of waste animal fat degrading bacteria from oil contaminated soil.

- ➢ Selection of the best fat degrader as well as biosurfactant producer among it on the basis of hemolytic activity, cell surface hydrophobicity and BATH assay.
- ➢ Identification of the isolated bacteria depending on biochemical tests and finally 16S rRNA gene sequencing analysis.
- ➢ Isolation and structural identification of the produced biosurfactant.
- ➢ Process optimization through statistically designed experiments to achieve highest biosurfactant yield.
- ➢ Evaluation of pharmaceutical application of the produced biosurfactant after purification.

12. Whether Objectives were Achieved   **Annexure – II**

13. Achievements from the Project   **Annexure – III**

14. Summary of the Findings (In 500 words)   **Annexure – IV**

15. Contribution to the Society   **Annexure – V**

16. Whether any PhD Enrolled/Produced out of the Project

Two students were enrolled PhD out of this project and one of them is under thesis writing stage.

1. Munna Bhattacharya done her PhD. registration, entitled "Biodegradation of Waste Mineral Oils for Environmental Production and Production of Value Added Products" (Registration No. 5655 Ph.D. (Tech.) Proceed/12, dated 03-12-2012).
2. Santanu Sana has joined as a Project Fellow on 1st October, 2012. His Ph.D. registration, entitled "Microbial Degradation of Animal Fat For Novel Product Formation" was done (Registration No. 4837Ph.D.(Tech.) Proceed/13, dated 14-08-2013).

17. No. of Publications out of the Project (Please Attach Re-Prints)   **Annexure – VI**

Evaluation of UGC Major Research Project entitled "Biosurfactant production from waste oils by microbes." F.No 41-501/2012(SR), dated 23.07.2012

The work done under this research project carried out under Prof. Sriparna Datta was a unique study of utilizing *Catla catla* fish fat as a carbon substrate for *Pseudomonas aeruginosa* C2 and *Bacillus stratosphericus* A15 for production of biosurfactants. Culture condition of the media was optimised to amplify the biosurfactant production using response surface methodology. The most remarkable parameters to influence the biosurfactant production was found as carbon and nitrogen ratio (C/N), carbon and phosphate ratio (C/P) and magnesium sulphate concentration. Using the optimised culture components biosurfactant yield enhanced about 3 times to that obtained normally and thereby helped in reducing the production cost of final product.

The kinetic study revealed a controlling mechanism on cell density dependant rhamnolipid formation. This study seemed to be the first mathematical model to produce rhamnolipid in a bioreactor utilizing animal fat as the cheap raw material for biosurfactant production. The biosurfactants i.e. rhamnolipid was produced by *Pseudomonas aeruginosa* C2 and BS15 produced by *Bacillus stratosphericus* A15 expressed potential activity of wound healing on rat using punch wound model. Healing activity was supported by a number of parameters like rapid wound closure due to connective tissue regeneration, thick epidermal layer and keratinocyte formation at the wound site, estimation of DNA, protein, hexosamine content of the regenerated tissue and TNF-α level of blood serum.

Antibacterial activity of rhamnolipid against *Staphylococcus aureus* ATCC25923 and *Escherichia coli* K 8813 respectively supported the healing activity by providing protection against bacterial infection at the wound site.

This research clearly demonstrated the cell membrane penetrating activity of rhamnolipid against both the tested Gram positive and Gram negative bacteria. As the traditional antibiotics are getting resistant day by day so this bacterial cell membrane disrupting and finally cell death activity of the biosurfactants could replace them.

The stability studies and potential value of CMC (46 mg L^{-1}) of the produced biosurfactant suggested that it has potentiality to express application under the extreme conditions in industries like paint, food, pharmaceutical and cosmetic, detergent, tannery and MEOR etc. in future.

Counter Signature

14-01-2019

Dr. Nakul Chandra Maiti
Principal Scientist / प्रधान वैज्ञानिक
संरचनागत जीवविज्ञान और जैवसूचना प्रभाग
Structural Biology & Bioinformatics Division
भारतीय रासायनिक जीवविज्ञान संस्थान
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
Indian Institute of Chemical Biology
(Council of Scientific & Industrial research)
कोलकाता-७०००३३ / Kolkata 700

REGISTRAR
UNIVERSITY OF CALCUTTA
31/12/2018

Prof. D. K. Bhattacharyya (Visiting)
School of Community Sc. & Tech.
IIEST, Shibpur

Research Work Summary

Currently most of the surfactants are chemically synthesized from petroleum industry[1] and they are used in various fields but nowadays biosurfactants have attracted the attention due to its biodegradable and environment friendly nature, biocompatibility, specific activity in extreme conditions of temperature, pH and salinity. Unfortunately biosurfactant cannot compete with synthetic surfactants due to their higher production cost. Hence attempts are made to produce biosurfactants from waste materials as substrate such as waste vegetable oil[2], soybean soapstock waste[3], sunflower oil soapstock waste[4] and various oil refinery wastes[5], distillery and dairy waste, potato process effluents (wastes from potato processing industry), cassava waste water (produced during the process of cassava flour), rice water (effluent from rice processing industry and domestic cooking), corn steep liquor and the waste materials produced during the processing of cereals, pulses and molasses[6].

Animal fat and plant derived fatty substances may solidify or become viscous between the temperatures of 32^0F to 150^0F (0^0C to 65^0C), and during their disposal, they separate from wastewater by gravity[7] as the density is lower than water. This leads to blockages in pipes and sewers in cold weather and increases pollution risk. One of the potential uses of the fats can be the production of biosurfactant using the process of microbial degradation of fats & oils. From literature only, Deshpande M. and Daniels L. *et al* claimed that *Candida bombicola* can grow on animal fat and produce surface active agent, sophorolipid[8]. So, animal fat is still unexplored and it can be used as substrate in production of biosurfactant.

We, therefore, aimed to utilize fish fat as substrate to produce biosurfactant using microbes and in doing so we have isolated a *Pseudomonas sp.* producing rhamnolipid and optimized the culture condition to obtain the maximum production of rhamnolipid.

Results of *Pseudomonas aeruginosa* DSM 50071

Screening of effective biosurfactant producer depending on surface active property

A clear zone around the colonies (Fig. 1) signified the hemolytic activity of the produced biosurfactant. A significant oil displacement ability of the produced biosurfactant of 3.42±0.81 cm diameter with respect to control (1.57±0.45 cm) indicated the production of biosurfactant from the tested strain. The cell adherence to hexadecane of 61.48±1.07 % was calculated.


Fig. 1 Growth of *P. aeruginosa* DSM 50071 on blood agar, lysis of erythrocytes can be indicated by the clear part on the plate.

Microbial isolation and identification

From the oil contaminated soil the isolated bacterial strain was identified as an aerobic, rod shaped gram negative bacteria which may be *Pseudomonas sp.* Sequence analysis of the 16S rRNA gene revealed it's homology with *Pseudomonas aeruginosa* strain DSM 50071. The phylogenetic tree generated using Neighbor-Joining method was provided in Fig. 2. The sequence has been submitted to GenBank with the accession no. NR 026078. Thus, our isolated strain C2 should be classified as a strain of *Pseudomonas* sp. C2[14]. This strain exhibited an ability to degrade animal fat as a good source of hydrocarbon to produce rhamnolipid.
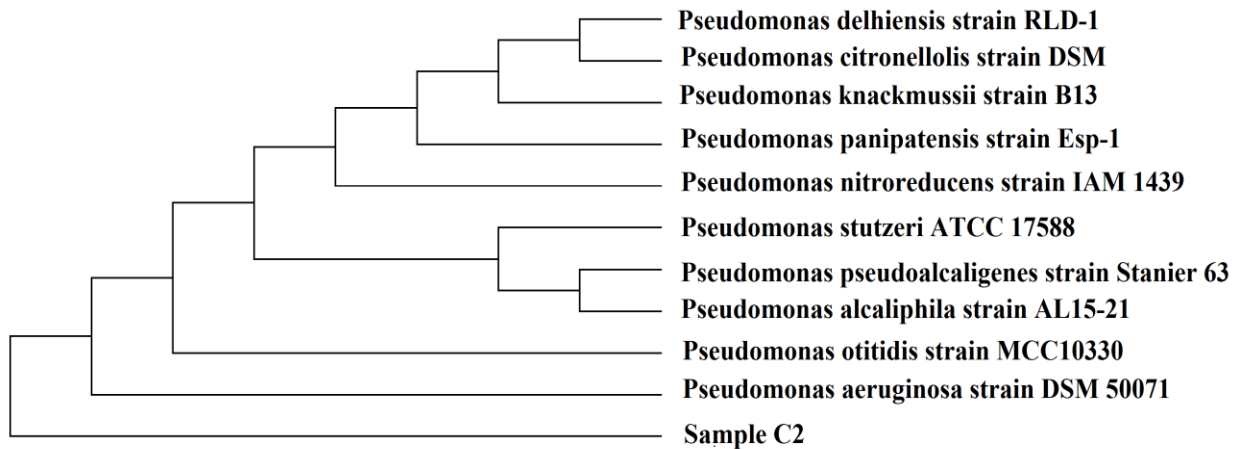
Fig. 2 Phylogenetic tree showing interrelationship of isolated strain C2 with closely related species of the different genera inferred from 16S rRNA sequences. The tree was generated using the Neighbor-Joining method

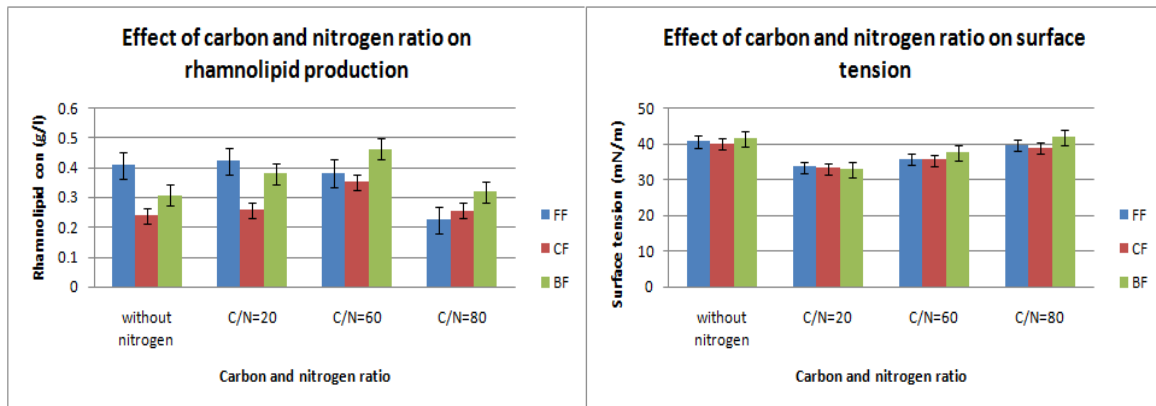**Rhamnolipid production from FF, CF and BF using BH media**

The rhamnolipid obtained from 20 g/l animal fat using BH media as the base media by shake flask method were 0.4205 g/l from Fish Fat (FF), 0.2565 g/l from Chicken Fat (CF) and 0.38 g/l from Beef Fat (BF). The surface tensions of the culture media were 33.4 mN/m from FF, 33.1 mN/m from CF and 32.68 mN/m from BF.
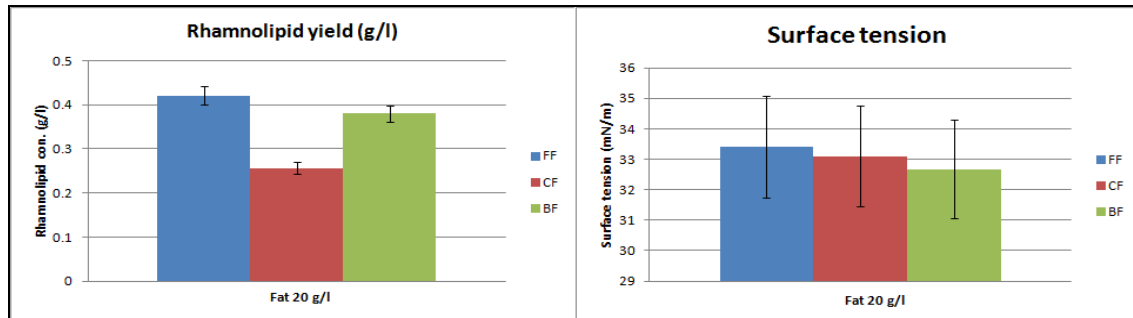
**Fig. 3** rhamnolipid production using BH media

Effect of carbon/nitrogen ratio

The carbon to nitrogen ratio markedly affected the production yield of rhamnolipid and the optimum C/N ratio was 20, which gave a rhamnolipid yield of 0.429 g/l from FF, 0.261 g/l from CF and 0.388 g/l from BF using glucose as carbon source and $NaNO_3$ as nitrogen source.

Fig. 4 Effect of carbon and nitrogen ratio on rhamnolipid production from FF, CF and BF

Effect of glucose

Glucose in BH medium influenced the rhamnolipid production. Rhamnolipid production reached maximum using glucose concentration of 15 g/l which degreased with further increasing concentration of glucose. In this optimum condition obtained rhamnolipid concentrations were 0.843 g/l from FF, 0.8535 g/l from CF and 0.8995 g/l from BF.
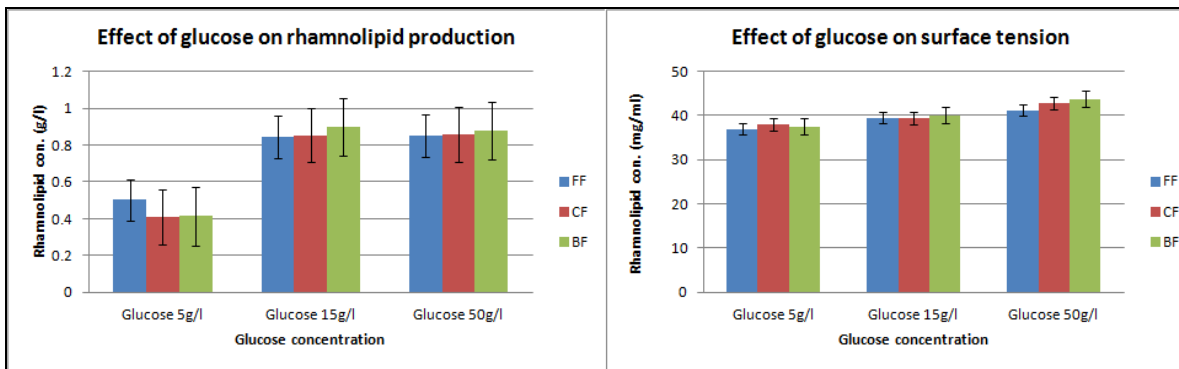
Fig. 5 Effect of glucose on rhamnolipid production and surface tension

Effect of MgSO₄

All the results of study indicate that the lowering in $MgSO_4$ influence the increase in rhamnolipid production and the optimum condition for rhamnolipid production achieved without $MgSO_4$.
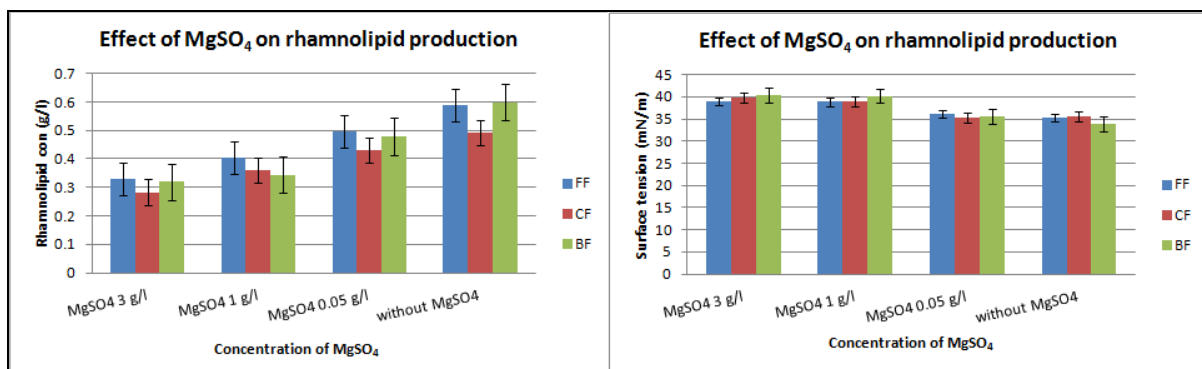
Fig. 6 Effect of MgSO₄ on rhamnolipid production and surface tension

Effect of Tween 80 and rhamnolipid

Tween 80 and rhamnolipid both have negative effect on rhamnolipid production may be due to the toxic nature of Tween 80 and in case of rhamnolipid, it inhibits further production of rhamnolipid.
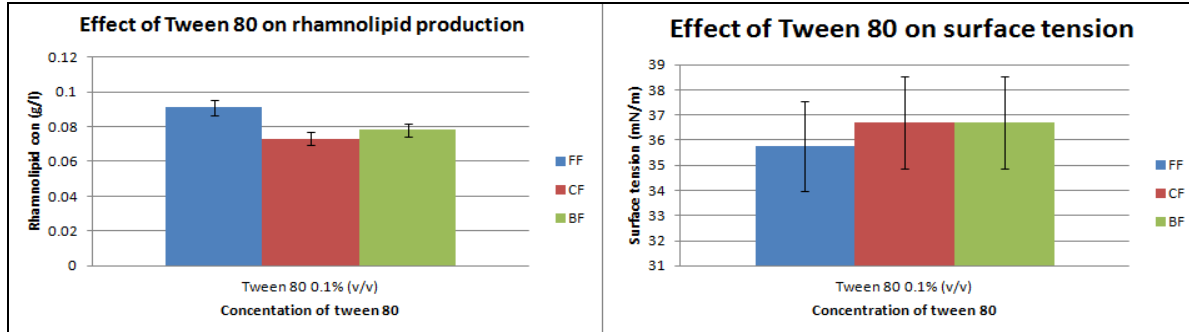

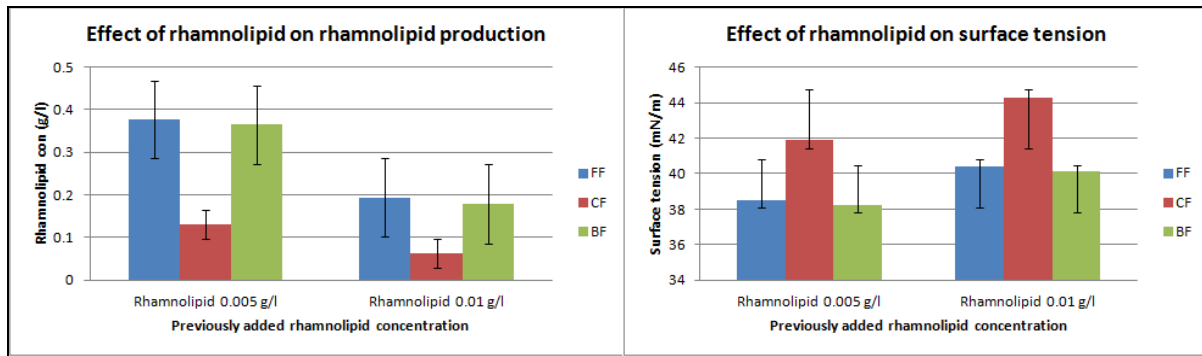**Fig. 7** Effect of tween 80 on rhamnolipid production and surface tension

**Fig. 8** Effect of previously added rhamnolipid on rhamnolipid production

Optimization of process parameter by Factorial design Study:

To produce highest amount of biosurfactant the optimum time required is 72 hour, the optimum pH 7.00, optimum inoculums concentration 1% v/v of bacterial inoculum of optical density 0.5 and optimum shaker speed 125 r.p.m.
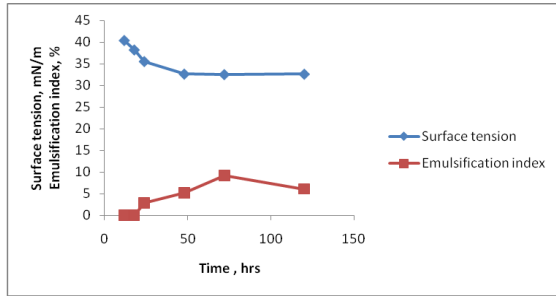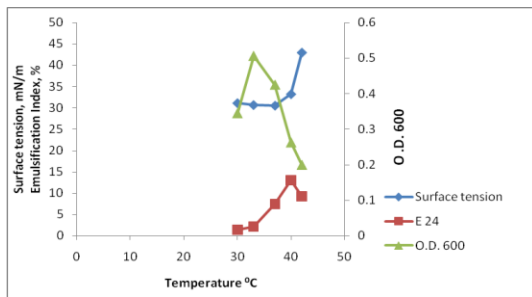
Fig. 9 Optimization of incubation time.

Fish fat = 2 vol%, Temp. = 37^{0}C, Inoculum vol. = 2%,

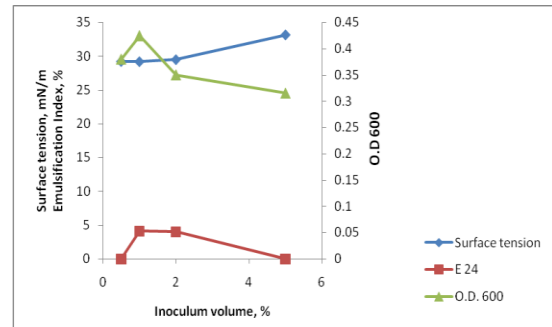Shaking speed = 125 r.p.m., pH = 7.00
r.p.m
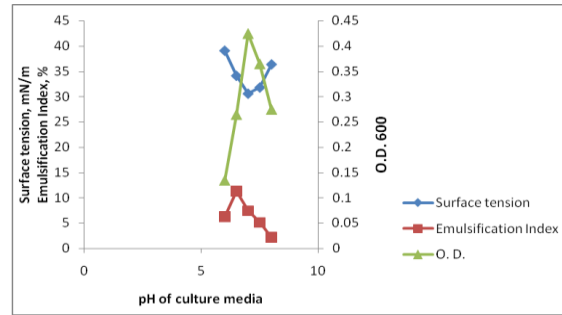
Fig. 10 Optimization of pH of culture broth.

Incubation time = 72 hrs., Fish fat = 2 vol%, Temp.

= 37^{0}C, Inoculum vol. = 2%, Shaking speed = 125

Fig. 11 Optimization of temperature. Incubation

time = 72 hrs., pH = 7.00, Fish fat = 2

Fig. 12 Optimization of inoculum vol. Incubation

time = 72 hrs., pH = 7.00, Temp. = 37^{0}C,

Fish fat = 2 vol%, Shaking speed = 125 r.p.m.
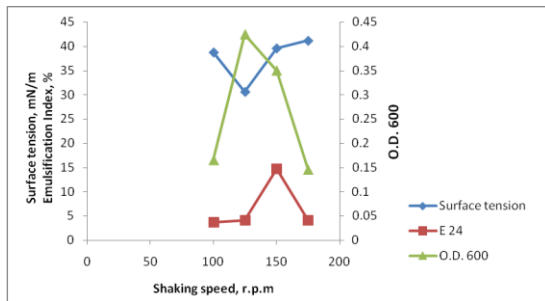
Fig. 13 Optimization of shaker speed. Incubation

time = 72 hrs., pH = 7.00, Temp. = 37^{0}C,

Inoculum vol. = 1%, Fish fat = 2 vol%
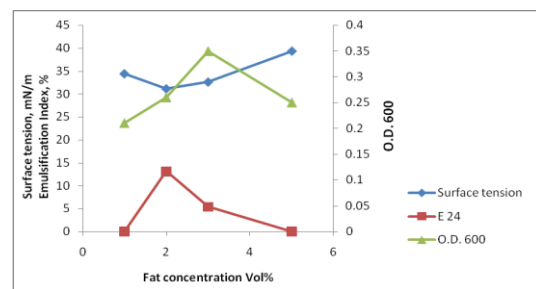
Fig. 14 Optimization of hydrocarbon volume. Incubation

time = 72 hrs., pH = 7.00, Temp. = 37^{0}C,

Inoculum vol. = 1%, Shaking speed = 125 r.p.m.

Response surface methodology:

The process parameters optimization was conducted by a series of experiments changing one variable at a time, keeping the other factors constant to evaluate the optimum condition for highest productivity of the biosurfactant. Six parameters were chosen aiming to obtain highest productivity of the biosurfactant viz. time of incubation, temperature, inoculum volume percentage, pH of the culture media, shaker speed, and hydrocarbon (fish fat) volume percentage. The ranges of the parameters studied are presented in Table – 1. Surface tension, Emulsification index and Optical Density (O.D.) were measured with variation of the parameter under study.

Table I Ranges of the factors chosen for response surface methodology

Factors	Ranges
Time (Hours)	12, 18, 24, 48, 72, 120
Temperature (°C)	30, 33, 37, 40, 42
Inoculum (vol %)	0.5, 2, 5, 10
pH	6, 6.5, 7, 7.5, 8
Shaker speed (r.p.m.)	0, 100, 125, 150, 175
Hydrocarbon (vol %)	1, 2, 3, 5

Response surface methodology using Box-Behnken design was used to evaluate the relationship between a set of independent variables (time, temperature, and pH) and their mutual interaction on surface tension reduction. This statistical optimization involves three important steps which are performed during the statistically designed experiment; these are estimating the coefficient and examining the accuracy of the observed value with respect to the model derived predicted value. Three level factorial designs were used to optimize the physical conditions for biosurfactant production. The design matrix and levels of controlled variables chosen in this study are shown in Table 1. The variables were coded according to the following equation

$$\chi_i = (X_i - X_i^*) / \Delta X_i \quad i = 1, 2, 3, \ldots, k$$

Where χ_i is the coded value of an independent variable, X_i is the actual value of an independent variable, X_i^* is the actual value at the centre point and ΔX_i is the step change value.

Table II. The ranges of culture parameters for biosurfactant production by C2 strain

		Levels		
		-1	**0**	**+1**
Factors	**Time (hr), A**	48	72	96
	Temperature (^{0}C), B	34	37	40
	pH, C	6	7	8

Mathematical relationship between the three variables can be predicted by the following quadratic polynomial equation

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2$$

Where Y is the predicted value for the changes in surface tension and biomass, β_0 is a constant, β_1, β_2, β_3, are linear coefficients, β_{12}, β_{13}, β_{23} are the cross product coefficients, and β_{11}, β_{22}, β_{33} are the quadratic coefficients. An ANOVA table was calculated to estimate the regression analysis of the independent variables individually and the p value less than 0.05 were considered as statistically significant. The software Design of Experiments (DOE++, Trial Version 8.0.7.1, Stat-Ease, Inc, USA) was used to optimize the optimum condition of surface tension reduction in terms of logical inputs to understand the effect of the variables individually and in combination. A total of 15 experiments were necessary to evaluate the regression coefficient. All the treatments were performed in triplicate and the average of the surface tension and optical density (O.D.) of the incubated culture media were used as the responses R_1 and R_2.

Table III. Box-Behnken design matrix with experimental and predicted values of Surface tension and optical density at 600 nm.

Trial	Time (hr)	Temperature (°C)	pH	Surface tension (mN/m)		Optical Density at 600nm	
				Experimental	Predicted	Experimental	Predicted
1	48.00	34.00	7.00	35.62	35.48	0.24	0.23
2	96.00	34.00	7.00	35.10	35.26	0.15	0.17
3	48.00	40.00	7.00	37.28	37.12	0.22	0.20
4	96.00	40.00	7.00	34.31	34.45	0.28	0.29
5	48.00	37.00	6.00	42.72	42.40	0.050	0.033
6	96.00	37.00	6.00	39.15	38.52	0.14	0.093
7	48.00	37.00	8.00	34.98	35.61	0.28	0.33
8	96.00	37.00	8.00	36.28	36.61	0.28	0.30
9	72.00	34.00	6.00	39.05	39.52	0.040	0.068
10	72.00	40.00	6.00	40.73	41.22	0.22	0.26
11	72.00	34.00	8.00	36.94	36.45	0.50	0.46
12	72.00	40.00	8.00	36.05	35.58	0.39	0.36
13	72.00	37.00	7.00	31.65	31.58	0.50	0.49
14	72.00	37.00	7.00	31.30	31.58	0.50	0.49
15	72.00	37.00	7.00	31.78	31.58	0.47	0.49

Table IV: Analysis of variance (ANOVA) for Response Surface Quadratic Model for the surface tension lowering capacity

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	150.36	9	16.71	39.12	0.0004	significant
A-Time	4.14	1	4.14	9.70	0.0264	
B-Temperature	0.34	1	0.34	0.80	0.4108	
C-pH	37.82	1	37.82	88.57	0.0002	
AB	1.50	1	1.50	3.50	0.1202	
AC	5.93	1	5.93	13.88	0.0136	
BC	1.65	1	1.65	3.85	0.1068	
A^2	15.47	1	15.47	36.22	0.0018	
B^2	14.12	1	14.12	33.08	0.0022	
C^2	80.25	1	80.25	187.93	<0.0001	
Residual	2.14	5	0.43			
Lack of Fit	2.01	3	0.67	10.64	0.0871	not significant
Pure Error	0.13	2	0.063			

The Model F-value of 39.12 implies the model is significant.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, C, AC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Table V: Analysis of variance (ANOVA) for Response Surface Quadratic Model for the OD_{600}

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob > F)	
Model	0.34	9	0.037	17.61	0.0028	significant
A-Time	$<0.45\times10^{-4}$	1	0.45×10^{-4}	0.21	0.6643	
B-Temperature	$<0.3403\times10^{-3}$	1	0.3403×10^{-3}	1.61	0.2609	
C-pH	0.12	1	0.12	58.39	0.0006	
AB	$<0.5625\times10^{-3}$	1	$<0.5625\times10^{-3}$	2.65	0.1642	
AC	$<0.2025\times10^{-3}$	1	$<0.2025\times10^{-3}$	0.96	0.3732	
BC	0.022	1	0.022	10.26	0.0239	
A^2	0.12	1	0.12	58.68	0.0006	
B^2	0.027	1	0.027	12.52	0.0166	
C^2	0.051	1	0.051	23.97	0.0045	
Residual	0.011	5	$<0.212\times10^{-3}$			
Lack of Fit	0.010	3	$<0.336\times10^{-3}$	13.01	0.0722	not significant
Pure Error	$<0.5167\times10^{-4}$	2	$<0.2583\times10^{-4}$			

The Model F-value of 17.61 implies the model is significant.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case C, BC, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Table VI: Model fitting values

| Model fitting values		Surface tension	O.D.
1	Coefficient of the variation	1.81	16.29
2	Multiple regression coefficient (R^2)	0.9860	0.9694
3	Adjusted R^2	0.9608	0.9144
4	The signal to noise ratio (adequate precision)	20.279	12.110

The analysis of variance (ANOVA) data shown in table 3 signifies that the F value is 39.12 and the Prob>F valus is 4×10^{-4} so the model is significant. There is only 0.04% chance that the model F value could occur due to noise. The Prob > F value also suggest that the independent variables time and pH, the interaction variables between time vs pH and quadritic components of all three variables i.e. time, temperature and pH have significant effect on the surface tension lowering capability.

The coefficient of variation is the ratio of standard error of estimate to the mean value of observed response. The lower value of coefficient of variation (1.81) shows more dependability upon the model. The multiple regression coefficient (R^2) value 0.9860 indicates a high degree of correlation between observed value and predicted value and here R = 0.9929 states that the model could explain upto 99.29% of the variability of the response. The signal to noise ratio of this model is 20.279 which is greater than 4, indicates that the model is a good fit.

Similarly the ANOVA data of Table 4 exhibited that the F value is 17.61 and the Prob>F value (28×10^{-4}) so the model is significant and there is only 0.28% chance that the model F value could occur due to noise. The Prob>F value also established that the independent variables time and pH, multiple variables time vs pH and the squared variables time, temperature and pH show significant effect on the biomass count. The coefficient of variation 16.29, signified that the model shows dependability. Multiple regression co-efficient (R^2) is 0.9694 and the R value is 0.9846 which demonstrate that the model can explain up to 98.46%. The adequate precision or signal to noise ratio is 12.110 which is greater than 4 that signifies the model is a good fit.

The effects of the interaction of the factors upon biosurfactant production were plotted against any two variables putting another variable at its middle value (i.e. coded value is 0). To obtain the interaction between the factors itself, the response surface graphs were plotted in all the possible direction. The interaction between the three factors *i.e.* time, temperature and pH and their effect on surface tension lowering capability is shown in Figure 15. Similarly Figure 16 illustrated the interactive effect of the factors itself and their impact on the biomass count.
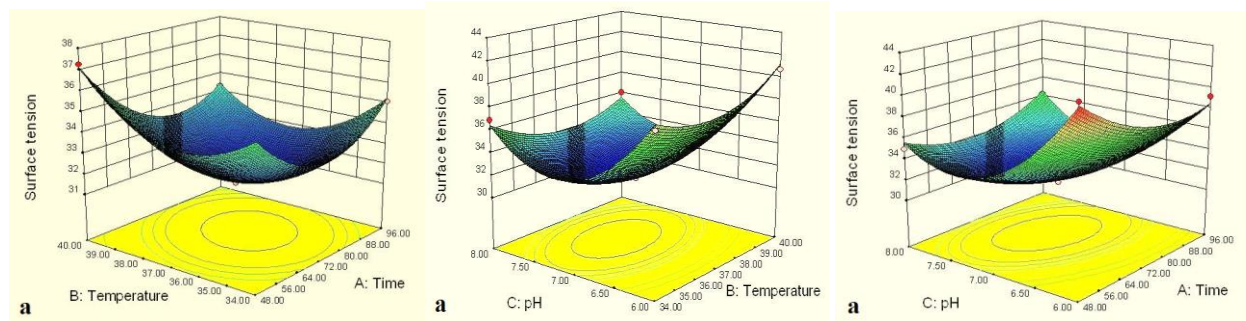
Fig.15 Surface tension lowering capability on 3D graphics for response surface optimization of time vs temperature and temperature vs pH and time vs pH
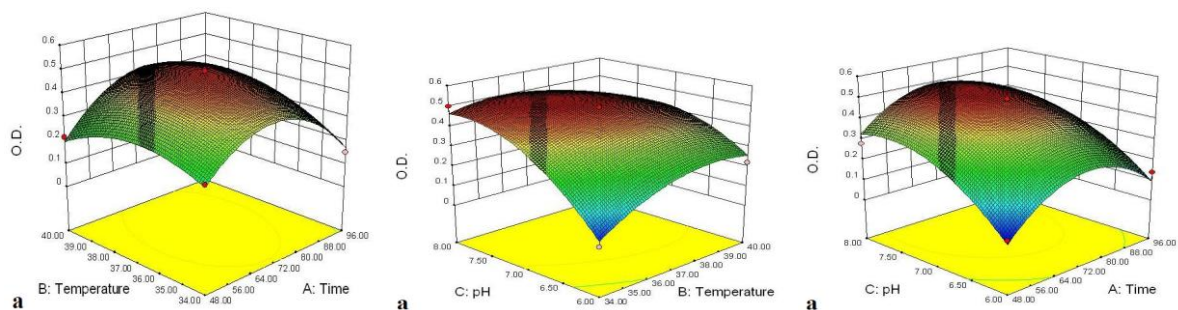
Fig. 16 Surface tension lowering capability on 3D graphics for response surface optimization of time vs temperature and temperature vs pH and time vs pH

Characterization of biosurfactant

The Molisch's test, phenol sulphuric acid test and Orcinol test indicated the presence of sugar moieties in the isolated molecule which may be rhamnose and the isolated biosurfactant could be rhamnolipid. The FT-IR analysis expressed the presence of rhamnose and long hydrocarbon chains. The appeared bands indicated the presence of CH_2 and CH_3 groups, C-O stretching bands rising from ester and carboxylic groups. The C-H and O-H deformation due to carbohydrates were also found.

The result of proton NMR spectroscopy for this purified biosurfactant at 400MHz clearly indicated that it was a glycolipid due to the presence of a long aliphatic chain (CH_2 at 1.24 ppm), CH_2 groups adjacent to carboxylic acid and ester groups (chemical shift at 2.33 ppm and 2.52 ppm) and presence of CH_3 group adjacent to CH_2 in aliphatic chain (chemical shift at 0.86 ppm). The signal at 5.33 signified the presence of OH in rhamnose ring. The signals also indicated the presence of β configuration for the two anomeric hydroxyl groups. All these results indicated the isolated biosurfactant to be rhamnolipid.

The mass spectrum of produced rhamnolipid by the *Pseudomonas aeruginosa* showed most intense signal at m/z 617.86 which confirmed the largest proportion of di-rhamnolipid (Rha-Rha-C_8-C_{10} and Rha-Rha-C_{10}-C_8) of calculated molecular weight m/z 621[9].

Stability study of the produced biosurfactant

The surface tension activity did not show any remarkable effect at a high temperature ($100\,^{0}$C) over different time intervals (0, 5, 10, 20, 40 and 60 min) and even after autoclaving thus it maintained its surface tension lowering property without any significant increase, therefore it was found that the biosurfactant mixture is thermally stable[10]. In addition, the salinity and pH stability analysis were carried out, revealing that, the biosurfactant conserves its ability to decrease surface tension up to 8 % (w/v) NaCl[11]. On the other hand, the surface active property was not affected after changing the pH values over a wide range from 2 to 8.

Pharmacological application of the produced rhamnolipid

The pharmacological activity in terms of excision wound healing effect of the produced rhamnolipid was examined after applying it on rat punch wound model. The wound healing experiment was conducted taking untreated group as negative control (NC), normal paraffin ointment treated group as ointment base (OB), rhamnolipid treated group (RL), and framycetin sulfate treated group (FS) as standard drug treatment.

Excision Wound Model

The rhamnolipid treated group expressed a good wound area reduction of 96 % after 10 days treatment in comparison to untreated control group. A significant healing effect of RL and FS treated group were observed compared to other groups.

The mean wound indices on days 3, 5, 7, and 10 of each of the treatment groups were considered for evaluation. On day 10, the RL and OB treated groups showed mean wound index of 1.33 and 2.33, respectively ($P < 0.05$), compared to untreated control (3). Mean wound indices of FS treated group (1.17) also showed significant reduction ($P < 0.05$) as compared to control.

Tensile Strength of regenerated tissue

A key parameter in healing involved regaining strength of the regenerated tissue and is directly related to collagen content of wounds. Collagen is the main element responsible for tissue integrity and provides a platform for reepithelialization[12, 13]. The mean tensile strength of the RL treated group was significantly greater ($P < 0.05$) than that of the untreated control group and OB group. Higher tensile strength of RL treated wounds indicated better healing than the NC and OB treated wounds. The increase in tensile strength of treated wounds may be due to the increase in collagen concentration. The greater the tensile strength, the better the healing. FS treated group also showed similar like tensile strength as compared to RL treated group.

Estimation of biochemical marker of the healed tissue

DNA, total protein and glucosamine content were indicative markers of cell growth after tissue injury[15]. The DNA content in wounds treated with RL and FS treated groups was significantly higher than in the NC group. However, DNA content of wound tissue of RL treated group was statistically higher than that of OB group. Similar phenomenon was noted in case of total protein content of wound tissues where RL and FS treated groups showed higher protein contents in comparison to the NC group and OB group.

Thus, the higher levels of the biochemical markers (compared to the untreated control) indicated cellular proliferation at the wound site and thereby faster healing of wound[16]. Interestingly, the levels of all of the selected biochemical markers were statistically higher in RL treated group than that of NC and OB treated group indicating faster and quality healing.

Dermal Toxicity Study

No irritation was observed following the 24 h dermal exposure of RL containing ointment over the skin of the test animals.

Results of *Bacillus stratosphericus* strain 41KF2a(T)

Screening of effective biosurfactant producer depending on surface active property

An effective biosurfactant producer, coded as A15 was selected on the basis of surface tension lowering capability from large number of isolated strains. The cell free supernatant showed highest reduction of surface tension from 71.02 mN/m to 32.2 mN/m i.e 54.66% as compared to other isolates.

The biochemical results indicated the selected strain as an aerobic, rod shaped, endo-spore forming gram positive bacteria with irregular shaped colonies having hemolytic activity (Fig. 17). Finally, 16S rRNA gene sequence (1441 bp) analysis of the isolated bacteria shown 97.8 % homology with *Bacillus stratosphericus* strain 41KF2a(T). The NCBI GeneBank accession number of the test bacteria is KU644139 and it should be classified as a strain of *Bacillus sp.* A15.

Fig.17. Haemolytic activity of A15 culture on blood agar media.

Optimization study

The biosurfactant production in terms of surface tension lowering capability and cell growth were strongly influenced by culture conditions like incubation time, temperature, pH and substrate concentration. The noticeable features of most microorganisms are their strong reliance on the culture conditions for production of secondary metabolites and biomass growth. The strain A15 generated maximum biosurfactant at pH 7.00, temperature 33 °C, 10 g/l substrate concentration and 120 hr incubation time.
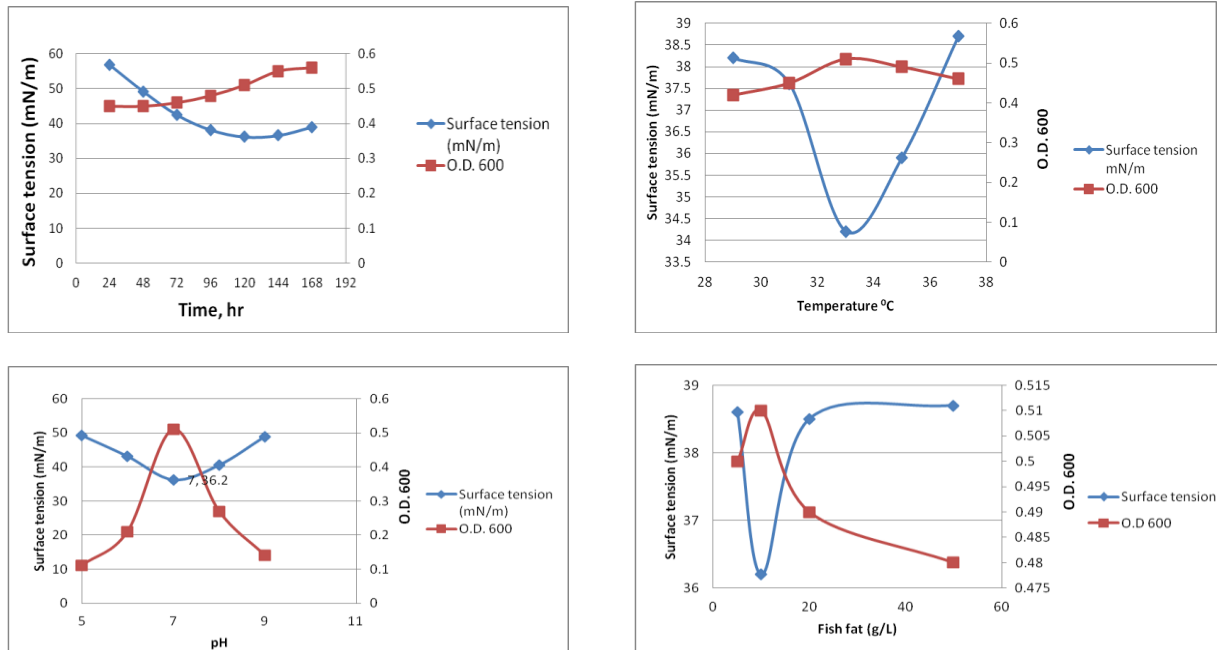
Fig. 18 Optimization of culture conditions

Kinetics of biosurfactant production

The biosurfactant production and surface tension lowering of the cell free supernatant was significantly dependent on the time of incubation. The surface tension dropped slowly during first 72 hr but after which it dropped rapidly and reached its lowest value (32.3 mN/m) at 120 hr of incubation time at exponential phase (Fig. 19). After 48 hr of growth, the lowering of surface tension started which continued till 120 hr of the incubation time along with the similar increase in biomass. Thus it indicated that the biosurfactant production from *Catla catla* fish fat occurred in the exponential growth phase of the *Bacillus sp* and it followed growth associated kinetics (19).
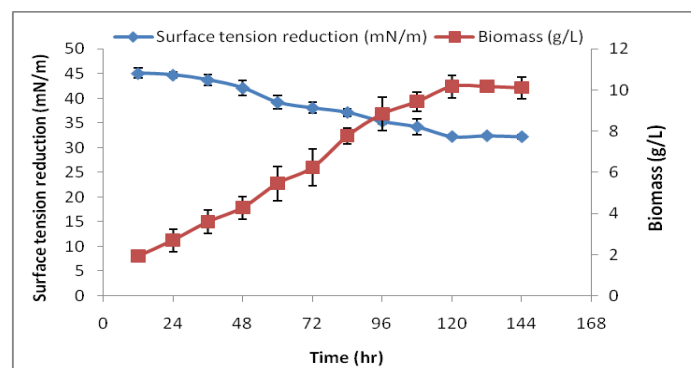
Fig. 19 The bacterial growth and surface tension profiles of the isolated *Bacillus sp*. bushnell hass medium at 37 °C, pH 7 and 100 rpm, shaking speed containing 10 g/l *Catla catla* fish fat. Data are mean±SEM of three individual experiments.

Structural identification of BS15

The IR spectra of BS15 expressed a characteristic of peptide with aliphatic chains[17]. The band at 1542 cm-1 expressed the presence of peptide by showing deformation mode of N-H bond combined with C-N stretching mode[18]. The IR band also indicated the presence of lactone carbonyl absorption due to presence of an ester carbonyl group[19]. All these results established that BS15 was lipopeptide due to containing of aliphatic chain in combination with peptide molecule.

The results of proton NMR spectroscopy of BS15 indicated the presence of amide (-CO-NH) functional group with a long aliphatic chain (CH_2 at δ 1.47 -1.18 ppm) containing α carbon proton (δ 3.52-5.30 ppm) and side chain protons. The NMR spectrum at 5.22 ppm signified the presence of ester carbonyl group of lactone ring in the structure of isolated biosurfactant[20]. The spectrum at δ 3.66 ppm suggested the presence of OCH_3 in the isolated molecule which was supported by previous reports[21] as they expressed the presence of methoxy group in Glu or Asp amino acid part. All the spectra showed similarity of BS15 with *Bacillus sp*. derived surfactin type biosurfactant.

The mass spectrometric analysis of the biosurfactant showed peaks at m/z 794, 695, 589, 486, 373 and 233 that signified the sequential losses of amino acid residues Val/Asp/Val/Leu/Leu/GluOMe present in the isolated biosurfactant produced from *Bacillus stratosphericus*. Though there are few differences in structural elucidation of the isolated lipopeptide type biosurfactant produced by *Bacillus stratosphericus* but it contained so many similarities with the previously reported other types of surfactin[22, 23].

Pharmaceutical application of BS15

Pharmaceutical application in terms of wound healing activity and antibacterial activity of BS15 was examined.

Wound healing activity

The wound healing experiment of BS15 was performed taking untreated group as negative control (NC), normal paraffin ointment treated group as ointment base (OB), BS15 treated group (BS), and framycetin sulfate treated group (FS) as standard drug treatment.

Excision Wound Model

The visual observation revealed the progress of healing of NC, OB, BS and FS groups respectively. The rate of healing was significantly higher in BS and FS groups in comparison to other two groups in terms of wound closure. No significant changes were observed on first 2 days of treatment for all the groups.

From day 3 the wound turned dark red in color which indicated the beginning of healing. The number of new blood vessels and the depth of dermal tissues at the wound site were significantly increased in BS and FS groups from the 5th day of treatment. Also swelling, redness and microbial attack was absent in those groups. Finally, complete wound closure and granulation tissue formation were achieved on 10th day of treatment. Conversely, the NC and OB groups were followed by pus formation, microbial attack and the wound remained open till the end of treatment.

The percentage wound closure of BS15 group after 10 days treatment established a promising result of 97.70%, which was even better than FS group (96.43%) (P<0.05). Whereas at the end of treatment, percentage wound contraction of OB (79.02%) group was near to NC (72.40%) group but much lower than the BS and FS group. So, significant healing effects were observed in BS and FS treated groups compared to other groups.

Histopathology of regenerated tissues

Hematoxylin and eosin stained sections of granulated tissue after 10 days treatment exhibited the tissue regenerating efficacy of the test BS in Fig 20. The presence of acute inflammatory cells and very few blood vessels as shown in NC group (Fig 20a). The dermal region of the hisotological picture of NC group showed a lack of hair follicles, vacuoles and keratinocyte indicated the absence of well developed regenerated tissue. The epidermal layer is not also well developed. The histological picture of the OB treated group (Fig 20b) showed formation of epidermis layer but the hair follicles and sebaceous glands were absent which signified an inadequate reepithelialisation. The histological pictures of BS (Fig 20c) and FS (Fig 20d) treated groups showed similarity in tissue reepithelialisation at the wound site. Both the group showed satisfactory formation of keratinocyte, thick epidermal layer with presence of hair follicles, sebaceous glands, vacuoles and higher number of intact cells in dermis layer. All these characteristics observed in the histopathology established a well developed skin tissue regenerating effect of BS and FS.

(a)　　　　(b)　　　　(c)　　　　(d)
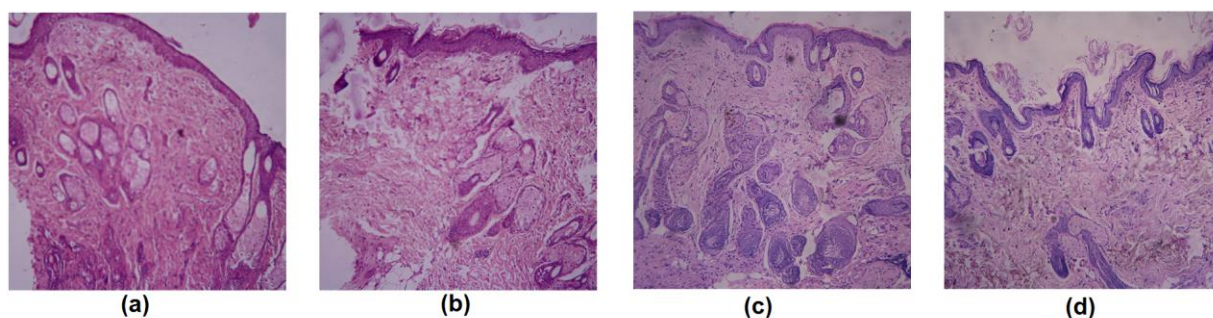
Fig. 20. Histopathology of skin tissue after 10 days (a) Granulation tissue of NC group, (b) OB group showing epidermis formation but lack of hair follicles and sebaceous glands, (c) FS treated group and (d) BS treated group showing well organized granulation tissue and epithelialisation. (magnification at 40x).

Antibacterial activity of rhamnolipid and BS15

Isolated rhamnolipid exhibited an inhibitory effect against a common skin bacterium *Staphylococcus aureus* ATCC6588 which is supported by the studies of other authors' also[24, 25].

The isolated BS15 exhibited an inhibitory effect against *Escherichia coli* ATCC 8739 which is supported by Ramachandran et al[26]. This additional activity of the isolated biosurfactant may help the healing from bacterial infection at the wound site.

References:

1. Banat, I. M., Makkar, R. S. and Cameotra, S. S. (2000) Appl. Microbiol. Biotechnol. 53, 495-508.
2. Haba, E., Espuny, M. J., Busquets, M. and Manresa, A. (2000) J. Appl. Microbiol. 88(3), 379-387.
3. Nitschke, M., Costa, S. G., Haddad, R., Gonçalves, L. A., Eberlin, M. N. and Contiero, J. (2005) Biotechnol. Prog. 21, 1562-1566.
4. Benincasa, M., Abalos, A., Oliveira, I. and Manresa, A. (2004) Anton. Leeuw. Int. J. G. 85, 1-8.
5. Bednarski, W., Adamczak, M. & Tomasik, J. (2004) Bioresourc. Technol. 95, 15-18.
6. Mukherjee, S., Das, P. and Sen, R. (2006) Trends. Biotechnol. 24(11), 509-515.
7. Wright-Pierce. (2006), Resource documents for the discharge of wastewater associated with food preparation establishments, Hadlyme Environmental Engineers, Connecticut, US.
8. Deshpande, M. and Daniels, L. (1995) Bioresourc. Technol. 54, 143-150.
9. Déziel, E., Lépine, F., Dennie, D., Boismenu, D., Mamer, O.A. and Villemur, R. (1999) Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. *BBA Mol Cell Biol L* 1440, 244-252.
10. Amani, H., Muller, M.M., Syldatk, C. and Hausmann, R. (2013) Production of microbial rhamnolipid by *Pseudomonas aeruginosa* MM1011 for ex situ enhanced oil recovery. *Appl Biochem Biotechnol* 170, 1080–1093.
11. Abdel-Mawgoud, A.M., Aboulwafa, M.M. and Hassouna, N.A. (2009) Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. *Appl Biochem Biotechnol* **157**, 329–345.
12. Agarwal PK, Singh A, Gaurav K, Goel S, Khanna HD, Goel RK (2009) Evaluation of wound healing activity of extracts of plantain banana (*Musa sapientum* var. paradisiaca) in rats. Indian J Exp Biol 47:32–40
13. Udupa AL, Kulkarni DR, Udupa SL (1995) Effect of *Tridax procumbens* extracts on wound healing. Int J Pharmacogn 33:37-40. doi:10.3109/13880209509088145
14. Sana S, Bhattacharya M, Datta S, Biswas D (2015) RSM study for the production of rhamnolipid using *Catla catla* fish fat. Int J Curr Microbiol App Sci 4:169-178

15. Bhaskar A, Nithya V (2012) Evaluation of the wound-healing activity of *Hibiscus rosa sinensis* L (Malvaceae) in wistar albino rats. Indian J Pharmacol 44:694–698. doi:10.4103/0253-7613.103252

16. Mohanty C, Das M, Sahoo SK (2012) Sustained wound healing activity of curcumin loaded oleic acid based polymeric Bandage in a rat model. Mol Pharmaceutics 9:2801–2811. doi:10.1021/mp300075u

17. Al-Wahaibi, Yahya, Sanket Joshi, Saif Al-Bahry, Abdulkadir Elshafie, Ali Al-Bemani, and Biji Shibulal. "Biosurfactant production by *Bacillus subtilis* B30 and its application in enhancing oil recovery." Colloids and Surfaces B: Biointerfaces 114 (2014): 324-333.

18. de Faria, A.F., Teodoro-Martinez, D.S., de Oliveira Barbosa, G.N., Vaz, B.G., Silva, Í.S., Garcia, J.S., Tótola, M.R., Eberlin, M.N., Grossman, M., Alves, O.L. and Durrant, L.R., 2011. Production and structural characterization of surfactin (C 14/Leu 7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. Process Biochemistry, 46(10), pp.1951-1957.

19. N. Bordoloi, B. Konwar, Colloids Surf. B 63 (2008) 73.

20. Liu XY, Yang SZ, Mu BZ. Production and characterization of a C-15-surfactin-Omethyl ester by a lipopeptide producing strain *Bacillus subtilis* HSO121. Process Biochem 2009;44:1144–51.

21. Kowall M, Vater J, Kluge B, Stein T, Franke P, Ziessow D. Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB 105. J Colloid Interface Sci 1998;204:1–8.

22. de Faria, A.F., Teodoro-Martinez, D.S., de Oliveira Barbosa, G.N., Vaz, B.G., Silva, Í.S., Garcia, J.S., Tótola, M.R., Eberlin, M.N., Grossman, M., Alves, O.L. and Durrant, L.R., 2011. Production and structural characterization of surfactin (C 14/Leu 7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. Process Biochemistry, 46(10), pp.1951-1957.

23. Liu X-Y, Yang S-Z, Mu B-Z. Isolation and characterization of a C12-lipopeptide produced by *Bacillus subtilis* HSO 121. J Pept Sci 2008;14:864–75.

24. Vatsa P, Sanchez L, Clement C, Baillieul F, Dorey S (2010) Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. Int J Mol Sci 11:5095-5108. doi:10.3390/ijms11125095

25. Haba E, Bouhdid S, Torrego-Solana N, Marqués AM, Espuny MJ, García-Celma MJ, Manresa A (2014) Rhamnolipids as emulsifying agents for essential oil formulations: antimicrobial effect against *Candida albicans* and methicillin-resistant *Staphylococcus aureus*. Int J Pharm 476:134-141. doi:10.1016/j.ijpharm.2014.09.039

26. Ramachandran, R., Chalasani, A.G., Lal, R. and Roy, U., 2014. A broad-spectrum antimicrobial activity of *Bacillus subtilis* RLID 12.1. The Scientific World Journal, 2014.

_____