Indigenous Biosurfactant Producing Strains for Potential Applications in Enhanced Oil Recovery

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by

POULAMI DATTA

(Roll no. 156152004)

Under the supervision of

Dr. Lalit M. Pandey & Dr. Pankaj Tiwari

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Centre for the Environment

Indian Institute of Technology Guwahati

Guwahati 781039, Assam, India

Indian Institute of Technology Guwahati

Centre for the Environment

DECLARATION

I, hereby declare that the content embodied in this thesis entitled "**Indigenous Biosurfactant Producing Strains for Potential Applications in Enhanced Oil Recovery**", is the results of investigations carried out by me at the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of **Dr. Lalit M. Pandey and Dr. Pankaj Tiwari** for the award of the Doctor of Philosophy. The content of this thesis, in full, or in parts, has not been submitted to any other University or Institute for the award of any degree or diploma. I wish to state that to the best of my knowledge and understanding nothing in this report amounts to plagiarism.

In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work described was based on the findings of other investigators.

Poulami Datta

Date: January 2022

Poulami Datta
Centre for the Environment,
Indian Institute of Technology Guwahati,
Guwahati - 781039, Assam, India.

Indian Institute of Technology Guwahati

Centre for the Environment

CERTIFICATE

This is to certify that the thesis entitled "**Indigenous Biosurfactant Producing Strains for Potential Applications in Enhanced Oil Recovery**" submitted by **Ms. Poulami Datta (Roll No.: 156152004)** for the award of the degree of Doctor of Philosophy is an authentic record of results obtained from the research work carried out under our supervision and guidance at the Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, India. The thesis has fulfilled all requirements as per the regulations of the Institute and has reached the standard required for submission. The work documented in this thesis has not been submitted to any other University or Institute for the award of any degree.

Dr. Lalit M. Pandey
Associate Professor
Thesis Supervisor
Department of Biosciences and Bioengineering
Indian Institute of Technology, Guwahati
Guwahati – 781039, Assam, India

Dr. Pankaj Tiwari
Associate Professor
Thesis Supervisor
Department of Chemical Engineering
Indian Institute of Technology, Guwahati
Guwahati – 781039, Assam, India

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Abstract

The isolation, screening and identification of potential indigenous biosurfactant-producing and oil-utilizing bacterial strains were carried out from the formation water as well as soil sample of the Assam oil reservoir. Among the isolated strains from the indigenous sources, *Bacillus subtilis* MG495086 and *Bacillus tequilensis* MK 729017 showed better surface-active properties as they lowered the surface tension (ST) to 30 ± 2 mN/m along with a high emulsification index (EI) of 70 ± 2 %. Their growth kinetics and stability studies were also performed. The produced biosurfactants were chemically identified using NMR, FTIR, LC-MS and HPLC and proved to be lipopeptide, Surfactin with very low critical micelle concentration (CMC) value. Response surface methodology based on the central composite design (RSM-CCD) experiments aimed to optimize the suitable carbon source percentage and the environmental parameters to maximize the biosurfactant (Surfactin) production in terms of surface tension (ST) reduction and biosurfactant concentration which was reduced to 29.85 mN/m and the maximum Surfactin concentration was determined to be 7.46 ± 0.39 g/L, respectively. The potential of the biosurfactants in oil degradation was also analyzed with their subsequent biosurfactant production capacity which was found to be very significant. *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 were able to degrade the hydrophobic substrate (light paraffin oil and glycerol) almost completely within 96 hours during the course of Surfactin production. Moreover, the isolated *Bacillus* strains were established to be suitable for both *ex-situ* and *in-situ* enhanced oil recovery (EOR) applications.

Surfactants can reduce interfacial tension (IFT) between water and residual oil, which improves the oil recovery factor. But one of the challenges of surfactant flooding is the adsorption phenomenon of surfactant onto the solid surfaces, which decreases its effectiveness in lowering the IFT for EOR application and leaves a negative impact on the process economics. Therefore, the adsorption behavior of Surfactin onto the model sand (silica) sample was discussed. HPLC

was used to quantify the amount of surfactant before and after the course of adsorption. The rock mineralogy along with the impact of aqueous media salinity and the experimental temperature was the major variables. EDX, XRD and BET analyses have been carried out to characterize sandstone samples. Both the kinetics and equilibrium adsorption data were obtained from the batch mode studies. Among alternate models, the Freundlich isotherm model, Elovich kinetics and intraparticle diffusion kinetics fit well to represent applied biosurfactant adsorption characteristics. Effect of contact time and temperature on the adsorption process was also analyzed. The thermodynamic feasibility of the adsorption process was studied to verify the spontaneity of the process as well. These findings provided newer insights into real-time biosurfactant adsorption characteristics, which are often ignored in conventional approaches and methodologies.

The suitability of the biosurfactant for core flooding studies and subsequent microbial enhanced oil recovery (MEOR) purpose was evaluated in terms of its oil washing efficiency, stability (thermal and halophilic) studies, interfacial activities and wettability alteration capability. The oil washing efficiency (80 ± 2 %) of the produced Surfactin was quite comparable with the commercial surfactants such as SDS, CTAB and Rhamnolipid. Surfactin improved wetting of hydrophobic rock surface from 90 ± 1 ° to 26 ± 1° and resulted in a lower interfacial tension (IFT) value of 0.32 ± 0.02 mN/m. The stability of the produced biosurfactants was studied over a wide range of temperature, pH, pressure and salinity and they were found to be stable even after their exposure for a longer period. Due to its thermal and colloidal stability, the biosurfactant was further endorsed to be employed for MEOR applications.

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Acronyms and Nomenclatures

ACN	Acetonitrile
API	American Petroleum Institute
ASP	Alkali-surfactant-polymer
ATR	Attenuated total reflection
BATH	Bacterial Adhesion to Hydrocarbons
BET	Brunauer-Emmett-Teller
BH	Bushnell has media
BLAST	Basic local alignment search tool
DNA	Di-oxy-ribonucleic acid
CSH	Cell Surface Hydrophobicity
CEOR	Chemical enhanced oil recovery
CMC	Critical micelle concentration
CTAB	Cetyl trimethyl ammonium bromide
DLS	Dynamic light scattering
EDX	Energy dispersive X-ray spectroscopy
EOR	Enhanced oil recovery
FESEM	Field emission scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
FW	Formation water
GI	Germination index
HPLC	High performance liquid chromatography
IFT	Interfacial tension
IOIP	Initial oil in place
JCPDS	Joint committee on powder diffraction standards

LC - MS	Liquid chromatography – mass spectrometry
LB	Luria bertani broth
MALDI - ToF	Matrix Assisted Laser Desorption Ionization – Time of Flight
MEOR	Microbial enhanced oil recovery
MSM	Mineral salt media
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
OD	Optical density
OOIP	Original oil in place
PPM	Parts per million
PV	Pore volume
RMSE	Root mean square error
RSM - CCD	Response surface methodology – central composite design
RNA	Ribonucleic acid
ROIP	Residual oil in place
SDS	Sodium dodecyl sulfate
ST	Surface tension
TFA	Trifluoroacetic acid
TGA	Thermal gravimetric analysis
WF	Water flooding
XRD	X-ray powder diffraction

Symbols

B	Temkin constant
C_e	Equilibrium surfactant concentration (mg/L)
C_o	Initial surfactant concentration (mg/L)
D	Oil drop width (mm)
EI	Emulsification index
K_1	First order constant (min^{-1})
K_2	Second order constant (g/mg min)
K_F	Freundlich constant
K_i	Intra particle diffusion constant (mg/g min)
K_L	Langmuir constant (L/mg)
K_T	Temkin constant (L/mg)
L	Length of oil drop (mm)
M	Mobility ratio
m/z	Mass to charge ratio
n	Freundlich constant
N_{cap}	Capillary number
O/W	Oil in water
q	Adsorption capacity (mg/g)
q_e	Adsorption at equilibrium time (mg/g)
q_t	Adsorption at time t (mg/g)
R	Universal gas constant (8.314 J/ mol. K)
R^2	Correlation coefficient
R_L	Langmuir separation factor

T	Temperature (K)
t	Time
V	Volume of surfactant solution (ml)
W	Weight of the adsorbent (mg)
W/O	Water in oil
ΔG	Gibbs free energy change (kJ/mol)
ΔH	Enthalpy change (kJ/mol)
ΔS	Entropy change (kJ/mol)
α	Initial adsorption rate (mg/g min)
β	Elovich constant (mg/g min)
δ	Chemical shift (ppm) in NMR
ρ_H	Density of heavier phase (g/cm^3)
ρ_L	Density of lighter phase (g/cm^3)
σ	Interfacial tension (mN/m)
θ	Half of the diffraction angle/Bragg angle

Chapter 1

Introduction

The contemporary global population growth, economic development and industrialization lead to the gradual concomitant reduction of petroleum from the conventional crude oil reserves. As a consequence, it is very important to understand, address and research the issues of increasing energy demand worldwide for fuels and oil derivatives. Although renewable energy sources have recently gained market but crude oil is still the major source of energy (Patel et al., 2015). In this direction, various unconventional methods have been explored to maximize the extraction of unrecovered crude oil (Wang et al., 2019). As a result, the conventional oil reservoir depletion accelerated further research in order to retrieve the remaining two-third of residual oil in place (ROIP) (Banat, 1995; Belyaev et al., 2004; Sen, 2008; Urum et al., 2004; Yernazarova et al., 2016). Consequently, with the increasing exploitation of unconventional crude reserves, the development and improvement of clean-alternative technologies are getting recognition with the fundamental establishment for unconventional hydrocarbon utilization. The microbial communities from various environments have been isolated and explored for their probable utilization in the oil recovery through flooding experiments (Al-Wahaibi et al., 2014).

The conventional enhanced oil recovery (EOR) mechanisms have disadvantages such as very high-temperature and complex procedures for thermal oil recovery. In chemical enhanced oil recovery (CEOR), the chemicals are quite expensive and impose serious environmental hazards. Therefore, scientists proposed some green alternative feasible approaches using biological molecules. Microbial enhanced oil recovery (MEOR) is such a type of EOR that uses microorganisms (indigenous or injected in the reservoir) or their metabolic products such

as biomass (Desai & Banat, 1997), biopolymer (Elshafie et al., 2017; Gudina et al., 2013), biogenic-acids (Fratesi, 2002), enzymes, bio-solvents (Bordoloi & Konwar, 2008; Fratesi, 2002), biogas (carbon dioxide, methane, hydrogen) (Nerurkar et al., 2012) and biosurfactants (Datta et al., 2018; Iglauer et al., 2010) to mobilize the residual oil. One of the major potential representatives for MEOR application is the biosurfactants. They contain hydrophobic and hydrophilic groups in the same biomolecule and accumulate on surfaces/interfaces of a system to reduce the surface tension (ST) and interfacial tension (IFT). Biosurfactants have some advantages over their chemical counterparts such as less toxicity, easy biodegradability, better stability in a wide range of pH, salinity and temperature and could be produced from renewable substrates. In the worldwide oil and gas energy sector, the demand for biosurfactants is gradually increasing because they could solubilize the petroleum constituents and assist in the recuperation of oil confined within reservoir rocks (Falatko & Novak, 1992). Among the wide diversity of biosurfactants, lipopeptides and glycolipids have been considered to be the most researched biosurfactants for MEOR. Several lipopeptides such as Surfactin, Iturin, Fengycin, Lichenycin and Bacillomycin have been produced by *Bacillus* sp., and studied for EOR applications (Geetha et al., 2018; Schaller et al., 2004; Sen, 2008). Surfactin is one of the most surface active biosurfactants, which is a cyclic lipopeptide compound containing seven amino acids in a lactone ring and bonded to a β hydroxyl fatty acid chain of length from C_{12}- C_{16} (IA Haddad et al., 2014).

There are very few literature reports on the microbes from the Assam oil reservoir and their role in *in-situ* and *ex-situ* EOR (Bordoloi & Konwar, 2008). The slower biodegradation of oils, poor yield of biosurfactants and economic feasibility are key challenges to be addressed for the practical implementation of MEOR (Chandankere et al., 2013). In order to address these bottlenecks, the present study is very relevant and significant to be carried out in Assam as well as other places, which are known for their abundance in oil reservoirs. So, the concept is to

isolate the microbes from the local indigenous sources so that they could be easily employed in the reservoirs for advanced research in oil recovery. The aim is also to ensure reasonably good biosurfactant concentration.

This research intends to understand the role of indigenous bacteria from the formation-water and soil samples of the Assam oil reservoir. The biosurfactant production and their potential applications in MEOR is the primary targets. Therefore, the surface properties (surface tension, interfacial tension, emulsification activity and wettability) are investigated thoroughly along with other stability studies and wetting properties.

Also, the current demand for biosurfactants in the worldwide marketplace is continuously increasing. According to Global Market Insights, 370,000 tons of biosurfactants were purchased by the industries in 2015. In 2018, the requirement reached 476,500 tons, which is equivalent to 2.21 billion USD. Further, by 2023 the requirement is expected to reach a worth of 2.69 billion USD (Brumano et al., 2017; Insights, 2016). Advanced investigations are required to develop newer visions and perspectives, novel alternative technologies and strategies to ensure economically profitable development with societal integrity, and ecological sustainability, safeguarding the future and quality of life for the next generations.

1.1.Objectives

With the aim to bridge the knowledge lacuna as discussed in Chapter 2 and to provide critical insight into the processes of biosurfactant induced microbial enhanced oil recovery, the objectives of the work are,

1. Isolation, Screening and Identification of Oil-Utilizing and Biosurfactant-Producing Microbes from Indigenous Sources of the Assam Oil Reservoir Field
2. Characterization and Optimization of the Produced Biosurfactants
3. Adsorption Behaviour of Biosurfactants onto Model Silica (sand) Surfaces
4. Suitability Assessment of the Potential Biosurfactants for Promising MEOR Applications

1.2.Organization of the Thesis

The thesis has been arranged into seven chapters based on the above-mentioned four objectives. A chapter-wise thesis outline is briefed as follows:

Chapter 2

This chapter is dedicated to the comprehensive review of existing works of literature on indigenous biosurfactant synthesizing microbes for their potential applications in enhanced oil recovery (EOR). The importance of EOR and the role of biosurfactant-producing strains along with their screening criteria and optimization approaches as well as suitability for oil retrieval have been discussed.

Chapter 3

This chapter describes the isolation of microbes from indigenous sources of Assam oil reservoir, their screening based on the oil utilization and biosurfactant producing competence followed by their identification. Bacterial growth patterns, suitable substrates and favorable growth conditions are elaborately discussed as well. The surface properties of these biosurfactant-producing strains are also investigated to analyze their promising potential for EOR applications.

Chapter 4

The biosurfactant-producing microorganisms along with their synthesized microbial surfactants are identified using various analytical techniques. Biosurfactant optimizations are conducted by statistical techniques considering the substrate concentration and environmental parameters; setting surface tension reduction as well as biosurfactant concentration as the response. The biosurfactant production along with hydrocarbon degradation is also estimated to establish their significance in bioremediation and waste/economic substrate to value-added product (biosurfactant) formation.

Chapter 5

This chapter discusses the adsorption phenomena of Surfactin onto model sand surfaces employing various adsorption equilibrium isotherms and adsorption kinetic models. The adsorption behavior is also monitored at different temperatures and contact time in order to imitate diverse reservoir conditions so as to understand the nature of the real-time adsorption process.

Chapter 6

Chapter 6 evaluates and endorses the produced biosurfactants for EOR applications depending on their suitability in terms of stability studies, oil washing, interfacial and other wetting properties. Their potential for *in-situ* and *ex-situ* MEOR is scrutinized by investigating their endurance at the higher thermal, barophilic and halophilic conditions as well as exploring oil washing efficiency, respectively. The kinetics of oil washing proficiency of the produced biosurfactants were critically examined and also compared with other chemical surfactants along with procured biosurfactant (Rhamnolipid) to signify their potential.

Chapter 7

Chapter 7 summarizes the overall conclusions of the present research work. The possible directions to extend the research work in the future are also suggested.

Chapter 2

Literature Review

This chapter reviews existing literature on indigenous biosurfactant-producing strains for their potential applications in enhanced oil recovery (EOR). The significance of EOR and the role of biosurfactant synthesizing microbes along with their screening parameters and optimization techniques as well as suitability for oil retrieval are discussed.

2.1.Elucidation and Significance of Enhanced Oil Recovery

Oil has been the most potential energy source so far and it will continue to contribute significantly to fulfill the future energy requirements as well. To progress the civic establishments, the energy aspects need to be addressed and researched (Pal et al., 2018b). The current scenario of the oil-based economy market is very uncertain (Elakkiya et al., 2020). Therefore, it is very essential to increase the recent production scale that can be obtained either by exploring new oil fields or by enhancing the retrieval from the existing oil reservoir fields. Petroleum reservoirs experience mainly three sequential steps of oil production viz: primary, secondary and tertiary recovery methods. Once the oil well completion is achieved the well is subjected to production. The primary method utilizes natural pressure to extract around 10 % of the initial oil present (IOOP) in the reservoir. In the next stage, oil production is improved by injecting water or gas which can further extract 20 – 30 % more oil. Altogether using primary and secondary techniques, only one-third of the oil (OOIP) can be retrieved. the significant amount of oil left within the reservoir after primary and secondary processes is termed as residual oil saturation (S_{or}) which is the target for various enhanced oil recovery (EOR) schemes. The schemes of various oil recovery methods especially EOR are shown in Figure 2.1.

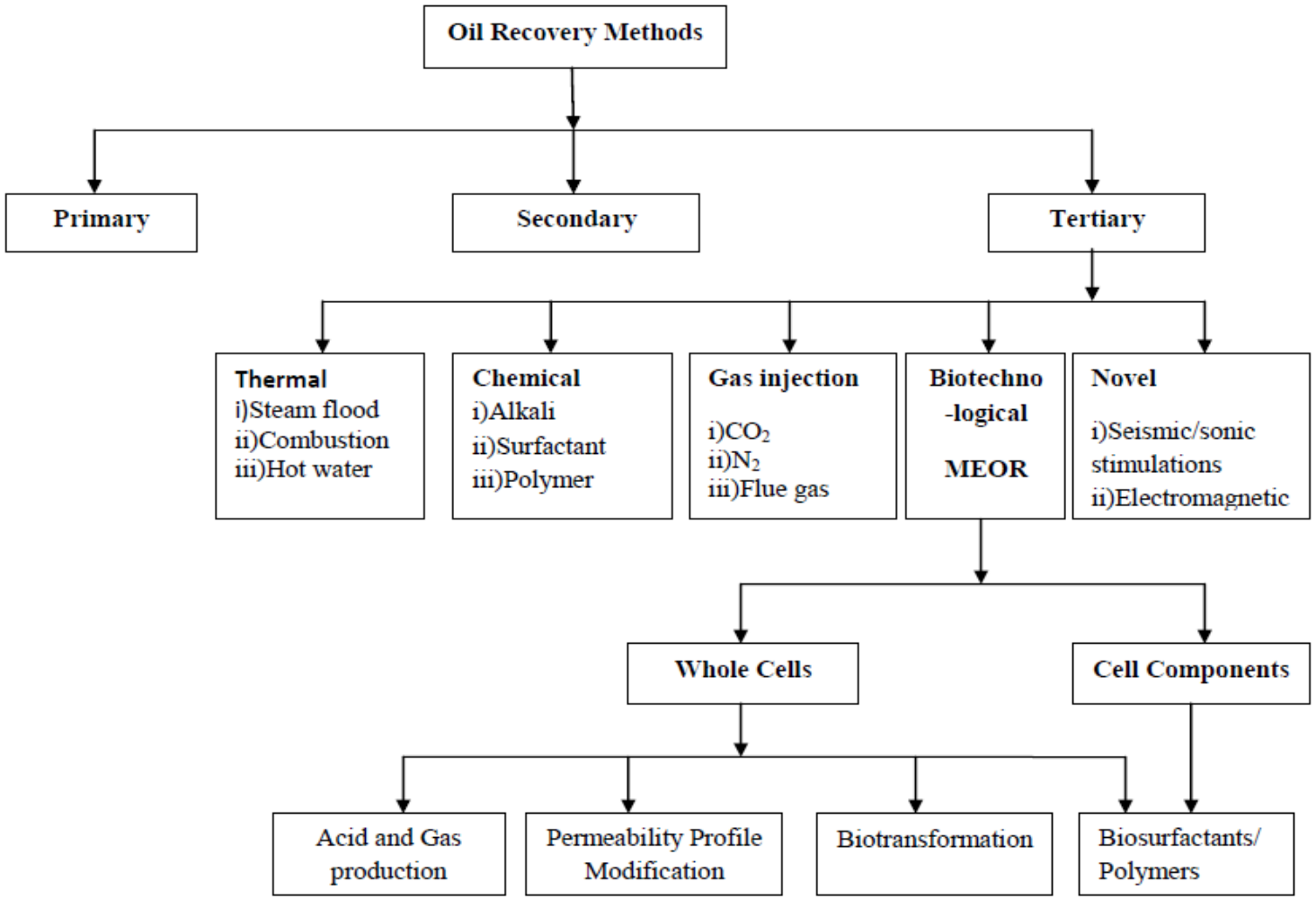


Figure 2.1. Schematic diagram of different types of Tertiary/Enhanced Oil Recovery. Adapted from Ref. (Sen, 2008) and (Bachmann et al., 2014) with permission from Elsevier.

2.1.1. Insights and Limitations of Thermal, Chemical and Gas Enhanced Oil Recovery

Tertiary recovery is an oil recovery methodology that is also recognized as enhanced oil recovery (EOR) consists of various technologies that mainly modify the mobility of fluids inside the oil reserve (Safdel et al., 2017). EOR may be primarily classified into thermal, miscible gas injection and chemical enhanced oil recovery (CEOR). CEOR includes primarily alkaline-surfactant and polymer flooding individually or their binary or ternary combinations are known as alkali-surfactant, surfactant-polymer or alkali-polymer flooding and alkali-surfactant-polymer (ASP) flooding (Das et al., 2017). However, employing these chemical agents lead to various environmental issues. Almost 60 % of used chemical surfactants go into the aquatic surroundings and cause enormous destruction to the flora and fauna of the marine environment. These non-biodegradable surfactants distress the environmental circumstances and create health hazards like dermatitis, respiratory problems, eye annoyance etc (Tmáková et al., 2016). Moreover, these conventional EOR technologies have other drawbacks as well such as high working temperature, a huge expense, not eco-friendly along with their complex operational procedures (Kamal et al., 2017; Thomas, 2008). Due to strict environmental curtailments, it is prime time to increase the exposure of green alternative agents in petroleum upstream and downstream industries (Geys et al., 2014; Kiran et al., 2010). Hence the researchers initiated to investigate the eco-friendly surfactants (natural surfactants and microbial surfactants) which could be obtained from natural sources like plants, microbes and fungi (Sharma & Melkania, 2017). Therefore some green substitutions such as organic compounds have been proposed and examined to access their feasibility for oil recovery applications (Tackie-Otoo et al., 2020).

2.2.Concept and Advantages of Microbial Enhanced Oil Recovery

Microbial enhanced oil recovery (MEOR) is a type of tertiary or enhanced oil recovery (EOR) improvement (Singh et al., 2014). The MEOR strategy uses the microorganisms or their produced metabolites such as biomass (Halim, 2015; Zheng et al., 2012), biopolymer (Couto et al., 2019; El-Hoshoudy & Desouky, 2018; Gao et al., 2018; Hong et al., 2019b; Jia et al., 2018; Tianyuan et al., 2019; Wang et al., 2018a; Zhao et al., 2018a), biogenic acids (Rathi et al., 2018), enzymes, bio-solvents (Wu et al., 2006), bio-gas (Anderson et al., 2017; Ansah et al., 2018; Ma et al., 2018; Rathi et al., 2018; Shabani & Vilcáez, 2017; Sugai et al., 2010; Vilcáez et al., 2018; Xu et al., 2019) and biosurfactants (Datta et al., 2018; Datta et al., 2020) to mobilize residual oil (Harner et al., 2011; Sen, 2008). Each of these metabolites has different roles in the process of EOR which are summarized in Table 2.1. In some of the cases, two or even more types of biometabolites have been combined in order to increase the oil retrieval (Dhanarajan et al., 2017; Pedraza- de la Cuesta et al., 2018; Qi et al., 2018; Souza et al., 2018b). The biological metabolites have also been combined with the chemical agents in order to maximize the oil recovery [1-3]. Even in some works of literature, the metabolites have been combined with nanoparticles (Al_2O_3, TiO_2, and SiO_2) to formulate nanofluids, emphasizing the synergistic effect, as a potential way to contribute to the energy sector by conducting EOR process [4, 5].

There are mainly two general strategies used in MEOR: stimulating indigenous microorganisms by supplementing with suitable nutrients or seeding reservoirs with selected microbes (plus amendments) to establish a new microflora with the desired functionalities. Extremophiles (thermophiles, barophiles and halophiles) generally fall into the biostimulation category while non-extremophiles are usually based on bioaugmentation (Dourado et al., 2015; Jones, 2006).

This biological approach of EOR i.e., MEOR is a substitutional oil exploration strategy that has a remarkable contribution for repossessing upto 50 % of the remaining oil. Additionally, this technique is comparatively easy to operate, reasonably less energy consuming, moderately cost-effective and environment-friendly approach which does not require any major changes in the field facilities and infrastructure to carry out this process (Lazar et al., 2007; She et al., 2019; Shibulal et al., 2014).

2.3.Mechanistic Insight and Benefits of Biosurfactant Assisted MEOR

Microbial enhanced oil recovery (MEOR) is an organic green alternative feasible approach using biological molecules for crude oil retrieval from the reservoir after the conventional oil recovery. Among the other microbial metabolites, one of the major potential bi-products for MEOR application is biosurfactants. Biosurfactants are a class of amphiphilic or amphipathic biomolecules containing both hydrophobic or non-polar and hydrophilic or polar moieties in the same biomolecule. Hence, they possess the capability of accumulating on surfaces/interfaces of a system and notably alter the free energy of these surfaces/interfaces (OSMAN et al., 2019; Putra & Hakiki, 2019; Rosen, 1989; Wood, 2019; Zhang et al., 2020a). Biosurfactants have specific multi-functionality and application versatility properties which assist them to contribute to oil recovery purposes. In the oil and gas energy sector, biosurfactants are utilized as a suitable candidate in the recuperation of oil confined within reservoir rocks. In comparison to the synthetic chemical surfactant counterpart, biosurfactants are secondary metabolites, produced by the metabolic activities of living organisms. Biosurfactants possess certain properties such as low eco-toxicity, higher biodegradability, biocompatibility and more eco-friendly. Biosurfactants require a milder production environment, they have a diverse structure with properties, possess greater stability in an extensive range of pH, salinity, and temperature, and have production prospects using economical renewable raw materials hence cost-effectiveness. These advantages allow their

utilization and possible replacement of chemically synthesized surfactants in various industrial operations (Fenibo et al., 2019; Sáenz-Marta et al., 2015). Introducing crude biosurfactants into the reservoir or reservoir-simulated systems, alongside surfactant-producing microorganisms improve the recovery performance efficiency because biosurfactants are well-tolerant to a wide range of physicochemical and environmental changes, such as high salinity (≤20 %), pH (2 – 12), and temperature (30 - 100 °C) (Jahan et al., 2020; Udoh & Vinogradov, 2019).

In the crude oil biodegradation approach, the complex constituents are transformed into simpler ones which change the traits of the crude oil, the viscosity is reduced and as a result, fluidity and recovery of crude oil are improved. In addition to that biosurfactants can be used in oil industries even in the crude form, they do not require a very high level of purity unlike food and health applications (Aitken et al., 2004). Biosurfactants can mobilize and retrieve the crude oil via mainly five different mechanisms; lowering oil viscosity, reducing oil-water and oil-brine IFT, altering wettability, emulsifying crude oil in the pH range of 5 to 6.5 and modifying porosity and permeability of the rock surfaces (Gao, 2018; Gao & Zekri, 2011; Karlapudi et al., 2018; Nikolova & Gutierrez, 2020; Saxena et al., 2017; Varjani & Upasani, 2017; Wood, 2019). Moreover, biosurfactants are also quite compatible with the reservoir brine and the adsorption rate of biosurfactants on the reservoir formation rock is very low which makes the microbial surfactants ideal candidates to be employed for the EOR (Pal et al., 2018a). The mode of action of biosurfactants to extract the entrapped oil from the reservoir rock pores by adapting various strategies has been illustrated in Figure 2.2.

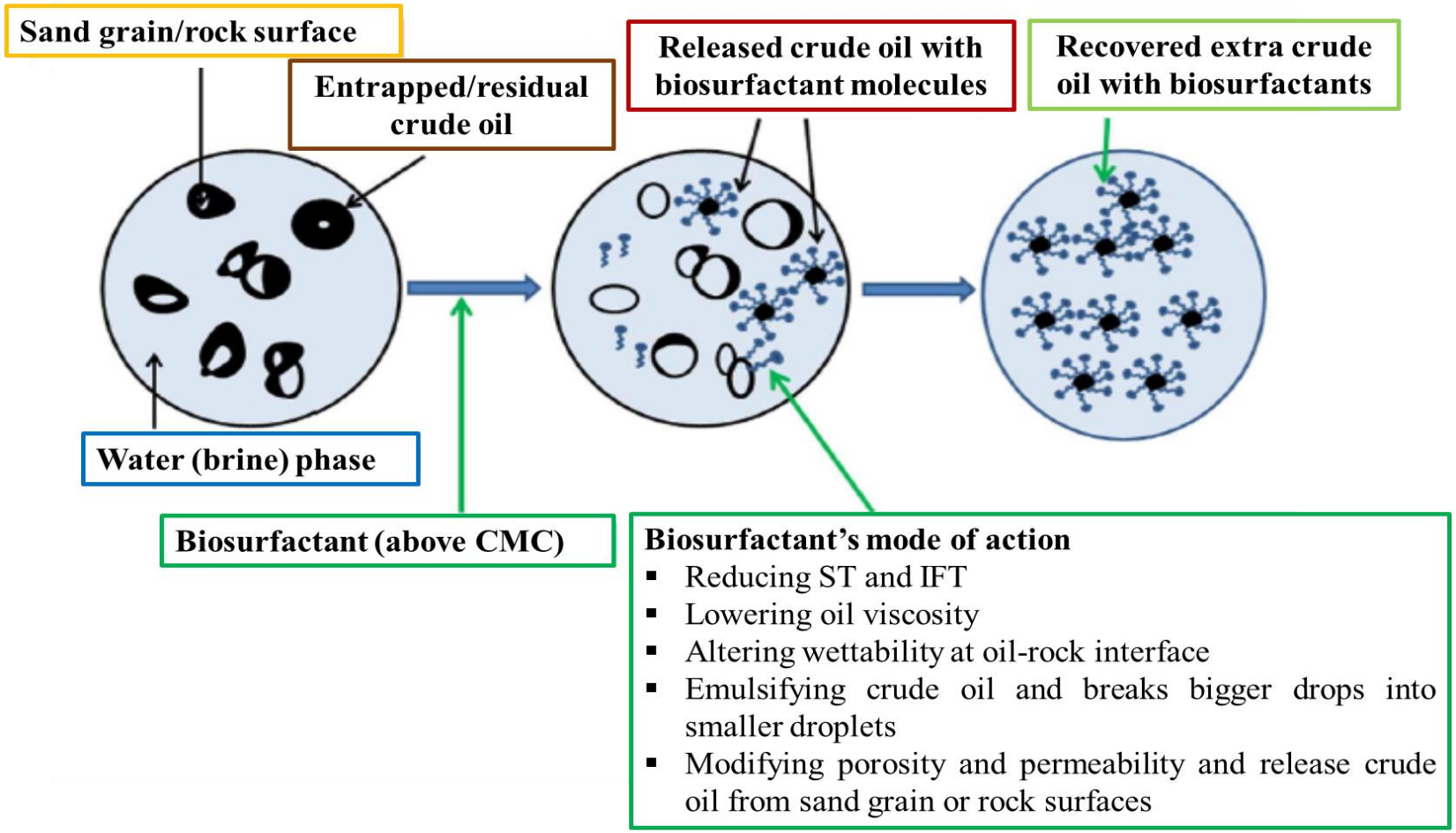


Figure 2.2. Mechanisms of biosurfactants to be suitable for MEOR. Adapted from Ref. (Geetha et al., 2018) with permission from Elsevier.

Table 2.1. Microbial metabolites production from different microorganisms and their roles in MEOR

Metabolite type	Microorganisms	Mechanisms involved in oil recovery	References
Biopolymers (xanthan, scleroglucan, polysaccharides)	*Bacillus polymyxa*, *Brevibacterium viscogenes*, *Leuconostoc mesenteroides*, *Xanthomonas campestris*, *Enterobacter* sp.	• Control the injectivity profile • Viscosity modification leads to mobility ratio alteration	(Desai & Banat, 1997; Gudina et al., 2013; Jack & DiBlasio, 1985; Sen, 2008)
Bio-acids (acetic, propionic, and butyric acids)	*Clostridium* sp., *Enterobacter aerogenes*	• Dissolve some of the minerals present in the formation rock. Rock dissolution increases the porosity, permeability of the reservoir and enhances oil mobility	(Fratesi, 2002; Kalish et al., 1964)
Bio-solvents (acetone, aldehydes, low-molecular-weight alcohols, ketones)	*Clostridium acetobutylicum*, *Clostridium pasteurianum*, *Zymomonas mobilis*	• Control the pressure within the reservoir by lowering the oil viscosity and direct the oil to flow in the proper direction • Emulsification and increase permeability	(Bordoloi & Konwar, 2008; Fratesi, 2002; Kalish et al., 1964)
Microbial biomass (cell debris)	*Bacillus licheniformis*, *Leuconostoc mesenteroides*, *Xanthomonas campestris*	• Lead to change in the wettability of the oil rock and play a key role in selective plugging • Contributes to oil degradation by reducing the viscosity	(Desai & Banat, 1997; Gudina et al., 2013; Sen, 2008)
Bio-gasses (CH_4, CO_2)	*Clostridium* sp., *Enterobacter aerogenes*, *Methanobacterium* sp.	• Gasses are produced by some kind of bacteria which causes pressurization within the reservoir and helps in EOR	
Biosurfactants (rhamnolipid, surfactin)	*Acinetobacter calcoaceticus*, *Arthrobacter paraffineus*, *Bacillus* sp., *Clostridium* sp., *Pseudomonas* sp.	• Interact with the crude oil by emulsifying the oil, decreasing the oil viscosity and increasing the oil flow, which results in the reduction in the interfacial tension (IFT) of the oil-water interface • Reduce the surface tension IFT significantly by aggregating at the interfaces between fluids of different polarities and mobilize the entrapped oil which enhances the ultimate oil recovery	(Banat, 1995; Cameotra & Makkar, 2004; Hosseininoosheri et al., 2016; Iglauer et al., 2010)

2.4.Classifications of Biosurfactant Mediated Microbial Enhanced Oil Recovery

Microbial community variation of the suitable production wells is very complex as well as diverse at the same time. The extensive collection of microbes deployed in MEOR can be classified broadly into two distinct types (Youssef et al., 2009). The first one is autochthonous or indigenous microorganisms already existing in oil reservoirs, and the second one is allochthonous or exogenous microorganisms which are developed purposely by injecting into reservoirs. Exogenous microbes are screened by employing reservoir-like conditions and followed by their injection into the reservoir to increase oil production by its propagation and metabolites (Cheng et al., 2006; She et al., 2019).

MEOR can be implemented mainly in two ways: either by injecting the externally (laboratory or industry) prepared metabolites into the reservoir (*ex-situ*) or by introducing suitable nutrients to stimulate the inherent microbes or stimulating them in some other way so that the native biosurfactant producing microorganism can function properly inside the oil reservoir (*in-situ*) (Bachmann et al., 2014; De Almeida et al., 2016; Jahan et al., 2020; Nikolova & Gutierrez, 2020; Niu et al., 2020; Safdel et al., 2017; Singh et al., 2007). However, both approaches have their characteristics with pros and cons. *In-situ* process expenses are less than the *ex-situ* one but its effect is not significant enough because of the harsh internal environmental conditions (high salinity and temperature) of the reservoir which is still one of the implications of this process. The *ex-situ* process has a short operating cycle and has better reservoir condition adaptability as well as a higher success rate (Banat et al., 2010; Geetha et al., 2018; Patel et al., 2015; Youssef et al., 2009; Zhang et al., 2020a). However, the production and purification costs are added in the *ex-situ* process which increases the overall cost involved.

The MEOR can further be categorized into two kinds of advanced types, those are genetically engineered MEOR (GMEOR) and enzyme-enhanced oil recovery (EEOR) which have also

been very significant during the consideration of upgradation of the MEOR process. GMEOR is primarily based on genetic engineering involving a series of technologies to manipulate microbe genes and other properties using biotechnology. This technique is used to resolve the constraints associated with the strains by integrating the genetic engineering strategies such as recombinant engineering, protoplast fusion and mutagenesis which incorporate excellent features of various strains to make a preferable substitute in order to overcome the survival issue inside the harsh reservoir conditions (Safdel et al., 2017). The advancements in genetic engineering tools and techniques offer the benefit of manipulating, engineering and producing novel strains that can withstand extreme environmental conditions and at the same time produce a considerable amount of metabolites by utilizing economic substrates (Patel et al., 2015).

2.4.1. *In-situ* MEOR

Oil reservoirs are one of the chief modules of the extensive biosphere, where intrinsic microbial inhabitants have survived over a prolonged period (Gao et al., 2018; Li et al., 2017). *In-situ* MEOR is carried out through the activation of the endogenous reservoir microflora, by incorporating a stimulator to reduce oil viscosity and improved mobility of crude oil for EOR [24]. In *in-situ* MEOR, the injection of bacteria (indigenous or specifically screened) or nutrients into the reservoir is followed by a shut-in period for few months. During this period, microbes synthesize several biometabolites which play an imperative task to extract the crude oil [1, 25, 26].

The implementation of the *in-situ* MEOR procedure is dependent on the choice of the reservoir type, the appropriate screening of prospective microbial community, the feasible functionality of the microbes in the internal reservoir environment, the amount of metabolites produced and the final consequences on mobilizing recovered oil and other cost-effective aspects (Jang et al.,

1983; Zhang et al., 2020b). Worldwide oil reservoirs are full of diversity in terms of temperature, salinity, traits of crude oil as well as the oil-water proportion of the reservoir. Because of the reservoir heterogeneity, the occurrence of microbes variation is obvious, when compared to the native population to the individual oil wells (Rabiei et al., 2013; Rathi et al., 2018).

The reservoir inhabitant microbes are usually extremophiles. Extremophiles can be further classified according to the environment in which they grow and survive; such as thermophiles and hyperthermophiles (microbes growing at high or very high temperatures, respectively), psychrophiles (microbes that grow best at comparatively lower temperatures), acidophiles and alkaliphiles (microbes optimally adapted to acidic or basic pH values, respectively), barophiles (microbes which grow best under pressure), and halophiles (microbes that require NaCl for growth). One of the major proficient approaches in MEOR is the biosurfactant production by intrinsic microorganisms which can survive in the existing extreme environment of oil reservoirs, possessing high thermal tolerance, salinity and depleted oxygen level, which are termed as allochtonous (Farias et al., 2018). The bacterial strains which could thrive in the high concentration of carbon dioxide are termed capnophiles (Rampelotto, 2013). Both gram-positive and Gram-negative thermophiles had been reported from thermal and nonthermal environments which can utilize hydrocarbon as their sole source of carbon and energy (PHILLIPS JR & PERRY, 1976). The reservoir microflora which could only utilize hydrocarbons as their substrate is termed as hydrocarbonclastic (Handaruni et al., 2020) such as *Acinetobacter*, *Bacillus*, *Escherichia coli*, *Micrococcus luteus*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Streptomyces*, *Vibrio*, *Xanthomonas maltophilia* (Mariano et al., 2007). Another major operational reservoir extremophilic taxonomic units included acetoclastic strains (converts acetic acid to methane) and hydrogenotrophic methanogens (utilize molecular hydrogen as their energy source) such as *Methanosaetaceae*, *Methanobacterium* and

Methanoculleus as well as thermophilic, thermotolerant, and/or spore-forming bacteria belonging to the families *Clostridiaceae* and *Thermotogaceae* (Kim et al., 2018).

There are primarily three phases for this *in-situ* study to transfer the technology from "lab scale to field scale" as described in Figure 2.3. In the initial phase, laboratory experiments are carried out with the indigenous or exogenous strains to analyze their ability for biosurfactant production in the reservoir condition which includes microbial isolation, screening and identification using modern molecular biology techniques. Then the suitable proportions of various nutrients are optimized under the environmental conditions of the oilfield of interest. In the second phase, bacterial growth kinetics, biosurfactant production kinetics, concerned oilfield mineralogy and previous flooding history, rheology of fluids is monitored. Thereafter, either cheaper nutrients or exogenous microbes are injected into the oil wells to produce biosurfactants internally. After the shut-in phase, oil wells are flooded with formation brine, then the propagation of indigenous or injected bacteria is tracked. In the final phase, depending on the pilot-scale studies, it is decided whether to proceed with the field applications or not (Geetha et al., 2018). The recent *in-situ* MEOR case studies have been elaborately tabulated with the majorly contributing microorganisms, their isolation and surface properties, produced biosurfactant yield along with the noteworthy oil recovery findings in Table 2.2.

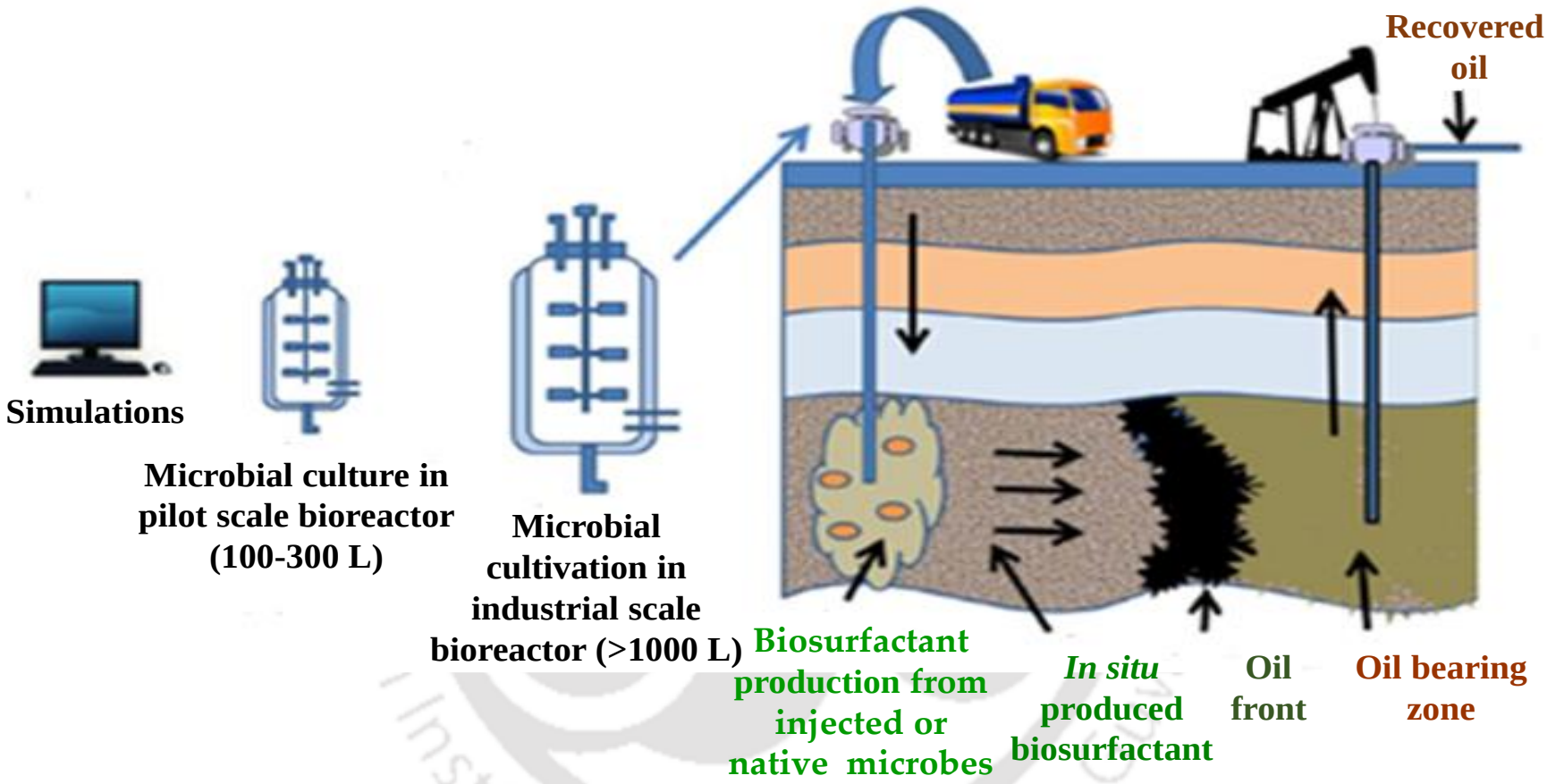


Figure 2.3. Illustration of sequential stages of biosurfactant mediated *in-situ* MEOR. Adapted from Ref. (Geetha et al., 2018) with permission from Elsevier.

Table 2.2. *In-situ* MEOR trials by biosurfactant-producing microorganisms isolated from various sites

Microbes	Sample and Isolation site	Employed Substrate	Metabolite yield	Surface properties	Additional outcomes	Reference
Alcaligenes faecalis	Formation water of carbonate reservoir, southwest Iran	Crude oil (ρ = 883 kg/m3, μ = 35 cp at 28 °C)	Biosurfactant	Reduce IFT to 8.4 mN/m, alter the wettability of reservoir rock from 156° to 86° and EI of 67 %	Total oil recovery of 49.4 % and 39.5 % in shut-in and quick flooding	(Najafi-Marghmaleki et al., 2018)
Pseudomonas aeruginosa DQ3	Daqing oil reservoirs, China	46.5 g/L glycerol, 4.5 g/L of $NaNO_3$	Rhamnolipid (228 mg/L)	Decreased ST to 33.8 mN/m and showed EI of 58 %	An additional 5.22 % of the oil was displaced	(Zhao et al., 2018b)
Bacillus subtilis	Production wells of Xinjiang Oil Field, China	Glycerol, glucose, molasses, corn steep powder, and vegetable oil	Lipopeptide (500 mg/L)	ST was reduced to 30 mN/m	Oil displacement efficiency of 16.71 % in core flooding	(Gao et al., 2016)
B. atrophaeus 5-2a, *B. aryabhattai* 6-2a, *B. amyloliquefaciens* 6-2c	Crude oil and soil samples from Ansai oilfield, Shaanxi Province, Northwest China	Maltose, glycerol and urea	Lipopeptide (1.01 g/L)	Reduced ST to 26.5 mN/m and EI 60 %	Removed 82.32–94.50 % of crude oil adsorbed on filter paper	(Zhang et al., 2016)
Pseudomonas aeruginosa SG	Xinjiang oilfield, China	70.3 g/L glycerol, 5.25 g/L $NaNO_3$	Rhamnolipid	Reduced ST and IFT to 33.3. and 2.14 mN/m at	8.33 % crude oil was displaced in	(Zhao et al., 2015;

				CMC of 80 mg/L, EI of 82.5 %	the core flooding experiment	Zhao et al., 2016)
Halomonas sp. MB-30	Isolated from a marine sponge *Callyspongia diffusa,* from the southeast coast of India	Engine gear oil	Glycolipid	Reduced ST to 30 mN/m and EI of 93 %	62 % of residual crude oil recovery from sand pack column (75 ml)	(Dhasayan et al., 2014)
Bacillus subtilis (309, 311, 573)	Brazilian oil field at depths of 300–400 m	Heating oil, viscous paraffin, Arabian Light and heavy oil	Produce lipopeptide (8-(86 mg/L)	CMC (20, 20 and 30 mg/L)	oil recovery of 6-25 % in Sand pack column (250 ml)	(Gudina et al., 2013)
Enterobacter cloacae PTCC 1798, *Enterobacter hormaechei* PTCC 1799	Formation water from oil reservoirs of southwest Iran	Molasses (10 g/L), Ammonium sulfate (1.06 g/L) and MIS dead crude oil	Produces biosurfactant (1.53 g/L)	Reduce ST and IFT to 31 and 3.2 mN/m, respectively	39.5 to 48.5 % oil recovery of OOIP	(Rabiei et al., 2013)
Rhodococcus ruber Z25	Formation brine in Daqing Oilfield, China	Paraffin oil or crude oil	Biosurfactant (12.95 g/L, 0.53 g/L in aerobic and anaerobic conditions)	Declined ST and IFT to 29.54 and 1 mN/m at CMC of 133 mg/L	8.88-25.78 % oil recovery in sand pack column	(Zheng et al., 2012)

2.4.2. *Ex-situ* MEOR

In the *ex-situ* MEOR, the metabolites are produced outside the reservoir and subsequently injected into the oil reservoir during the flooding process. The *ex-situ* MEOR also comprises three phases for the technology transfer from laboratory to original oilfield as shown in Figure 2.4. In the first phase, laboratory studies are carried out by isolating biosurfactant-producing microorganisms from relevant environmental sites. Then the surface activities like surface tension (ST), interfacial tension (IFT) and emulsification index (EI) measurements are conducted and the other analyses like the chemical characterization of the produced biosurfactant, wettability alteration capability and critical micelle concentration (CMC) values are evaluated. The stability and adsorption studies at a wide range of pH, temperature and salinity are examined as well to optimize the biosurfactant production. In the next phase of pilot-scale studies, computer simulations and mathematical models are framed after supervising the upstream scale production and downstream separation, product recovery and transportation of the metabolite to the oilfield. A longer shut-in period is not required as there is no acclimatization step involved. Finally, the success rate in the field upscale is validated from the data gathered from the previous experiments (Geetha et al., 2018).

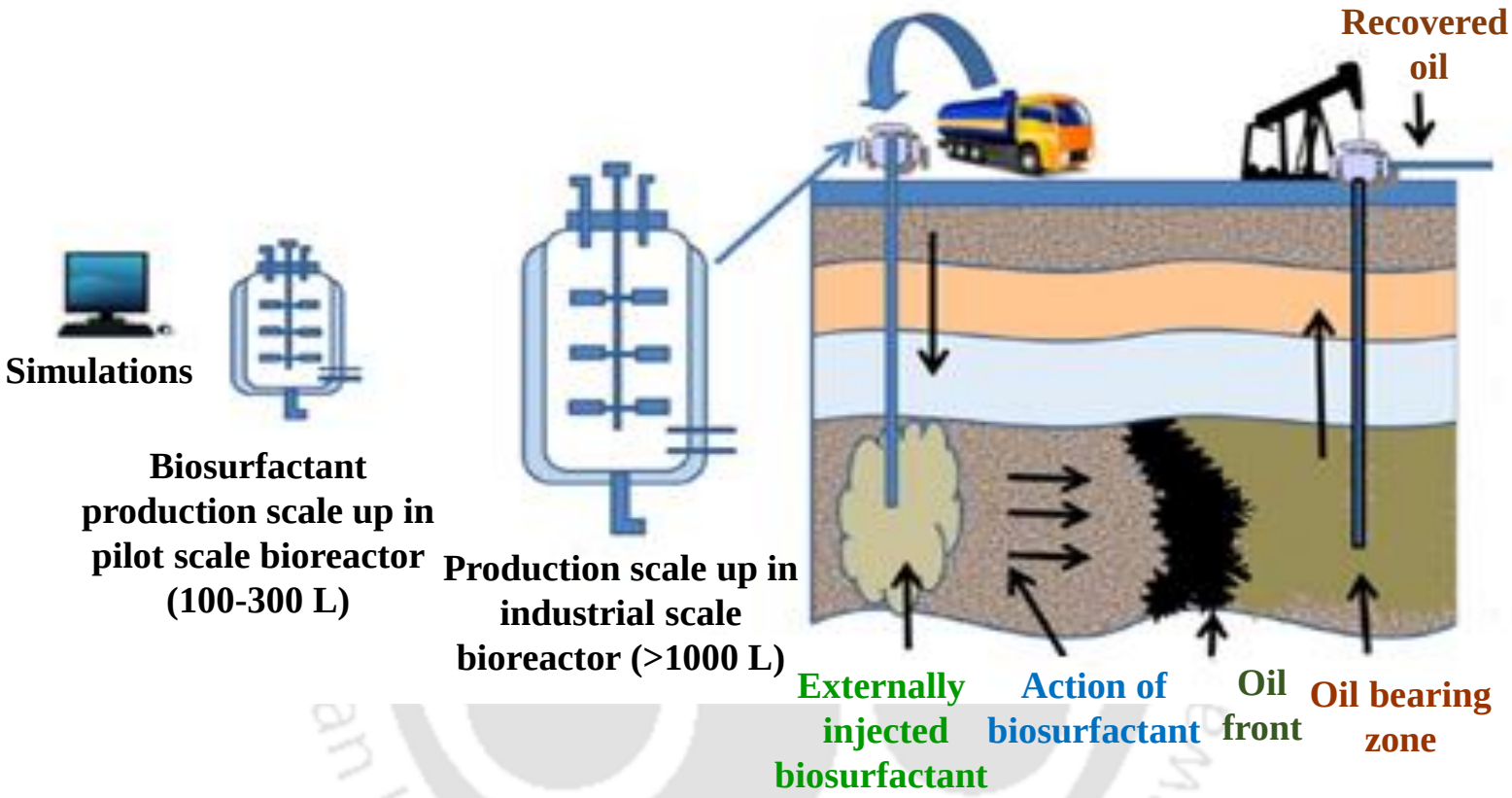


Figure 2.4. Schematic of successive phases of biosurfactant induced *ex-situ* MEOR. Adapted from Ref. (Geetha et al., 2018) with permission from Elsevier.

Wang *et al*., isolated a thermotolerant biosurfactant-producing strain XT-1 from a high pour-point reservoir, identified as *Bacillus subtilis* and examined its applicability for exogenous-MEOR (e-MEOR) to combat the challenge of long acclimatization period in the indigenous MEOR technology. The strain XT-1 could produce biosurfactant at an extensive thermal condition from 20 °C to 57 °C, proving its high degree of thermal stability at the maximum biosurfactant concentration of 830 mg/L. Microbial core flooding trials employing three different types of crude oil at 35 °C, 45 °C and 55 °C at three different blocks indicated significant IFT reduction as well as augmented oil recovery of 7 - 13 % by XT-1 revealing immense prospective for e-MEOR. Several other microbial strains including *Acinetobacter junii* BD, *Pseudomonas aeruginosa* L6-1 and *Bacillus subtilis* M15-10-1 were also isolated from oil reservoirs and scrutinized as appropriate exogenous microbes for MEOR (Wang et al., 2018b). Joshi *et al*., carried out the Surfactin production by five *Bacillus* strains in a bench-scale bioreactor and scrutinized its potential for EOR using sand pack columns. The ST and IFT were declined to 28 mN/m and 5.8 – 0.5 mN/m, respectively within 10 hours, controlling the pH from 6.8 to 7.2 at ambient temperature. The concentration of crude biosurfactant produced by *Bacillus* strains was 0.08 –1.1 g/L having CMC values in the range of 19.4 – 39 mg/L. Residual oil recovery of 30 – 34 % was obtained which proved the strain to be promising for *ex-situ* MEOR (Joshi & Desai, 2013). Two biosurfactant synthesizing strains, *Bacillus amyloliquefaciens* SAS-1 and *Bacillus subtilis* BR-15 produced lipopeptides, surfactins having molecular weight of 1007, 1021, 1035 and 1049 Da, containing a mixture of several isoforms. The biosurfactant showed exceptional EI of 60 – 78 % with hydrocarbons, ST (20 – 22 mN/m) and constancy at broad temperature (4 – 100 °C) and pH (4 – 10) range. The biosurfactants generated by both the strains corresponded for almost 57 % and 66 % EOR respectively and improved (75 – 94 %) engine oil biodegradation by microbial consortia,

which were additional threefold as compared to the control (22 – 31 %) in sand pack column experiments (Sharma et al., 2018b). The cyclic lipopeptides synthesized by *B. subtilis* 32811 reduced the ST from 68 mN/m to 25 mN/m along with IFT of water-oil from 25.6 to 4.6 mN/m. The optimum cultural temperature for *B. subtilis* was reported to be 40 °C, however, the strain was not suitable to be used for the oil displacement in acidic conditions. Table 2.3 summarizes the recent *ex-situ* MEOR cases with the contributing microbes along with their isolation from hydrocarbon-contaminated sites, their surface activities, optimum production of biosurfactants and their performance for the oil recovery purposes.

Table 2.3. *Ex-situ* MEOR case studies by microorganisms isolated from various sites (Saravanan et al., 2020)

Microbes	Samples and Isolation sites	Employed substrates	Metabolite yield	Surface properties	Additional findings	Reference
Pseudomonas sp. TMB2	Oil contaminated soil of Tejpur, upper Assam, India	Crude oil from ONGC, Jorhat, Assam, India	Glycolipid (2.8 g/L)	ST and IFT were declined to 33.4 to 0.8 mN/m at CMC of 120 mg/L, wettability change from 75° to 42°	39 - 55 % residual oil recovery in the core flooding experiment	(Haloi et al., 2020)
Pseudomonas aeruginosa	Crude oil contaminated soil, Brazil	Petroleum (API 21.9 °) collected from Brazilian oilfield	Rhamnolipid (260 mg/L)	EI 69 %, CMC 127 mg/L, reduced ST to 35.26 mN/m	50.5 % total oil recovery among which 12 % contributes to MEOR	(Câmara et al., 2019)
Candida tropicalis MTCC230, *B. subtilis* MTCC2423	IMTECH Chandigarh, India	Four strokes engine oil (Castrol ACTIV 4T)	Surfactin	Decreased ST 32 mN/m to at CMC of 32.5 mg/L	Additional oil recovery of 39.8 % in sand pack column	(Das, 2018)
Pseudomonas aeruginosa NCIM 5514	Crude oil contaminated soil from Central Tank Farm (CTF) of Ankleshwar Asset, ONGC, Gujarat, India	Crude oil from Ankleshwar CTF (API gravity: 35.77°)	Rhamnolipid	Viscosity reduction from 1883 cp to 1002 cp and ST to 29.7 mN/m	8.82 % recovery of residual oil by flooding in Cerro-metal pack Berea core	(Varjani & Upasani, 2016a; Varjani & Upasani, 2016c)
Bacillus licheniformis W16	Soil sample near the Omani oil well	Light crude oil from Petroleum Development Oman (PDO)	Lichenysin-A (0.52 g/L)	Reduced ST and IFT to 24.3 and 2.47 mN/m, wettability alteration from 56° to 19°	24 - 26 % residual oil recovery by Berea sandstone flooding system	(Joshi et al., 2016)

Bacillus subtilis R1	Kutch desert (Gujarat, India)	Light crude oil	Surfactin and Fengycin (0.7 g/L)	Lowered ST to 29 mN/m at CMC of 20 mg/L	33 % additional oil recovery in sand pack glass column	(Jha et al., 2016)
Bacillus atrophaeus 5-2a	Oil-contaminated soil in the Ansai oilfield, Northwest China	Crude oil from a depleted oil well (Hua-20-4) in Ansai oilfield	Lipopeptide (0.77 g/L)	EI of 61.8 % and decrease ST to 26.5 mN/m	Recover 90 % crude oil adsorbed on sand	(Zhang et al., 2016)
Lysinibacillus chungkukjangi	The sludge of rice bran oil processing, India	Kerosene	Lipopeptide	Decreased ST to 27.9 mN/m	Recovered upto 90 % of entrapped kerosene in sand pack column	(Bhardwaj et al., 2016)
Acinetobacter junii BD	Oil wells at Xinjiang Oilfield, Northwest China	Crude oil	Rhamnolipid	ST was reduced to 30.75 mN/m	9.6 - 13.4 % improve in total oil recovery in glass micromodel	(Dong et al., 2016)
Bacillus licheniformis ATCC 14580, yeast *Candida albicans* IMRU 3669	Microbiological Resource Center (MIRCEN), Cairo, Egypt	Crude oil from Niage 1 oilfield Badr El-din Petroleum Company	Surfactin (1 g/L) and Sophorolipid (12 g/L)	EI of 96 % and 65 %; ST to 36 and 45 mN/m for bacteria and yeast, respectively	16.6 % and 8.6 % oil recovery by bacterial and yeast strains, respectively in the sand pack column	(El-Sheshtawy et al., 2016)
Bacillus licheniformis	Water samples from Niage field, West desert, Egypt	Crude oil	Surfactin (1 g/L)	EI of 96 %, ST reduction upto 36 mN/m	Oil recovery of 16.6 % in sand pack column	(El-Sheshtawy et al., 2015a)
Bacillus licheniformis R2	Oil-contaminated site in Kutch desert, near Kandala port, Gujarat, India	Heavy crude oil (API 15.54°) supplied by IRS, Ahmedabad, India	Lichenysin-A (1.1. g/L)	Reduce ST and IFT to 28 and 0.53 mN/m, respectively at CMC of 19.4 mg/L	37.1 % additional oil recovery from Berea sandstone cores at 80 °C	(Joshi et al., 2015)

Geobacillus toebii R-32639	Handil reservoir, Indonesia	Crude oil from Handil Field of Kutei Basin, East Kalimantan	Biosurfactant	Lower the IFT and oil viscosity by 25.3 % and 14.1 %, respectively	Average oil recovery factor of 14.27 % in core flooding	(Fulazzaky et al., 2015)
Bacillus amyloliquefaciens TSBSO 3.8	Petroleum contaminated drilling mud and water from Brazil	Crude oil from a virgin off-shore field, Atlantic Ocean, Rio de Janeiro, Brazil	Surfactin	EI of 63 % and reduced ST and IFT to 28.5 and 11.4 mN/m, respectively	43 % oil recovery and total petroleum hydrocarbon (TPH) of 1200 µg/L in the column experiment	(Alvarez et al., 2015)
Pseudomonas sp. SWP-4	WCO contaminated sludge sample from a local sewer	Heavy crude oil from Zhongyuan oilfield, China	Rhamnolipid (6.87 g/L)	CMC 27 mg/L, reduced viscosity from 26,300 mPa s to 550 mPa s (40 °C) and ST and IFT to 24.1 and 0.9 mN/m	Additional oil recovery of 24.4 %	(Lan et al., 2015a; Lan et al., 2015b)
Bacillus subtilis B30	Petroleum contaminated soil samples from Oman	Heavy crude oil (15.9°) and light crude oil (API 36.51°) provided by PDO	Surfactin (0.5 g/L)	EI of 50 % and decline ST and IFT to 26.63 and 3.79 mN/m	Enhanced light oil recovery by 17–26 % and heavy oil recovery by 31 % in Berea sandstone core-flooding	(Al-Wahaibi et al., 2014)
Acinetobacter baylyi ZJ2	Crude oil-contaminated soil, China	Crude oil from Zhongyuan oil field (Henan, China)	Lipopeptide (0.09 g/L)	ST and IFT were decreased to 35 and 15 mN/m	28 % additional residual oil was recovered	(Zou et al., 2014)
Bacillus subtilis (309, 311, 573)	Brazilian oil field	Arabian Light oil	Surfactin (931, 980 and 2288 mg/L, respectively)	ST was reduced to 28 mN/m and EI was 52.7 %	Oil recovery 19- 22 % in sand-pack column	(Pereira et al., 2013)

Enterobacter cloacae PTCC 1798, *Enterobacter hormaechei* PTCC 1799	Formation water collected from oil reservoirs in the southwest of Iran	MIS dead crude oil	Biosurfactant (1.53 g/L)	Reduce ST and IFT to 31 and 3.2 mN/m, respectively	9.85 - 12 % oil recovery of ROIP by core flooding test	(Rabiei et al., 2013)
Serratia rubidaea SNAU02	Hydrocarbon-contaminated soil from Cuddalore, Tamilnadu, India	Engine oil	Rhamnolipid	Declined ST to 34.4 mN/m	85 % and 92 % adsorbed oil in the sand was recovered	(Nalini & Parthasarathi, 2013)
1. *Pseudomonas aeruginosa* WJ-1, 2. *Bacillus subtilis* H10, 3. *Rhodococcus erythropolis* Z25	Formation water of the Chinese petroleum reservoir	Crude oil (density 898 g/L) of Xinjiang reservoir	1. Biosurfactant (2.66 g/L) 3. Biosurfactant (1.56 g/L)	1. EI of 80 % ; declined ST to 22.5, 27 and 29.5 mN/m; IFT to 1.85, 2.87 and 4.45 mN/m for 3 strains; CMC were 30, 50 and 70 mg/L, respectively	14.3 %, 10.4 % and 7.2 % recovery of residual oil in three core flooding systems, respectively	(Xia et al., 2011)
Pseudomonas aeruginosa MTCC7815, *P. aeruginosa* MTCC7814, *P. aeruginosa* MTCC7812, *P. aeruginosa* MTCC8165	Oil fields (Lakuwa, Gelekey, Rudrasagar) Assam asset basin, ONGC, Assam, India	Crude oil	Rhamnolipid (5 g/L)	Decreased ST to 30 mN/m at CMC of 110 mg/L	10-15 % additional recovery of residual oil by sand pack glass column at 70° – 90 °C	(Bordoloi & Konwar, 2008)
Bacillus subtilis 20B	Fermented food sample (rice – idly batter)	Crude oil (API 25°)	Surfactin	Reduced ST and IFT to 29.5 and 4.5 mN/m with EI of 77- 80 %	30.22 % recovery of residual oil by sand pack column	(Joshi et al., 2008)

2.5.Elucidation of Screening Parameters to Identify Suitable Biosurfactant Producing Strains

The biosurfactant production by microbes can be screened through a variety of methods including oil-displacement test, surface tension (ST) and/or interfacial tension (IFT) measurements, drop collapse test, critical micelle concentration (CMC) value measurement and emulsification index (EI) determination (Al-Bahry et al., 2013). The stability studies can also be performed by analyzing their stability in a wide range of environmental conditions in terms of the screening parameter constancy.

A strain *Bacillus licheniformis* JF-2 was isolated from oil-field injection water which could grow upto 10 % NaCl, at temp upto 50 ºC and in the pH range 4.6 - 9 and secret biosurfactant, Lichenysin under anaerobic conditions (Jenneman et al., 1983). Among thermophilic halophiles, bioemulsifier was extracted from *Methanobacterium thermoautotrophicum* which was able to grow upto 80 °C and active over a wide range of pH (5 - 10) at a very high salt concentration (de Acevedo & McInerney, 1996). Many bacterial species that can grow and survive at high salt concentrations are named halophilic strains. *B. subtilis* and *B. licheniformis* can survive in 5 % salinity and upto 50 °C temperature (Daryasafar et al., 2016). Methanogenic *Methanocalculus halotolerans* also from an oil well showed maximum growth upto 20 % salinity and 45 °C temperature (Ollivier et al., 1998). After screening more than 30 different bacterial isolates from hot water springs for their capability to synthesize biosurfactants under thermophilic conditions, the biosurfactant production by *Bacillus stearothermophilus VR-8* was reported (Gurjar et al., 1995). The produced biosurfactant was found to be completely stable at 80 °C for 30 minutes while at 90 to 100 °C 60 % emulsification index was determined and 5 % NaCl could cause 8 % loss of surface activity (Singh & Desai, 1989).

Yakimov *et al*., studied the potential of several strains of *Bacillus licheniformis* in EOR and reported that these strains (BNP29, BNP36, BAS50 and Mep132) produced a significant

amount of a surfactant similar to Surfactin at moderately elevated temperatures of 55 °C and salinities up to 12 % NaCl (Yakimov et al., 1997). Makkar and Cameotra screened two strains of *Bacillus subtilis* that demonstrated a high capacity for biosurfactant production at thermophilic conditions (Makkar & Cameotra, 1998). McInerney *et al*., investigated the ability of over 200 strains of *Bacillus subtilis*, *B. licheniformis*, *Bacillus mojavensis* and *Bacillus sonorensis* for biosurfactant production and compared their surface activity under anaerobic conditions at 5 % salinity (McInerney et al., 2005). In another work, halo-thermotolerant strain, *B. licheniformis* ACO1 showed a high capacity for bioemulsifier production at temperatures and NaCl concentrations upto 60 °C and 180 g/L, respectively. The optimum NaCl concentration, pH and temperature for bioemulsifier production were reported as 4 % (w/v), 8, and 45 °C, respectively (Dastgheib et al., 2008). Among thermophilic halophiles, bioemulsifier was isolated from *Methanobacterium thermoautotrophicum* which could grow upto 80 °C and be active over a wide range of pH (5 - 10) at a very high salt concentration (upto 200 g/L) (Trebbau, 2005).

The biosurfactant production potential of a microbial consortium of halo-thermotolerant *Enterobacter cloacae* and *Pseudomonas* sp. (ERCPPI-2) isolated from heavy crude oil-contaminated soil of south Iran, has been investigated under extreme environmental conditions which displayed excellent screening properties such as oil spreading and emulsification index. This consortium was able to grow at temperatures upto 70 °C, pressures upto 6000 Psi, salinities upto 15 % (w/v) in the pH range 4 - 10. The ERCPPI-2 could reduce surface and interfacial tensions to 31.7 and 0.65 mN/m from the original values of 58.3 and 16.9 mN/m, respectively and emulsified the available heavy crude oil up to EI 83.4 % with a biosurfactant concentration of 1.74 g/L. The results of the core-flooding tests at simulated reservoir conditions demonstrated 27 % oil recovery efficiency due to the injection of the cell-free biosurfactant solution (Darvishi et al., 2011).

Marinobacter hydrocarbonoclasticus was isolated from the upper segment of an oil well in Vietnam, which grew on high salinity (5 % NaCl) and degrade n-hexadecane, pristane along with some other crude oil components (Huu et al., 1999). *B. subtilis*, *P. aeruginosa*, and *Bacillus cereus* isolated from oil-contaminated sites of Iran, were able to withstand harsh reservoir conditions (120 °C, pH 4, 25 g/L salinity) and reduction in ST from 72 to about 26 mN/m were observed due to the production of biosurfactants (Amani et al., 2010; Bachmann et al., 2014). *Bacillus mojavensis* was employed in the Masjed-I Soleyman carbonate field (Iran) having a pressure of 3 – 4 MPa and temperature of 42 °C which reduced the ST to 26.7 mN/m within 12 hours of incubation. This strain was also employed in Talara offshore oil fields (Peru) having 5.8 – 300 MPa, 55 °C and 45 % in residual oil were obtained. In the Tatariya carbonate oil field (Russia), *Clostridium* increased the oil production by 28 % – 46 % (Sakthipriya et al., 2017). The low-temperature heavy oil fields (Russia) accommodated a microbial community that was competent enough to produce oil-displacing metabolic compounds. Aerobic bacteria *Rhodococcus erythropolis* HO-KS22 and *Gordonia amicalis* 6-1 isolated from Russian oil reservoirs reported to oxidize heavy crude oil to produce biosurfactants which considerably reduced ST and IFT as well as had shown prospects for paraffin degradation, MEOR and the hydrocarbon bioremediation (Nazina et al., 2020). *Bacillus licheniformis* was isolated from the Zilaei oil reservoir in southwest Iran which could grow optimally and produce biosurfactant at 50 °C which reduced the ST from 72 to 23.8 mN/m and the IFT from 36.8 to 0.93 mN/m (Daryasafar et al., 2016). This same species was further isolated from the Niage field of the Western Desert, Egypt, which presented considerably good surface properties of maximum emulsification index of 96 % and ST reduction to 36 mN/m when incubated for 72 hours at 45 °C (El-Sheshtawy et al., 2015b). The microbial community composition of indigenous microbes in Gulf of Mexico beach sands denoted the predominance of *Gammaproteobacteria* and *Alphaproteobacteria* as the chief contributor in oil biodegradation (Kostka et al., 2011). *Kosmotoga olearia*, a thermophilic,

heterotrophic, anaerobic bacterial strain was reported from the Troll B oil platform in the North Sea which could perform well in the temperature range of 20 to 80 °C, 5.5 - 8 pH, and 10 - 60 g/L salinity (DiPippo et al., 2009). Moderately thermophilic and halophilic SRB, *Petrotoga halophila*, have been isolated from an offshore oil well in Congo, West Africa capable of hydrocarbon degradation (Miranda-Tello et al., 2007). Nine bacterial groups have been found in the oil samples from Brazilian oil reservoirs, such as *Acinetobacter*, *Arcobacter*, *Bacillus*, *Halanaerobium*, *Leuconostoc*, *Marinobacter*, *Streptomyces*, *Propionibacterium* and *Streptococcus* (Sette et al., 2007; Souza et al., 2014). A fungal strain (*Neosartorya fischeri*) isolated from Venezuela and a bacterial isolate (*Garciaella petrolearia*) from Mumbai, India could grow well utilizing asphaltenes substrate and favorably degraded asphaltenes and aromatics in crude oil (Lavania et al., 2012; Uribe- Alvarez et al., 2011). Among several isolated spore-forming bacteria from soil samples of Oman oil fields, an autochthonous strain, *Paenibacillus ehimensis* BS1 was reported to improve heavy oil recovery due to the great endurance of its dormant spore cells in stressful conditions with long dormancy period, in high temperature, drying and presence of acid. The isolate exhibited utmost growth at elevated heavy oil concentrations within four days of incubation. Biotransformation of heavy crude oil (API 4.57°) to light aliphatic and aromatic constituents and its prospects in EOR was accessed aerobically and anaerobically (Shibulal et al., 2017). However, *Pseudomonas aeruginosa* DQ3 strain remained the most dominant in Daqing oil reservoirs and could produce biosurfactant maximum of 228 mg/L of biosurfactant anaerobically at reservoir temperature (42 °C) which was sufficient because the minimum biosurfactant concentration necessary for mobilizing the entrapped oil from the sandstone reservoir was already known to be only 10 mg/L (Youssef et al., 2007; Youssef et al., 2013). *Brevibacillus brevis* and *Bacillus cereus* were also employed at Daqing low permeability high-temperature oil field at 65 °C which could reduce 40 % oil viscosity. *Enterobacter*, *Bacillus licheniformis* were utilized at the Fuyu oil field in China (1.95 – 2.95 MPa, 28 °C), which could increase oil recovery by two folds (Sakthipriya et al., 2017).

G. amicalis strain LH3 which was isolated from oil-contaminated water samples of Jidong oilfield, China could degrade 18 % (2 % w/v) paraffin at a rate of 4.4 mg/d under anaerobic conditions after 10 days of cultivation at 40 °C with 5 % NaCl. The strain could also reduce oil viscosity by 45 % and degraded 10.5 % (w/w) oil under aerobic conditions after 7 days of cultivation (Hao et al., 2008). Two *Pseudomonas aeruginosa* strains (Gx and Fx) were isolated from oil-contaminated soils of Yanchang oilfield, China; which produced biosurfactants using crude oil heavy components as their substrate. The prospects of Gx and Fx in oil displacement was examined by degrading capability pure asphalt and crude oil asphaltenes where almost 10 % of pure asphalt and 59–72 % of crude oil asphaltenes were biodegraded utilizing bacterial supernatants showing the oil-spreading diameter from 15 to 17 cm. The lighter fractions (saturates and aromatics, maximum 11 %) content augmented while the heavier fractions (resins and others, maximum 75 %) contents were reduced in the degraded oil compared to the controls along with the oil viscosity (35 °C) reduction to nearly half from 76.5 m Pa·s (Gao et al., 2017).

2.6.Structural Classifications of Biosurfactants Those Contribute to MEOR

Biosurfactants have been primarily classified into five types i.e., glycolipids, phospholipids and fatty acids, lipopeptides and lipoproteins, polymeric and particulate biosurfactants (Pacwa-Płociniczak et al., 2011; Roy, 2017; Singh et al., 2014; Varjani & Upasani, 2017; Zhang et al., 2020a). The glycolipid includes Rhamnolipid (Abdel-Mawgoud et al., 2011; Henkel et al., 2012; Reis et al., 2011; Soberón-Chávez et al., 2005), Sophorolipid, Trehalolipid, Cellobioselipid and Mannosylerithritol lipids (Geys et al., 2014; Kitamoto et al., 2009; Reis et al., 2013); lipopeptides can be of Surfactin (Chen et al., 2015), Lichenysin, Iturins, Viscosin, Subtilisin, Fengycin, Putisolvin and Polymixin depending on their amino acid sequence (Bezza & Chirwa, 2015; Jacques, 2011); fatty acids/phospholipids/neutral lipids such as Phosphatidyl-ethanolamine, Spiculisporic acid and polymeric biosurfactants are Liposan, Emulsan, Alasan

and Biodispersan (Desai & Banat, 1997; Fenibo et al., 2019; Sen, 2010; Silva et al., 2014) which have been largely investigated for their crucial role in EOR.

The chief representative biosurfactants for EOR with their produced microorganisms are: *Bacillus* produces Surfactin (Pereira et al., 2013), Iturins and Lichenysin (Bonmatin et al., 2003; Halim et al., 2017), *Pseudomonas* produces Rhamnolipid (Bhardwaj et al., 2013; Bordoloi & Konwar, 2008; Dobler et al., 2016; Makkar et al., 2011; Souza et al., 2014; Varjani & Upasani, 2017), *Acinetobacter* produces Emulsan (Suthar et al., 2008) and Alasan (Mujumdar et al., 2019; Navon-Venezia et al., 1995), *Rhodococcus* produces Viscosin and Trehaloselipids (Silva et al., 2014), *Candida* produces Sophorolipid (Saborimanesh & Mulligan, 2015). Many yeast strains such as *Candida tropicalis*, *Geotrichum candidum*, *Galactomyces pseudocandidum*, *Aureobasidium pullulans* and *Galactomyces geotrichum* were found to produce glycolipids and form stable emulsions, reduce ST which made them applicable for oil industries (Brumano et al., 2017; Eldin et al., 2019; Mulligan, 2005; Sáenz-Marta et al., 2015; Yalçın et al., 2018). In MEOR, glycolipids along with lipopeptide biosurfactants are assumed to have huge commercial and industrial prospective which directed towards extensive recognition (Bachmann et al., 2014). Among the wide diversity of biosurfactants, surfactin and rhamnolipid have been considered to be the most researched biosurfactants for core flooding studies (Kubicki et al., 2019; Niu et al., 2020; OSMAN et al., 2019; Patel et al., 2015; Zhang et al., 2020a).

Surfactin is a cyclic lipopeptide consisted of beta-hydroxy fatty acids along with seven amino acid residues, produced by various strains of *Bacillus* sp. (*Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus mojavensis*, *Bacillus pumilus* and *Bacillus tequilensis*) and is investigated to be one of the influential biosurfactants for its exceptional surface properties that is necessary to mobilize the entrapped oil [12-14]. The synthesized Surfactin contains a mixture of its isoforms which are formed because of the alterations in the

carbon chain length and branching of its hydroxyl fatty acid components. The fatty acid alkyl chain of surfactin may differ lengthwise mostly starting from 13 to 15 carbon atoms. The sequence of amino acids has been typically reported to be Glu-Leu-Leu-Val-Asp-Leu-Leu [13]. Surfactin is renowned to be resistant to the autoclaving temperature which indicates that this molecule can maintain and retain its native property even after sustaining at so elevated thermal conditions in oil reservoirs. Although, Surfactin has not been examined much with other environmental parameters existing in oil reservoirs such as high salinity which possibly impede positively or unconstructively in the process of MEOR [15].

2.7.Optimization of Culture Conditions for Biosurfactant Production

The main schemes to obtain the optimum biosurfactant production could be through various modes (i) evaluation of suitable substrates and good productivity by focusing on appropriate microbes, nutritional balance and the use of inexpensive or waste substrate to minimize the initial raw material expense involved in the process; (ii) development of proficient bioprocess, including optimization of the culture conditions and cost-effective separation technique to maximize recovery; and (iii) synthesis and utilization of hyper-producing mutant or recombinant strains for enhanced yields (Makkar et al., 2011).

The primary strategies for optimization of biosurfactant production include the bioprocess engineering perspectives mainly statistical approaches, bioreactor scaling up, efficient process development for biosurfactant purification and recovery. The productivity of microbial processes could be enhanced by majorly two ways. In the first approach, inexpensive raw materials are employed as alternative substrates for the production of the desired final product which indirectly includes the manipulation of biosynthetic pathways using mutagenesis or recombinant strains for overproduction. The second approach focuses on various designs and tailored optimization techniques for improving the performance of bioprocesses that include

experimental designs, process optimization, response surface models, mathematical models for kinetic and process control (Eswari et al., 2019).

2.7.1. Optimization of Biosurfactant Production Employing Suitable Economical Substrates

Almost 10 – 30 % of the total biosurfactant production cost is dedicated to raw materials therefore, by using another substitute approach, the cost-effectiveness concern could be significantly improved by using commonly accessible, abundant, affordable, cheap and renewable substrates like agricultural and food waste as fermentation feedstock because this would furthermore improve the ecological and economic sustainability of the biosurfactant production methods as shown in Table 2.4 (Mukherjee et al., 2006). Various carbon sources in the growth media influence the composition of produced biosurfactants. There are two types of cost-effective carbon sources, those are water-soluble substrates, such as molasses, glucose, sucrose, starch-rich wastes and glycerol, as well as water-immiscible substrates, such as hydrocarbons, oils, and edible oily wastes (Makkar & Cameotra, 2002). Hydrophobic compounds such as crude oil, diesel have been reported to be more preferable renewable substrates for biosurfactants production because they can improve the production of the biomolecule (Vijaya et al., 2013). The ability of bacteria to utilize hydrocarbons as the substrate to produce biosurfactants could serve the purpose of bioremediation of hydrocarbon-contaminated environments. Among the bio-resources, waste vegetable oil, waste cooking oil such as residual frying oil which is produced from worldwide restaurants, are being utilized for the production (Ibrahim, 2018; Lan et al., 2015b; Makkar et al., 2011; Oliveira & Garcia-Cruz, 2013; Sharma et al., 2019a). Nitrogen is crucial for microbial growth and biosurfactant production. There are different types of nitrogen sources for the production of biosurfactants such as yeast extract, urea, peptone, ammonium sulfate, ammonium nitrate and sodium nitrate

and apparently concentration of the nitrogen source plays a fundamental role in the optimization of biosurfactant production (Tripathi et al., 2018).

Indian economy is majorly dependent on agro-industries, producing a large amount of agro-industrial wastes which could be most suitable as a substrate for biosurfactant production (Banat et al., 2014). The use of cheaper, renewable raw materials from different industries such as agro-industrial waste and bi-products included crop residues (sugars, molasses, plant oils, oil wastes, starchy substances, lactic whey), dairy industries (Curd whey, cheese whey, whey waste), distillery wastes (industrial effluents), animal fat, food processing industries (frying edible oil, olive oil, potato peels, rape seed oil, sunflower, vegetable oils), fruit processing industries and oil industries (olive oil-mill wastewater, palm oil mill, peanut cake, effluent, soybean cake, soapstock, waste from lubricating oil) have been mentioned and reviewed comprehensively by several researchers (Banat et al., 2014).

Bacillus pseudomycoides BS6, isolated from an oil-contaminated soil, was identified to be an effective producer of lipopeptide using soybean oil waste as the sole source of carbon and energy and showed good interfacial characteristics. The CMC of the purified lipopeptide was only 56 mg/L and could reduce the ST of water from 71.6 mN/m to 30 mN/m with an emulsification index of approximately 62.8 - 94.2 % (Li et al., 2016). Surfactin and Iturin were produced by two *Bacillus subtilis* strains (KP7 and I0-1a), respectively by growing them on media constituting renewable natural resources such as brewery wastewaters from the beer industry (barley malt BW#4 and wheat malt BW#6). The media contained 2 % beet molasses, apple peels extract supplemented with 0.25 % of yeast extract or 0.25 % peptone and similarly supplemented carrot peels extract. The productivity (140.6 and 428.7 mg/L) and the structural diversity of synthesized lipopeptides were observed to be dependent on the medium composition (Paraszkiewicz et al., 2018).

Table 2.4. Enhancement of biosurfactant production employing various economic substrates

Strain	Isolation site	Biosurfactants (g/L)	C, N Source	CMC (mg/L)	References
Bacillus subtilis AB2.0	Beach sediment sample, Brazil	Surfactin (0.099)	10 % (v/v) glycerol in tryptic Soy broth	16.23 ± 0.19	(Alvarez et al., 2020)
B. subtilis RSL 2	Oil contaminated sludge of IOCL, Noonmati, Assam, India	Surfactin (5.2)	1 % (v/v) crude oil, 1.5 % yeast extract	500	(Sharma & Pandey, 2020)
Pseudomonas aeruginosa TEN01	Petroleum industry, India	Rhamnolipid	9 % (w/v) cassava waste, 1.2 % (w/v) of glycerol, 0.3 % (w/v) $NaNO_3$	NA	(Elakkiya et al., 2020)
Pseudomonas aeruginosa HAK01	Urban waste of Tehran	Rhamnolipid (2.07)	20 g/L sunflower oil, 1g/L yeast extract, 3 g/L $NaNO_3$	120	(Khademolhosseini et al., 2019)
Pseudomonas aeruginosa	Artificially oil-contaminated soil	Rhamnolipid	125 g/L of glycerol, 3.86 g/L $NaNO_3$	127	(Câmara et al., 2019)
Bacillus licheniformis DS1	Crude oil sample from wellhead of oil reservoir, Sumatra	Lipopeptide (0.4)	2 % (v/v) non-sterile crude oil, 0.1% (w/v) yeast extract	157.5	(Purwasena et al., 2019)
Pseudomonas sp. G3	Formation brine from petroleum reservoir of South Sumatra	Rhamnolipid	Heavy crude oil, 0.1 % (w/v) yeast extract	730	(Astuti et al., 2019)
Streptomyces sp. DPUA1566	Lichens from the Brazilian Amazon region	Lipoprotein Bioelan (1.9)	10 g/L soybean waste frying oil, 20 g/L corn steep liquor	800	(Santos et al., 2019)

Bacillus subtilis YB7	Polymer dumpsite, Chennai, India	Surfactin (2.62)	Waxy crude oil and long-chain paraffin	20 - 60	(Sakthipriya et al., 2015)
Serratia rubidaea SNAU02	Hydrocarbon-contaminated soil of Tamil Nadu, India	Rhamnolipid	29.31 g/L Mannitol, 2.06 g/L yeast extract	NA	(Nalini & Parthasarathi, 2013)
B. subtilis R1, *B. licheniformis* R2, *B. subtilis* 20B, *Bacillus* HS3	Oil-contaminated desert site, formation water, fermented food, and hot water spring	Surfactin and Lichenysin (0.08 – 1.1)	Glucose, date molasses 30 g/L, yeast extract, NH_4NO_3, $NaNO_3$	19.4 – 39	(Joshi & Desai, 2013)
Streptomyces species B3	Marine sediment samples from the West coast of India	Glycolipid	2 % (w/v) sucrose, 10 g/L yeast extract	110	(Khopade et al., 2012)
Brevibacterium aureum MSA13	Oil contaminated soil	Brevifactin Lipopeptide (18)	Molasses, wheat bran and 1 % olive oil, 2 % acrylamide	NA	(Kiran et al., 2010)
Pseudomonas sp. ANBIOSURF-1	Municipal sewage sludge	Rhamnolipid	Coconut oil (0.4 % w/v), $NaNO_3$ (4 g/L)	52	(D Albino & Nambi, 2010)
Pseudomonas aeruginosa	Petroleum contaminated soil of different Assam oil reservoirs	Rhamnolipid (0.23 – 0.5)	Pyrene or fluorine, Phenanthrene (100 mg/L) and 2 g urea 2 g, $(NH_4)_2SO_4$	100 – 110	(Bordoloi & Konwar, 2009)
P. aeruginosa strain BS2	Oily sludge	Rhamnolipid (0.92)	Curd whey and distillery waste	NA	(Dubey & Juwarkar, 2004)

2.7.2. Selection of Efficient Downstream Processing

A proficient and cost-effective bioprocess is the basic establishment for every profit-making biotechnology industry. Therefore, bioprocess expansion is the preliminary step towards the commercialization of all biotechnological products, including biosurfactants. Biosurfactant yield improvement requires optimal addition of media components along with the selection of the optimal culture conditions which would induce the maximum or optimum productivity. Similarly, competent, fast and reasonable downstream processing techniques and methods are needed for the maximum product recovery (Muthusamy et al., 2008). Since two-third of the entire biosurfactant production expense is generally associated with downstream collection, separation and purification processes. It is very significant to explore and substitute the traditional method with competent, feasible and cost-effective alternative analytical production techniques for extraction, recovery and purification of biosurfactants on a commercial scale (Heyd et al., 2008; Mukherjee et al., 2006). The criteria to select a specific recovery method include: (1) the expenditure associated with the extraction method, which would be included in the price of the end-biosurfactant as well, (2) the probable function of the final product, which control the purity level of the biosurfactant, and (3) the sustainability of the method to the specific industrial fermentation practice (Abdel-Mawgoud et al., 2011). The conventional methods for recovery of microbial surfactants are acid precipitation, solvent extraction, centrifugation and filtration. Lipopeptides are generally precipitated by acid and extracted using methanol (Banat et al., 2010; Satpute et al., 2010). Production and purification of biosurfactants were performed mainly depending on their charge, solubility in suitable solvents. The most common biosurfactant recovery methods are acid precipitation and solvent extraction. Biosurfactant solubility and activity are critically affected by the pH with effective pH ranging between 4 and 10. At pH $\leq$ 4 many biosurfactants were found to precipitate probably because their isoelectric point is near pH 4 (Al-Bahry et al., 2013). The solvent for

precipitating the active fraction is selected based on the biosurfactant-producing species. Mostly the biosurfactants are less soluble in water due to their complex structure hence solvent mixtures like chloroform-methanol, dichloromethane-methanol, acetone, butanol, pentene, hexane, ether, acetic acid and ethyl acetate are used (Makkar et al., 2011; Mata-Sandoval et al., 1999). Often a single downstream processing technique is not sufficient for biosurfactant recovery and purification. In such cases, multi-step sequential recovery strategy with proper purification steps is more effective that helps to obtain the product at the required level of purity (Muthusamy et al., 2008). For biosurfactants commercialization; enhancement of production capacity, the bio-separation methods and the rate of product removal are crucial which involved the integration of rapid separation methods along with efficient production steps (Najmi et al., 2018). Various proficient and economic downstream processing techniques for maximum biosurfactant recovery and purification with their relative advantages have been illustrated in Table 2.5.

Table 2.5. Suitable downstream recovery methods for the biosurfactants with their relative advantages (Mukherjee et al., 2006; Muthusamy et al., 2008)

Process	Biosurfactant	Biosurfactant Property for Separation	Instrument/ apparatus/ setup required	Advantages	References
Acid precipitation	Surfactin, Rhamnolipid, Lichenysin	Acidification of biosurfactants neutralize their charge and make them insoluble at low pH values (2)	No set-up required	Low cost, efficient in crude biosurfactant recovery	(Datta et al., 2018; Déziel et al., 1999; Sen & Swaminathan, 2005; Varjani & Upasani, 2016b)
Organic solvent Extraction (acetone, ethyl acetate, chloroform and methanol)	Rhamnolipids, Trehalolipids; Sophorolipids; Liposan	Biosurfactant molecules are precipitated by acidification but soluble in organic solvents due to their hydrophobic moiety	No set-up required	Efficient in crude biosurfactant recovery and partial purification, reusable nature	(Banat et al., 2010; Heyd et al., 2008; Reiling et al., 1986; Varjani & Upasani, 2016b)
Solvent extraction (Using Methyl tertiary-butyl ether)	Glycolipids	Biosurfactants get dissolved in organic solvents because of the hydrophobic group in the molecule	No set-up required	Less toxic than Conventional solvents, reusable, economical	(Kuyukina et al., 2001; Philp et al., 2002; Shavandi et al., 2011)
Ammonium sulfate precipitation	Rhamnolipids, Emulsan; Biodispersan; Lipopeptides	Salting-out of the polymeric biosurfactant or protein-rich biosurfactant	No set-up required	Effective for precipitation of high molecular weight (polymeric) biosurfactants	(Banat et al., 2010; Varjani & Upasani, 2016b)
Centrifugation	Glycolipids	Insoluble biosurfactants get precipitated because of centrifugal force	Centrifuge is required	Reusable, effective in crude biosurfactant recovery	(Nitschke & Pastore, 2006)
Foam fractionation	Surfactin; Rhamnolipid	Due to the surface activity of biosurfactants, frothing happens which form foam	Specially designed bioreactors that	Useful in continuous biosurfactant recovery	(Chen et al., 2006; Heyd et al., 2008; Najmi et al., 2018; Rangarajan &

			facilitate foam recovery during fermentation	procedures, high purity of the product	Clarke, 2016; Rangarajan & Sen, 2013; Sarachat et al., 2010; Winterburn et al., 2011)
Membrane ultrafiltration	Glycolipids; Surfactin	Biosurfactants form micelles above their CMC, which are trapped by polymeric membranes	Ultrafiltration units with a porous polymer membrane	Fast, one-step recovery, high level of purity	(Najmi et al., 2018; Ramnani et al., 2005; Sen & Swaminathan, 2005)
Adsorption on polystyrene resins	Lipopeptides; Glycolipids; Mannosylerythritol Lipids (MEL)	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvents	Polystyrene resin packed in glass columns	Fast, one-step recovery, high level of purity, reusability	(Reiling et al., 1986)
Adsorption on wood-activated carbon	Lipopeptides; Glycolipids; MEL	Biosurfactants are adsorbed on activated carbon and can be desorbed using an organic solvent	No setup required, can be added to culture broth, can also be packed in glass columns	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture	(Dubey et al., 2005)
Ion-exchange chromatography	Glycolipids	Charged biosurfactants are attached to ion-exchange resins and can be eluted with the proper buffer	Ion-exchange resins packed in columns	High purity, reusability, fast recovery	(Abadi et al., 2009; Reiling et al., 1986)
Size exclusion chromatography	Lipopeptide	After desorption on activated carbon, biosurfactant was separated from other impurities	Sephadex gels in column	Biosurfactants are purified from various impurities	(Benitez et al., 2010; Rangarajan & Clarke, 2016)
Reverse Phase - High-Performance Liquid Chromatography	Lipopeptide	Resolve lipopeptides as isoforms depending on the isocratic/gradient mode of elutions	Biocompatible column with fast protein liquid chromatographic system	Recovery of ultra-purified biosurfactant	(Datta et al., 2018; Rangarajan & Clarke, 2016; Razafindralambo et al., 1993)

2.7.3. Statistical Approaches to Optimize the Biosurfactant Production Culture Conditions

The production expenditure of biosurfactants can be addressed and adjusted by employing suitable experimental designs based on statistical approaches. This would reduce the time and effort through appropriate planning, execute the experimental runs, establish optimal conditions by evaluating the effect of factors and build mathematical models. Experimental design assists in proposing trials by varying different experimental conditions simultaneously and investigating their influences on biosurfactant production. The classical optimization mechanism works by changing one independent variable at a time while fixing the others at a preset level which is enormously time-consuming and exploits chemicals for a large number of variables and requires numerous experimental trials to determine the optimum level. The optimum conditions for biosurfactant production and other biotechnological processes can be predicted by experimental trials as well as by using some statistical approaches such as Taguchi design of experiments (DOE), one factor at a time (OFAT), response surface methodology (RSM), Box-Behnken design (BBD) and Placket-Burman design (PBD), etc. Optimizing all the affecting parameters by statistical experimental designs can effectively eliminate these issues of a single factor optimization process collectively by a statistical experimental approach such as response surface methodology (RSM) which is the most extensively used method for culture medium optimization (Joshi et al., 2007). The most significant factors during the biosurfactant production process are the cell growth and conversion of substrate to biosurfactant during the bioprocess. These studies are also affected by various media compositions and other process parameters. RSM assist in optimizing the media and process parameters for the production of biosurfactants and has been successfully employed in various other prospects of biotechnology to optimize bacterial growth media and culture conditions which were dependent on the experimental data (Almansoory et al., 2017; Eswari et al., 2019;

Kim & Kim, 2020; Ohadi et al., 2017; Souza et al., 2018a). Some of the experimental design methods are central composite designs (CCD), orthogonal designs, and factorial designs. These optimization algorithms establish the relationship among the variables as well as with the response, which is known as response surface models that optimize the medium compositions along with the bioprocess conditions. The statistical approach-based optimization of various biosurfactants production using different substrates in diverse environmental conditions has been summarized in Table 2.6.

Astuti *et al*., performed the characterization and biosurfactant production using *Pseudoxanthomonas* sp. G3, a thermophilic bacterium from a heavy oil reservoir, was further simulated for MEOR application in the sand-packed column. Strains were screened depending upon some qualitative (oil-displacement assay) and semi-qualitative (emulsification index and IFT computation) parameters. Total 32 isolates were obtained after screening, among them *Pseudomonas* sp. G3 could produce the maximum biosurfactant which also showed a high EI of 73 % with light crude oil and lowered the IFT from 12.9 to 9.7 mN/m with an effectual CMC value of 0.73 g/L. The FTIR verified the glycolipid nature of the crude biosurfactant extract which was proved to be stable at the high thermal condition and in moderately halophilic surroundings and sustain over an extensive pH range. The most favorable condition for biosurfactant production was analyzed using the RSM-BBD model which studied the interactive effects of the parameters such as pH range 2 – 12, temperature 40 - 120 °C, and with 10 % (w/v) saline condition. The sand-packed column experimentation with biosurfactant flooding displayed 20 % additional oil recovery, which designated its potential in MEOR purpose (Astuti et al., 2019).

The prospective of biosurfactant producing *P. aeruginosa* PBS for MEOR was analyzed via the optimization of suitable medium constituents along with the process variables through two sets of experimental runs designed by RSM-central composite rotatable design (CCRD)

(Sharma et al., 2018a). The highest biosurfactant concentration was achieved with 2 % fresh inoculum in MSM (pH 7), supplementing 2.17 % sodium citrate as C-source and 0.5 % yeast extract as N-source, after 48 hours of incubation at 30 °C/150 rpm. Under optimized conditions, biosurfactant concentration was improved additionally threefold which turned out to be 2.65 g/L as compared to 0.82 g/L under earlier conditions. The biosurfactant was identified as a glycolipid consisting of four rhamnolipid congeners (Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_8-C_{10}, Rha-Rha-C_{12}-C_{10}/Rha-Rha-C_{10}-C_{12}, Rha-C_{10}-C_{10}) and extremely competent for oil recovery purpose displaying a great decline in ST from 72 to 24 mN/m, massive hydrocarbons emulsification capability (50 – 60 %) along with better stability at an extensive range of temperature (4 – 100 °C) and pH (4 – 10) as well as an exceptional (56.18 ± 1.59 %) additional oil recovery in lab-scale sand-pack column (Sharma et al., 2018a).

Sharma *et al*., optimized the biosurfactant production from *Bacillus subtilis* RSL 2 using RSM-CCD at pH 4, 25 °C, consuming 1 g/L crude oil as the sole substrate for a week, which was reported to be 3.6 ± 0.3 g/L. The produced biosurfactant characterized to be lipopeptide having a CMC value of 500 mg/L could enhance surface wettability of hydrophobic substrate by increasing surface energy from 30 ± 1 to 35 ± 1 mJ/m^2 as an indication that this strain could be further utilized in MEOR purpose. The impact of the produced biosurfactant on microbial oil degradation was studied by sequential and simultaneous modes. The presence of biosurfactant not only enhanced the oil bioavailability but also improved oil-microbes interaction when the simultaneous mode was applied. This approach increased overall oil degradation (72 %) and biosurfactant concentration (5.2 g/L) by 1.6 folds than the sequential mode (Sharma & Pandey, 2020).

Table 2.6. Statistical model-based optimization of media components and environmental parameters for biosurfactant production

Optimization technique	Microbes	Isolation sites	Experimental parameters & their ranges	Optimized media components	Optimized environmental conditions	Optimized outcomes	References
RSM-PBD and CCD	*Bacillus aryabhattai* ZDY2	Crude oil contaminated soil from Karnataka, India	$NaNO_3$ (0.75-1.5 %), KCl (0.1-0.22 %), NaCl (0.1-0.22 %), KH_2PO_4 (0.34-0.68 %), $MgSO_4 \cdot 7H_2O$ (0.05-0.1 %), yeast extract (0.05-0.1 %), glucose (1-2 %) and crude oil (1-2 %)	Crude oil 4 %, yeast extract 0.7 % and $NaNO_3$ 3 %	Stability upto 100 °C, at pH 5–10 and upto 8 % NaCl concentration	2.51 folds increase in lipopeptide production (8.86 g/L)	(Yaraguppi et al., 2020)
RSM- 3^3 full-factorial design	*Bacillus pumilus* IJ-1	Oil contaminated site from Taean, South Korea	NaCl (0, 0.5, 1 %, w/v), tryptone (0, 0.5, 1 %, w/v), and temperature (15, 20, 25 °C)	0.6 % (w/v) NaCl and 0.8 % (w/v) tryptone	Temperature 21.8 °C	54 % ST reduction was obtained (26 mN/m)	(Kim & Kim, 2020)
Biosurfactant concentration determination in the parameter range	*Agrobacterium fabrum* SLAJ731	Core sample of Assam oilfield	pH (4–10), incubation temperature (20–60 °C), C-source (Glucose, Sucrose, Glycerol, Molasses, Crude oil, and Hexadecane); N-source (Yeast extract, Urea, Ammonium sulfate, and Sodium nitrate)	Glucose: yeast extract (2:1)	pH 6, 30 °C	5.77 g/L lipopeptide with EI 65 %	(Sharma et al., 2019b)
RSM-CCRD, 3-level fractional factorial design	*Planococcus* sp. MMD26	Southwest coast of India	pH (4–9), incubation temperature (10–50 °C), NaCl (0.5–3.5 %) and incubation period (24–144 hours)	Glucose 4 %, ammonium nitrate 1%	pH 7, 30 °C	50 % EI after 48 hours due to glycolipid	(Hema et al., 2019)

RSM-CCD	*Bacillus subtilis* N3-1P	Hydrocarbon contaminated site of Atlantic Canada	Brewery waste (2-10 v/v), NH_4NO_3 (2-10 g/L), pH (4.75-8.75), agitation speed (0-400 rpm), temperature (18-42 °C)	7 % (v/v) brewery waste, 6.22 g/L ammonium nitrate	pH of 6.4, 150 rpm, and 27 °C	ST 27.31 mN/m and EI 63.11 % and 657 mg/L biosurfactant	(Moshtagh et al., 2019)
Taguchi-OFAT experiment	*Achromobacter* sp. TMB1	Crude oil contaminated soil of Tezpur, Assam	Temperature (23-60 °C), pH (4-10), C-sources glucose (1-3 % w/v), sucrose, kerosene, n-hexadecane, n-tridecane, and n-pentadecane N-sources $NaNO_3$ (3 % w/v), urea, NH_4Cl, ammonium sulphate, ammonium acetate and yeast extract (0.05-0.5 % w/v)	Glucose (1 %), $NaNO_3$ (0.05 %) and 0.05 % yeast extract	30 °C, pH 7.2 and 48 hours incubation	90.12 % of TPH removal after 20 days	(Haloi & Medhi, 2019)
OFAT	*Paenibacillus* sp. D9	Oil contaminated soil from Durban, South Africa	pH (4-10), temperature (25-60 °C) C-sources n-paraffin, n-dodecane, n-hexadecane, Sunflower oil Canola oil, Sucrose, Glycerol, Diesel fuel, n-tetradecane, Engine oil N-sources Ammonium sulfate, Sodium nitrate, Yeast extract, Urea, Peptone, Potassium nitrate, Beef extract C:N ratio 0.14 to 7	2 % (v/v) diesel fuel, 2 % (w/v) $(NH_4)_2SO_4$, C:N (3:1), 4 mM $MgSO_4$, and 1.5 % inoculum size	pH 7, 30 °C	4.11 g/L lipopeptide	(Jimoh & Lin, 2019)

Biosurfactant concentration determination	*Brevibacillus* sp. AVN 13	Crude oil spilled soil from Tamil Nadu, India	pH (4-8), temperature (30-70 °C), C-sources fried and normal sunflower oil, used engine oil, sesame oil, palm oil, normal and fried coconut oil N-sources peptone, yeast extract, ammonium nitrate, KNO_3 and $NaNO_3$	1 % (v/v) used engine oil and 0.5 % (w/v) KNO_3	pH 7, temperature 40 °C	76 % EI and 1.28 g/L lipopeptide was achieved	(Vigneshwaran et al., 2018)
OFAT and RSM-CCD	*Serratia marcescens*	Hydrocarbon contaminated soil in Melaka, Malaysia	pH (5-9), incubation time (24-168 hours), temperature (20-40 °C), agitation (20-200 rpm) Glycerol (3-7 %), peptone (2-6 g/L), $(NH_4)_2SO_4$ (3-7 g/L)	5 % glycerol, 4 g/L peptone and 5 g/L $(NH4)_2SO_4$	pH 8, temperature 30 °C, salinity 1 % and 200 rpm for 120 hours of incubation	1.5 g/L biosurfactant production minimum ST of 28 mN/m	(Almansoory et al., 2017)
Two-level fractional factorial design	*Acinetobacter junii* B6	Oil contaminated soil sample from Southwest Iran	Crude oil (1-5 %), $NaNO_3$ (0.2-2 g/L), inoculum (1-3 %), temperature (25-37 °C), aeration rate (150-300 rpm)	$NaNO_3$ (2 g/L), Iranian light crude oil 5 % inoculum size 2 %	25 °C, 300 rpm aeration	ST value was reduced to 38 mN/m from 65 mN/m	(Ohadi et al., 2017)

2.8.Recent Core Flooding Investigations Utilizing Biosurfactant Systems

Core flooding experiments have been described to simulate and assess reservoir conditions for the validation of MEOR practices. Four types of core setups are commonly used for oil recovery purposes such as natural reservoir cores, artificial cores, micro-models and sand-packed columns (Sun et al., 2011). Core flooding experiments are used to investigate the mobilization of residual oil due to microbial activity or its metabolite release both in the lab and field scale. The standard or native cores (collected directly from selected reservoirs) are connected to a continuous flow system and known as core flooding apparatus.

Inherent microbes isolated from the oil reservoir could be incubated easily in hefty batches in the laboratory imitating the reservoir environment and consequently injected into the reservoir. *B. licheniformis* was isolated from Zilaei oil reservoir of southwest Iran which could consume crude oil (API 37°) at 50 °C and subsequently produce glycolipid. The core flooding studies were executed in the sandstone core specimen (porosity of 16 % and permeability of 7.5 mD) and permitted to nurture for 7 days and then the glycolipid (18.1 mg/L) was introduced which resulted in 13.7 % of OOIP and reduce the oil viscosity by 41 % along with ST and IFT to 23.8 and 0.93 mN/m, respectively at optimum conditions (Daryasafar et al., 2016). The prospects of Pumilacidin synthesized by *Bacillus safensis* CCMA-560 (isolated from a mangrove microbiota) were analyzed for increasing oil retrieval via a sustainable economic eco-friendly approach in lab-scale MEOR. A lower CMC value of 96 $mg.L^{-1}$, the thermal consistency and 20 % ST reduction and 56 % IFT decline were observed which addressed the suitability of the biosurfactant in oil recovery. Core flooding tests were performed in Berea sandstone rock plug (4.16 cm × 3.81 cm) with 20.7 % porosity and 118.29 mD permeability also scrutinized the influence of this MEOR injection on the rock properties. The findings revealed that an alternating inoculation of a 1.3 CMC (30 % excess

of the CMC) biosurfactant solution with 3 wt % brine could permeate through the porous media and subsequently migrating to the water/oil interface and lower the IFT, and augment the oil recovery factor by 13 %. The total recovery factor was calculated to be 64 %, which proved the process efficiency to be elevated because of the suitable properties of the biosurfactant while interacting with the oil (de Araujo et al., 2019). *Bacillus licheniformis* TT42 was isolated from Tuva-Timba hot water spring, Gujarat, India. It could produce Lichenysin and reduced ST and IFT to 27 and 0.05 mN/m at CMC of 22 mg/L as well as resulted in 10.64 % additional oil recovery in core flooding experiments (Suthar & Nerurkar, 2016). MEOR column experiment was carried out with Surfactin (99.6 mg/L) producing *Bacillus subtilis* AB2.0 (isolated from sediment of beach in Rio de Janeiro, Brazil) using light crude oil (API 33.8°) at various temperatures of 39 °C, 55 °C and 71 °C in high salt concentration of 52.5 g/L (Alvarez et al., 2020). The oil recovery was found to be enhanced from 16.7 (39 °C) to 24 (55 °C) times higher than the control (without Surfactin) experiment.

This research gap between lab-scale and field-scale MEOR was addressed by conducting long-term large-scale field trials along with laboratory experiments to prove the feasibility of MEOR on an industrial scale. Halim *et al*., investigated the effect of microbial (*Bacillus licheniformis* 421) injections in Danish North Sea limestone reservoirs. A significant rise of 1 - 2.3 % and 7 - 8.8 % additional oil recovery was observed in homogenous cores (Ho 1, Ho 2, and Ho 3 having a porosity of 25.3 % to 42.9 %, 13.9-37.1 PV and the permeability from 0.5 mD to 6.4 mD) with (7.5 cm × 3.8 cm) and heterogeneous limestone cores (He 1 and He 2 having 17.4-19.4 PV) of (4.9 cm × 3.8 cm), respectively (Halim et al., 2015). Subsequent research highlighted the correlation of microbial growth and metabolite profile with the increase in oil production under anaerobic conditions. IFT

reduction to 0.6 mN/m and emulsion formation (6 - 29 %) was observed due to the presence of Lichenysin G which made the process feasible to conduct MEOR (Halim et al., 2017).

A reservoir-inhabiting facultative strain, *Luteimonas huabeiensis* sp. nov HB-2, was isolated from Baolige oilfield (heavy oil reservoir), which could grow at 35 - 50 °C (internal reservoir temperature) by consuming hydrocarbons, generate biosurfactants and simultaneously reduced ST and IFT of oil-water as well as oil viscosity with very low CMC and good emulsification activity. The produced cyclic lipopeptides could degrade the elongated hydrocarbons chain (C_{20} - C_{35}) of the crude oil and convert them into shorter chains (C_{10} - C_{19}) through metabolic activity, followed by the enhancement in oil mobilization. The consequence of MEOR was analyzed in a laboratory-scale core flooding column model (100 cm × 25 cm) for 14 days at 40 °C and 80 Bar pressure, possessing 140 ml PV, 29.2 % porosity, 0.59 μm^2 permeability, exhibiting an 11 % increase in average oil reclamation, that was followed by a field trial concerning two water injection wells (B51-91 and B51-13) and 8 oil production wells over 16 months, exhibited incredible enhancement in the average single well oil production from 0.48 ton/day (without MEOR) to 1.77 ton/day with the maximum attainment of 9.5 ton/day by MEOR and the cumulative EOR was reported to be 2300 tons from this field trials. These field trials anticipated a firm establishment for industrialized relevance in large-scale in Baolige oilfield. The research findings comprising both lab and field experiments could also be potentially implemented in other oilfields having a comparable geological and physical setting, to implement it in an even more large-scale MEOR process (Ke et al., 2018b). The performance of six facultative anaerobic bacterial isolates (from the Baolige oilfield of China) and their synergistic effect were assessed in the laboratory conditions and two consortia were formulated by combining *Rhodococcus* sp. JH and *Bacillus licheniformis* LC; another one with the combination of *Bacillus subtilis* HB3, *Pseudomonas aeruginosa* Z-2 and

Arthrobacter IV. The core flooding was conducted in a core tube (50 cm × 2.5 cm) possessing porosity of 26.45 % and 25.88 %, permeability of 0.23 and 0.25 μm^2, respectively. These two consortia reduced the oil viscosity by 35 % and 56 % and improved the oil recovery by 9.1 % and 13.2 % in core flooding, whereas the single strain could recover only 7 % to 8.7 %, respectively. In pilot-scale trials (conducted in four blocks - B19, B38, B48, B51), cumulatively 2.10×10^5 tons of crude oil was extracted from 169 production wells after 78 injections over 43 months using the selected consortia (Ke et al., 2018a). The contributing chief biosurfactant-producing microorganisms isolated from various terrestrial and aquatic environments worldwide, their maximum biosurfactant concentration and improvement of surface properties, their role in different types of core flooding set-up along with the improved oil retrieval have been tabulated in detail in Table 2.7.

Table 2.7. Detailed analysis of current core flooding experiments along with the contributing microbes and the biosurfactant systems

Microbes	Isolation sites	Produced metabolites	Surface properties	Core flooding substrates	Core properties (L×D in cm), Porosity, Permeability	Flooding type (conditions), (Pore volume)	Recovery	Reference
Consortium of *Enterobacter cloacae* and *Enterobacter hormaechei*	Formation water of Southwest Iranian oil reservoir	Biosurfactant (1.53 g/L)	ST and IFT reduction to 31 and 3.2 mN/m, respectively	MIS crude oil (API 35.93°), Gachsaran crude oil (24.2°)	Dolomite core (8 × 2.54), 14 %, 11 mD	*In-situ* (40 and 60 and 80 °C, 7 days), (8.1, 7.9 and 7.78 cc)	53.2 %, 56.2 % and 61.33 % of OOIP	(Hosseini & Tahmasebi, 2020)
Enterobacter aerogenes B19 and *Bacillus cereus* ISU-02	Formation water from Basra, South Iraq	Biosurfactant and bioemulsifer	ST was reduced to 27.61 and 28.93 mN/m	Crude oil (API 29°)	Sandstone core plug, (16 % and 24.8 %), (197 and 843 mD), respectively	*In-situ* (40 °C for 14 days), (4 and 4.4 cc)	34 % and 66.9 % oil recovery, respectively	(Hamzah et al., 2020)
Pseudomonas sp. TMB2	Oil-contaminated soil of Tezpur, upper Assam, India	Rhamnolipid (2.8 g/L)	ST and IFT were declined to 33.4 to 0.8 mN/m, CMC of 120 mg/L, wettability change from 75° to 42°	Light crude oil from ONGC, Jorhat, Assam, India	Sandstone core plug (8.9 × 3.74), 19.7 % - 22 %,	*Ex-situ* flooding (70 °C), (19.25-21.5 cc)	10.7 – 16.7 % additional oil recovery, residual oil recovery of 39 - 55 %	(Haloi et al., 2020)
Fungal strain *Trichoderma* sp. MK116452	Seri Chermin filling station, Brunei Darussalam	Glycolipid (6.33 g/L)	EI of 17.82 % and 7.3 cm oil displacement diameter	Crude oil	Sand pack column (50 g sand mixed with 15 mL crude oil in 100 mL Erlenmeyer flask)	*Ex-situ* biosurfactant flooding	63.33 % oil recovery in which only 16 % due to water-flood	(Shivanand et al., 2020)

Bacillus subtilis 22.2	NA	Surfactin	IFT of 0.056 and 0.110 mN/m for Oil-1 and Oil-2, Wettability from 71° to 35°	Two stock tank crude oil (API 27.3 and 41.6 °)	Sandstone core BS1(10.2 × 3.8), 21 %, 292 mD, BS2 (10.2 × 3.8), 21.5 %, 153mD	*Ex-situ* (25 °C)	1.3 - 5 % additional oil recovery	(Hadia et al., 2019)
B. licheniformis	Hydrocarbon contaminated soil from Beijing, China	Lichenysin-A (1.1 g/L)	ST and IFT reduction to 26.21 and 0.26 mN/m, CMC 21 mg/L, EI 66.4 %, wettability from 50° to 17°	Light crude oil was provided by Xinjiang oilfield, China	Sand pack column (4.5×5×17.5)	*Ex-situ* flooding, (59-62 ml PV)	32 % additional oil recovery	(Ali et al., 2019)
Bacillus licheniformis D S1	Oil reservoir in Indonesia	Lichenysin A	CMC 157.5 mg/L, EI = 94.28 %	Light crude oil from oil reservoir wellhead in Sumatra, Indonesia	Berea sandstone core (7.4 × 4.4), 12.84 %	*Ex-situ* flooding, (50 °C for 12 hours), (14.84 mL)	5.4 % additional oil recovery	(Purwasena et al., 2019)
Bacillus licheniformis W16	Soil sample near the Omani oil well	Lichenysin-A (0.52 g/L)	Reduced ST and IFT to 24.3 and 2.47 mN/m, wettability alteration from 56° to 19°	Light crude oil (API 36.51°) from Petroleum Development Oman (PDO)	Berea sandstone core (7.6 × 3.8) (18–22 %), (250–260 mD)	*In situ* (60 °C) (17-19 cc)	24-26 % residual oil recovery	(Joshi et al., 2016)

2.9.National Scenario of Biosurfactant Assisted MEOR

In India, mainly Cyclic microbial recovery (CMR) and Microbial selective plugging recovery (MSPR) techniques were employed for MEOR [44]. The Oil and Natural Gas Corporation (ONGC) Limited, in collaboration with The Energy and Resources Institute (TERI, New Delhi) and the Institute of Reservoir Studies (IRS), Ahmedabad, conducted some field trials by employing a Huff and Puff process and using an indigenously developed MEOR technology based on a consortium of halophilic, barophilic, thermophilic (upto 90 °C) and anaerobic extremophiles (*Clostridium* type *Thermoanaerobacterium* sp. and *Thermococcus* sp.) isolated from the candidate reservoirs and supplementing 3 % molasses as a nutrient. The field trials were carried out in 109 wells of 9 different fields of ONGC and 8 wells of Naharkatia oilfield of OIL, Duliajan, Assam. Total oil recovery of 61,000 m^3 was achieved after 6 - 8 months of operation (Patel et al., 2015; Woodward, 2006). The indigenously formulated IRS consortiums IRSM-1 and IRSM-2 were anaerobic thermophilic and halophilic (3 % salinity) bacterial mixture comprising small cocci and short rods (1.5 – 2.0 μm), with pH tolerance of 6 – 8.5 and upto 65 °C thermal tolerant. This microbial system could produce useful metabolites like fatty acids, biosurfactants and biogases in the oil fields. The field trials for MEOR through huff and puff were conducted in Badarpur (3 wells), Kosamba (1well) and Padra (1well) of the Mehsana asset belonging to Cambay basin. IRS further developed two more anaerobic consortia, NJS7-91 and NJS4-96, which were hyper-thermophilic (grew at 91 °C and 96 °C) and halophilic (grow in 7 % and 4 % salinity) and prepared from the microbes of formation waters of Nandej and Sobhasan wells of Ahmedabad and Mehsana oil fields (Patel et al., 2015).

Biosurfactant producing hyper-thermophilic *Clostridium* sp. N-4 was isolated from a high-temperature oil reservoir and produced biosurfactant over a wide range of pH (5–9), salinity (0–

13 %) at high temperature (80 - 100 °C) and optimum production of 1 g/L was observed at pH 7, 96 °C with 4 % salinity. The biosurfactant, characterized as glycoprotein reduced the ST by 32 mN/m at a CMC value of 100 µg/ml. The emulsion forming ability of the produced glycoprotein was examined with crude oils collected from different oil wells (from Shobhasan, Kalol (K3 and K4 crude oil), Gandhar and Viraj reservoirs in Gujarat state, India) and it showed almost 100 % when tested against three of the five types of crude petroleum oils along with 55 and 85 % against remaining two crude oils. The ability of *Clostridium* sp. N-4 in EOR was assessed in sand pack glass column studies in which mobilization of 17.15 % of residual oil saturation was observed with the crude biosurfactant flooding whereas 36.92 % of the residual oil was recovered after 14 days of flooding with N-4 microbial culture under the depleted high-temperature oil reservoir mimicking environmental conditions (Arora et al., 2019).

2.10. Global Scenario of Biosurfactant Mediated MEOR

The efficiency of the MEOR process depends on the subsequent parameters; formation temperature, crude oil viscosity, permeability, brine salinity, water cut, API gravity of crude oil, pH, pressure, residual oil saturation, depth, porosity, wax content and microbial content and diversity of the reservoirs (Sen, 2008). The constructive outcome or the success rate of the MEOR process significantly depends upon the microbial consortia present in reservoirs along with the reservoir category. The MEOR progression involves primarily hydrocarbon-consuming microbes (Saravanan et al., 2020). The suitable reservoir conditions to implement the MEOR process were described in various previous pieces of literature which could be generalized as the temperature below 93 °C, salinity 100,000 mg/L and permeability 75 mD (Yernazarova et al., 2016). The inoculum size, oxygen availability was also found to affect the MEOR process through cellular activities. MEOR has been attempted in more challenging reservoirs in China, i.e., high

temperature, high salinity, low permeability and heavy oil. In some of the field trials, reservoir temperature reached 80 °C, salinity as high as 46,000 mg/L, oil viscosity as high as 43,000 cP, and reservoir permeability as low as 25 mD. Field assessments proved that MEOR achieved accomplishments even under such adverse conditions (Gao, 2018). Due to the variation in the reservoir environmental conditions, different countries such as the US Department of Energy (US-DOE) (Bryant & Douglas, 1988), China National Petroleum Corporation (CNPC) (Guo et al., 2015; She et al., 2019) and Institute of Reservoir Studies (IRS), India (Patel et al., 2015) proposed standard parameter ranges for oil well selection to carry out MEOR process. The detailed information of the screening parameters of the reservoirs for an individual country to perform the MEOR process is represented in Table 2.8. Worldwide the MEOR trials are conducted by Microbial flooding recovery (MFR 33 %), Cyclic microbial recovery (CMR 27 %), Microbial selective plugging recovery (MSPR 18 %) as well as other methods are 22 % [43].

In-situ MEOR was carried out in various Chinese oilfields and the succeeding oil retrieval amount was very significant such as Fuyu (3392 tons in a year), Daquing (Sabei, Bohetai, Toutai, Chao-50, Lamadian, Fang-6 and Pubei block-1873 tons extra oil recovered), Dagang (Kongdian-2, Gangxi block), Xinjiang, Liaohe (Leng-43, Jing-35), Jingbian, Shengli (Luo-801, Zhan-3, Binnan and NG-3, Shan 12-block 108- 146 tons oil recovered in 6 months), Zhongyuan, Qinghai, Baolige, Chunfeng and Changqing (Maling block- 2500 tons extra oil recovered) oilfields. The reservoir characteristics and the probable success rate were also analyzed which was determined to be more than 70 % for MEOR which have been discussed elaborately in the previous works of literature (Gao, 2018; Gao & Zekri, 2011; Safdel et al., 2017). Pilot-scale testing of MEOR was carried out in the Daqing oilfield of north China in recent decades to analyze suitable reservoir conditions and application requirements. By the end of 2012, cumulative oil increments reached 1.2×10^5 tons

using MEOR in the Daqing oilfield (Chaoyanggou, Lamadian, Songfangtun and Sabei block). Single-well microbial huff-and-puff in 518 wells yielded a cumulative incremental oil production of 6.3×10^4 tons, and 10 projects with 45 well patterns adopted microbial flooding and profile modification to achieve a cumulative incremental oil production of 5.7×10^4 tons (Le et al., 2015). Among the San Andres project (Texas, USA), Tupungato-Refugio project (Medoza, Argentina), Xinjaing project (China), 2.5 % and 13 % oil recovery improvement were achieved for the first two projects, respectively (Nnaemeka et al., 2018).

Biosurfactant-producing strain *Pseudomonas aeruginosa* WJ-1, isolated from the formation water of Xinjiang oil reservoir was employed for its comparative investigation of *in-situ* and *ex-situ* MEOR. The strain was exhibited to be proliferating inside the columns, produced higher biosurfactant concentration (2.66 g/L) and reduced IFT. Smaller average diameters of emulsified oil droplets were determined in *in-situ* conditions than those of the *ex-situ* condition however, analogous wettability modification was measured in both the modes. The flooding tests in sand-pack columns proved that the recoveries in *in-situ* and *ex-situ* were 7.5 % OOIP and 4.6 % OOIP, respectively after 10 days. Therefore, the *in-situ* strategy was proved to have better potential in improving oil recovery in contrast to the *ex-situ* manner which suggested stimulating indigenous microbes rather than injecting microbial metabolites for implementing MEOR technologies (Cui et al., 2017a).

Two spore-forming strains: *Bacillus subtilis* AS2 and *Bacillus licheniformis* AS5, isolated from oil-saturated soil samples from a heavy oil (13.3 °API) field, Oman, were examined for their biotransformation potential i.e., converting heavier fractions of oil to lighter fractions for convenient oil recovery in both manners. In the flask scale aerobic biodegradation tests, MSM medium supplemented with glucose showed the highest biomass as well as crude oil

biodegradation whereas in anaerobic *in-situ* surroundings in Berea sandstone core-flooding trials, an additional 2.9 % and 3.1 % of residual oil saturation was recovered by *B. subtilis* AS2 and *B. licheniformis* AS5, respectively after 7 days of incubation. By increasing the incubation period and inoculum concentration for strain AS5, the oil recovery could be improved to 5 %. Furthermore, in glucose supplemented *ex-situ* MEOR, oil recovery was augmented to 16.4 % by AS5 (Al-Sayegh et al., 2017).

Table 2.8. Detailed characteristics of oil reservoir fields worldwide to carry out MEOR trials

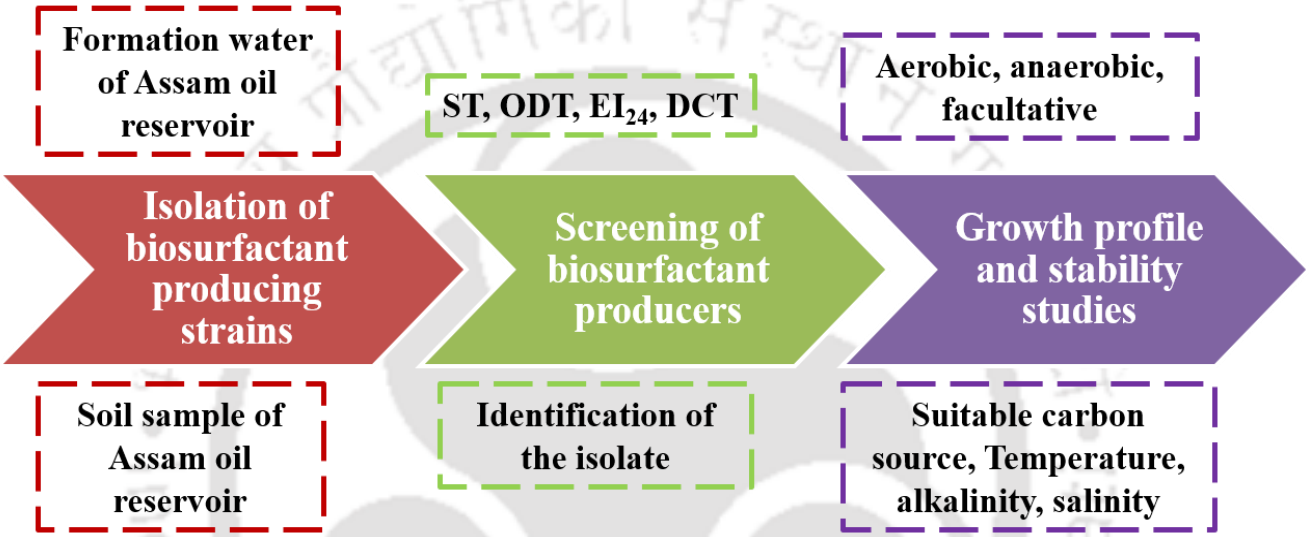
Countries	Parameters											
	Reservoir lithology	**pH**	**Temperature (°C)**	**Permeability (mD)**	**Porosity, %**	**Depth (m)**	**Pressure, (kg/cm^2)**	**Water cut, %**	**Salinity, (g/L)**	**Visco-sity, cP**	**°API gravity**	**References**
IRS	Sandstone	6-9	< 90	> 50		< 2400	< 300	30-90	< 10	< 20	> 20	(Patel et al., 2015; Safdel et al., 2017)
US DOE	Sandstone		< 71	> 100		< 3048			< 10		18-40	(Bryant & Douglas, 1988; Niu et al., 2020)
CNPC	Sandstone	6-8	30-60	≥ 150	17-25	2500		60-85	≥ 100	30-150		(Gao, 2018; Guo et al., 2015; He et al., 2018; She et al., 2019)
Iran	Carbonate		60	50	19.5	1450	1200	20		2000	14	(Kamari et al., 2014; Sari et al., 2019)
Argentina (Tupungato Refugio)	Sandstone		71	300	18	1800	100	63.5	42	9	28	(Gao & Zekri, 2011; Nnaemeka et al., 2018)
Norwegian Petroleum Directorate	Carbonate		61-155	1-20,000	11-35	1300-4208			14-273	0.1-4.83		(Awan et al., 2008; Safdel et al., 2017)
Romanian Academy (Bragadiru field)				150-300		780			60-300	9		(Sen, 2008)

2.11. Conclusions

The microbial activity in the lab investigations cannot be anticipated to be similar for the field scale; which makes it quite impractical to estimate the outcomes of the MEOR process in the fields. The disclosed challenges consist of (i) the deficiency of standard numerical models that can predict the MEOR process and assess the mechanism compared to field upscale, (ii) the insufficiency of techniques to monitor the behavior of the targeted microbes in oil reservoirs, (iii) activation of indigenous microbes by incorporating only economically feasible substrates into oil reservoirs and (iv) finally incapability to effortlessly search and screen appropriate bacteria to synthesize preferred metabolites required for EOR [30, 31]. Thus, focused research work should be performed based on the perception of the reservoir attributes, biochemical and physiological features of microbial community and economic aspects which are the prior requirements for MEOR practices to upscale the oil recovery [32]. Both industry and academia need to continue multidisciplinary and collaborative research on microbiology, petroleum engineering and process engineering to resolve the knowledge gaps or limitations which obstruct the implementation of MEOR process [33].

Chapter 3

Isolation, Screening and Identification of Oil-Utilizing and Biosurfactant-Producing Microbes from Indigenous Sources of the Assam Oil Reservoir Field



(*Bioresource technology* 270, 439-448, 2018 (Datta et al., 2018) and *Journal of Petroleum Science and Engineering* 195, 107612, 2020 (Datta et al., 2020))

This chapter discusses the isolation of microbial strains from indigenous sources of Assam oil reservoir, their screening based on the oil-utilizing capability and biosurfactant producing potential followed by their identification. The bacterial growth pattern was elaborately studied. Suitable carbon and nitrogen sources were chosen by employing various possible economical substrates for growth. Then the optimal favorable conditions for the biosurfactant production were predicted by analyzing their stability in a wide range of environmental parameters. The surface activities of these biosurfactant-synthesizing strains were further scrutinized with the aim of analyzing their promising potential for enhanced oil recovery applications.

3.1.Introduction

An extensive review has been carried out in Chapter 2 regarding the implications of conventional oil recovery and hence the significance of enhanced oil recovery (EOR) with the insight of microbial enhanced oil recovery (MEOR) by employing biosurfactants (Banat, 1995; Belyaev et al., 2004; Sen, 2008; Urum et al., 2004). In MEOR, suitable microbe-generated products (metabolites) are utilized to retrieve the residual oil from the depleted reservoirs (Hong et al., 2019a) which have been acclaimed worldwide for their economical, sustainable and eco-friendly perspectives (Safdel et al., 2017). The EOR scheme with the help of biosurfactants is considered to be one of the recent strategies. Biosurfactants are surface-active diverse groups of molecules that mainly consist of lipopeptides and lipoproteins, glycolipids, fatty acids, phospholipids, natural lipids, polymeric and particulate biosurfactants (Desai & Banat, 1997; Kiran et al., 2010). They can reduce the surface tension (ST) and interfacial tension (IFT) and simultaneously contribute to the wettability alteration of the surface (Halim et al., 2017) as well as enhance the emulsion formation (Dorobantu et al., 2004). Primarily glycolipid (Rhamnolipid) producing *Pseudomonas aeruginosa* and lipopeptide (Surfactin) synthesizing *Bacillus subtilis* have been reported to be majorly contributing in MEOR trials at laboratory and pilot scales because they possess excellent surface-active properties to mobilize entrapped oil (Sen, 2008).

Various biosurfactant producing strains have been isolated from different oil reservoir fields worldwide (Table 3.1) such as *Enterobacter cloacae, Enterobacter hormaechei* from formation water of southwest of Iran oil reservoirs (Rabiei et al., 2013), *Pseudomonas aeruginosa* SG from Xinjiang oilfield China (Zhao et al., 2016), *Bacillus subtilis* RI4914 from formation water of Brazilian oil field (Fernandes et al., 2016), *Bacillus licheniformis* from water samples from Niage field of Egypt (El-Sheshtawy et al., 2015a), *Pseudomonas aeruginosa* (WJ-1), *Bacillus subtilis*

(H10) and *Rhodococcus erythropolis* (Z25) from formation water of Chinese petroleum reservoir (Xia et al., 2011), and *Bacillus subtilis* B30 from petroleum-contaminated soil samples in Oman (Al-Wahaibi et al., 2014). This indicated the presence and suitability of distinct biosurfactant-producing microorganisms in various geographical locations (Balan et al., 2017).

The availability of diverse biosurfactant-synthesizing strains has accelerated the exploration of oil-utilizing and biosurfactant-producing strains from the indigenous sources of the Assam oil reservoir. Easily available, sustainable and economical raw materials were employed as the substrate (Dhanarajan et al., 2017) for bacterial growth. The surface properties such as surface tension (ST), interfacial tension (IFT) reduction capability (Al-Wahaibi et al., 2014; Alvarez et al., 2015; Pereira et al., 2013), emulsifying activity, oil displacing ability and drop collapsing capacity were studied to screen the potential biosurfactant producing strains. Biosurfactant productions using the identified strains have been studied under different growth conditions (media, pH, temperature, salinity). Their sustainability was also accessed for *in-situ* and *ex-situ* conditions (Gudina et al., 2013; Rabiei et al., 2013). In most of the previous studies, the biosurfactant yield was not so remarkable and it took a longer duration for the maximum oil degradation so the biosurfactant production by employing cheap and easily available substrates was monitored throughout in order to achieve the optimum production of the value-added product (biosurfactant).

Table 3.1. Summary of various microbial strains isolated from different geographical locations with their surface properties

Strains	Isolation site	Optimum culture condition	Yield	Surface tension, CMC	Oil degradation %	References
Acinetobacter baylyi ZJ2	Crude oil-contaminated soil sample in China	pH 6 - 7, salinity 4 %	NA	35 mN/m	NA	(Zou et al., 2014)
Bacillus licheniformis	Oil reservoir, Egypt	pH 7, 10 % salinity	1 g/L	36 mN/m	NA	(El-Sheshtawy et al., 2015a)
Bacillus licheniformis Y1	Dagang Oilfield, China	40 °C, 30 g/L NaCl, pH 7	NA	27.26 mN/m, 40 mg/L	60.2 % in 5 days	(Liu et al., 2016a; Liu et al., 2016b)
Bacillus methylotrophicus USTBa	Petroleum reservoir in Northeast China	2 % crude-oil at 35 °C and 180 rpm after 192 h	1.8 g/L	28.0 ± 0.1 mN/m	92 % crude oil degradation in 2 weeks	(Chandankere et al., 2013) (Chandankere et al., 2014)
Bacillus subtilis, *Pseudomonas aeruginosa*, and *Rhodococcus erythropolis*	Formation water of Chinese petroleum reservoir	pH 5, 20 g/L salinity	2.66 g/L	22.56 mN/m	NA	(Xia et al., 2011)
Corynebacterium variabile PG-Z	Persian Gulf, Iran	ONR7a medium	NA	43.4 mN/m	82 % after one week	(Hassanshahian et al., 2014)
Pseudomonas aeruginosa 112	Brazilian oil field	Corn steep liquor (10 %) and molasses (10 %)	3.2 g/L	30 mN/m, 50 mg/L	55 ± 3.4 % in 144 h	(Gudiña et al., 2015)
Pseudomonas sp. SWP- 4	Sludge sample from a local sewer, China	4 % crude oil in MSM at 30 ºC, 150 rpm for 72 h	6.87 g/L	22.7 mN/m	90 % n-alkane degradation in 7 days	(Lan et al., 2015b) (Lan et al., 2015a)
Virgibacillus salaries KSA-T	Jeddah region, Saudi Arabia	40 °C, pH 9, 4 % NaCl	1.64 ± 0.25g/L	30 mN/m	NA	(Elazzazy et al., 2015)

3.2.Materials and Methods

3.2.1. Chemicals and Reagents

For bacterial isolation, the formation water, reservoir soil and crude oil (API gravity 27.06º) samples were collected from the upper Assam oil reservoir field, India. The detailed characterization of the samples has been carried out previously in our lab and reported earlier (Saha et al., 2017b). The same formation water and soil samples were utilized for isolation purposes. Crude oil (API gravity of 27.06º) was utilized as the sole carbon source during the course of isolation. The major chemicals and reagents used were of analytical grade quality. Bushnell haas (BH) media (M350-500G), Luria bertani (LB) media (M1245-500G), mineral salt media (MSM) (M1864-500G), NaCl (GRM031-500G), NaOH (GRM467-500G), KNO_3 (GRM402-500G), *n*-hexadecane (RM2238-100ML), glucose (MB037-500G), light paraffin oil (GRM1310-500ML), heavy paraffin oil (GRM6362-500ML), glycerol (MB060), agar powder (GRM026), *n*-hexane (AS097), ethyl acetate (AS051), hydrochloric acid (AS004) and agar powder (GRM026-500G) were procured from HiMedia Laboratories, India.

3.2.2. Isolation and screening of crude oil-utilizing microbial isolates

Bacterial isolation from the formation water sample of the Assam oil reservoir was carried out by dilution plating method depending upon their crude oil utilizing property. Bushnell haas (BH) media supplemented with 1 % (v/v) crude oil (Assam crude oil) as the sole carbon source, was used for the isolation purpose (Gudiña et al., 2012). For the isolation of bacterial strains from the soil sample of the Assam oil reservoir, the crude oil utilizing ability of the isolated strains was investigated by gradually increasing the crude oil concentration from 1 to 5 % (v/v). The screening tests were performed for the isolated strains which could withstand 5 % (v/v) crude oil concentration. The screening of these isolates was carried out further based on their surface

properties such as surface tension (ST) measurement, oil displacement activity, drop collapse test, emulsification index and biosurfactant concentration determination (Datta et al., 2018).

3.2.2.1.Surface tension measurements

Isolated strains were incubated in MSM with 1 % NaCl and 1 -5 % crude oil at 37 ºC and 180 rpm for 96 hours. Then the culture was centrifuged at 12,000 rpm for 15 minutes and the cell pellet was discarded. The cell-free supernatant was taken for the surface tension measurement using a tensiometer (Make: Dataphysics, Model: DCAT 11EC) at 25 °C by Du Nouy ring method (Gudina et al., 2010; Margaritis et al., 1979).

3.2.2.2.Oil displacement test

An oil displacement test was performed to determine the ability of the biosurfactant to form a clear zone. Distilled water (~20 ml) was poured into the Petri plate and a thin layer of the oil was prepared over the water surface. Then 10 µl cell-free supernatant was added to it and immediately a clear zone was observed. In the control experiment, 10 µl of water was used in place of the biosurfactant-containing solution. The diameter of the clear zone on the oil surface correlates to the biosurfactant oil displacement activity (Morikawa et al., 1993).

3.2.2.3.Drop collapsing test

To know the capability of the cell-free supernatant to deform the oil droplet, drop collapsing test was performed. Approximately 2 µl of light paraffin oil was added to 96 well microtiter plates. The plate was equilibrated at 37 ºC for 1 hour. Then 5 µl culture supernatant was added to the surface of the oil. 5 µl distilled water was added as the control experiment. The plates were observed after 1 min. The positive result indicates that the supernatant can collapse the oil drop (Kiran et al., 2009; Youssef et al., 2004).

3.2.2.4.Emulsification index (EI) determination

The emulsification index measures the effectiveness of a biosurfactant at different temperatures, pH and salinity. To measure the emulsification index, a homogenous hydrophobic agent was used to make the system less heterogeneous in terms of the properties of the liquid involved. Crude oil is hydrophobic with compositions in the wide range of carbon numbers which is specific to a reservoir. Thus, hexadecane was chosen as a model hydrophobic agent. Hexadecane of analytical grade also eliminates the possibility of any bacterial contamination.

The emulsification activity was determined by the mixing of an equal volume of hexadecane and cell-free supernatant. The mixture was homogenized in a vortex for 2 minutes by vigorous shaking and then kept idle for 24 hours at room temperature. The emulsification activity was determined as followed by Equation (3.1) (Cooper & Goldenberg, 1987).

$$EI\ \% = (\text{Height of the emulsified layer}/\text{Total height of the liquid column}) \times 100 \qquad (3.1)$$

3.2.3. Identification of screened microbial isolates

After the strain isolation, depending upon the screening parameters, three and two of the seven strains were identified from the formation water sample and the soil sample, respectively and the best ones were chosen to carry out the further studies. HiPurA bacterial genomic DNA purification kit (HiMedia) was used for isolating the genomic DNA in the laboratory. Then 16S rRNA sequencing was performed using universal primers 27F (forward seq) and 1492R (reverse seq) by Europhins, India. The obtained specific sequences were compared with known 16S rRNA using BLAST. Sequence alignment was performed by Clustal Omega and, the phylogenetic tree was constructed using neighbor-joining methods through MEGA version 6.0 (Dastager et al., 2009).

3.2.4. Bacterial growth profile and biosurfactant production

The growth kinetics study of the selected strain was carried out in Luria bertani (LB) media to analyze the change in bacterial biomass or cell population over a certain period of time in a culture in aerobic (at 37 ºC, 180 rpm agitation), facultative (at 37 ºC, without agitation) and anaerobic (at 37 ºC) conditions. Seed culture (1 % v/v) was used for the growth experiments. The absorbance (optical density, OD) data were measured at 600 nm using Cary 100 UV–vis spectrophotometer for 96 hours at an interval of 4 hours (Najafi et al., 2011). In these three conditions, the subsequent surface tension readings were also measured every 8 hours to ensure continuous biosurfactant production and suitable readings are reported. The subsequent biosurfactant concentrations were also measured every 12 hours.

Separation of the produced biosurfactant from cell-free culture broth was performed by acid precipitation followed by solvent extraction. The cell-free supernatant was obtained by centrifugation at 13,000 rpm for 10 minutes, which was acidified at pH 2 with 2 M HCl. Then liquid-liquid extraction was performed by mixing the supernatant with an equal volume of ethyl acetate and the sample was kept for shaking where two layers were formed: bottom aqueous layer and top organic (ethyl acetate) layer. The top organic layer containing the biosurfactant was separated after extraction and transferred to a round bottom flask connected to the rotary evaporator to remove the solvent. After the evaporation, the concentrate was obtained and referred to as crude biosurfactant (Radzuan et al., 2017).

3.2.5. Estimation of suitable carbon sources and favorable culture conditions

The influence of various environmental parameters on biosurfactant production was accessed in terms of surface tension reduction and crude biosurfactant concentrations. The effect of various carbon sources on the bacterial growth and biosurfactant production were analyzed by

supplementing the bacterial culture with glucose, hexadecane, crude oil, glycerol, light and heavy paraffin oils as the carbon source at the concentration range of 1 - 5 % (v/v, for glucose w/v). The experiments were conducted for 96 hours incubation at pH 7, 37 ºC and 180 rpm agitation in mineral salt media (MSM) with 1 % NaCl. Specifically, the effect of pH, temperature and salinity was examined by growing the bacteria in the media with previously optimized carbon and nitrogen sources for 96 hours (Table 3.2). For the pH optimization, the growth of the selected strain from the soil sample was maintained at 37 ºC with 1 % NaCl and the pH was varied from 3 to 11. Likewise, the temperature optimization was carried out in the range of 25 ºC – 65 ºC at previously optimized pH with 1% NaCl. Finally, the optimum salinity was determined by growing the bacterial culture from 0 – 60 g/L in the previously optimized pH and temperature.

At the end of the experiments, the minimum surface tensions or biosurfactant concentrations were determined and the optimum carbon sources were chosen to continue the next set of experiments.

Table 3.2. Summary of the culture conditions of the bacterial growth experiments

Parameter	Range	Culture conditions
pH	3 - 11	3 % light paraffin oil, 37 ºC, 1 % NaCl
Temperature	30 - 70 ºC	3 % light paraffin oil, pH 9, 1 % NaCl
Salinity	0 - 30 g/L	3 % light paraffin oil, 60 ºC, pH 9

3.3.Results and Discussion

3.3.1. Isolation, screening and identification of efficient biosurfactant-producing strains

The bacterial strains were isolated from the formation water and soil sample of the Assam oil reservoir field by the dilution plating method (Datta et al., 2018) using BH media. Throughout the isolation from formation water, the strains were supplemented with 1 % (v/v) crude oil as the carbon

source and almost seven bacterial colonies were observed in the petri-plates which were screened depending on better surface-active properties (ST, oil displacing diameter, drop collapsing activity and EI). The more tolerance to higher concentrations of crude oil, the better would be the crude oil utilizing capability as well as biosurfactant production ability. During the isolation from the soil sample, the strains were initially supplemented with 1 % (v/v) crude oil as the carbon source and almost fifteen bacterial colonies have appeared in the petri-plate. The number of bacterial colonies decreased from 15 to 7 at a crude oil concentration of 5 %. So, the gradual increase in crude oil concentration was considered a kind of primary screening for efficient biosurfactant-producing strains. These isolates were also screened for their ability to produce biosurfactants by utilizing crude oil as a carbon source. The obtained results of the screening parameters for the seven isolated strains from the formation water as well as soil sample are summarized in Table 3.3 and Table 3.4, respectively.

Table 3.3. Summary of the screening methods for isolated strains from formation water

Strain No.	Surface Tension (mN/m) ± S.D.	Oil Displacement Test (cm) ± S.D.	Drop Collapse test	EI ± S.D. (%)
1	39.38 ± 0.03	5.3 ± 0.14	yes	45.86 ± 0.8
2	44.77 ± 0.96	4.5 ± 0.28	no	33 ± 3.46
3	48.28 ± 0.74	3.7 ± 0.21	no	25.87 ± 1.62
4	40.52 ± 0.39	4.3 ± 0.21	no	38.2 ± 1.7
5	37.49 ± 0.31	5.7 ± 0.07	yes	57.2 ± 1.6
6	38.72 ± 0.07	4.5 ± 0.14	yes	47.33 ± 2.51
7	**35.92 ± 0.13**	**6.6 ± 0.21**	**yes**	**71.87 ± 1.02**

The last three strains (5, 6, 7) of Table 3.3 showed comparatively better surface-active properties hence the molecular characterization of these strains was performed by amplifying and sequencing the 16S rRNA and compared with the database of known 16S rRNA sequences. Two strains (strains 6 and 7) belonged to *Bacillus* sp. and the other (strain 5) was identified to be *Stenotrophomonas* sp. The sequences of the strains were submitted to the GenBank sequence database of the National Centre for Biotechnology Information (NCBI). The strains 5, 6 and 7 in Table 3.3 were identified with the GenBank accession number, *Stenotrophomonas* sp. MG 520349, *Bacillus subtilis* MG 520348 and *Bacillus subtilis* MG 495086, respectively.

The surface tension of the supernatant of *Bacillus subtilis* MG495086 (Strain 7) was reduced to 35.92 ± 0.13 mN/m and 6.6 ± 0.21 cm clear zone was observed during the oil displacement test. The emulsification index (EI) was also found to be the highest (71.87 ± 1.02 %) for this strain among all the other strains mentioned in Table 3.3. Hence, it could be interpreted from the experiments that due to better surface-active properties feature, the *Bacillus subtilis* MG 495086 is the best among all these isolated strains and was chosen as the potential strain for further studies. The phylogenetic tree of *Bacillus subtilis* MG 495086 is shown in Figure 3.1.

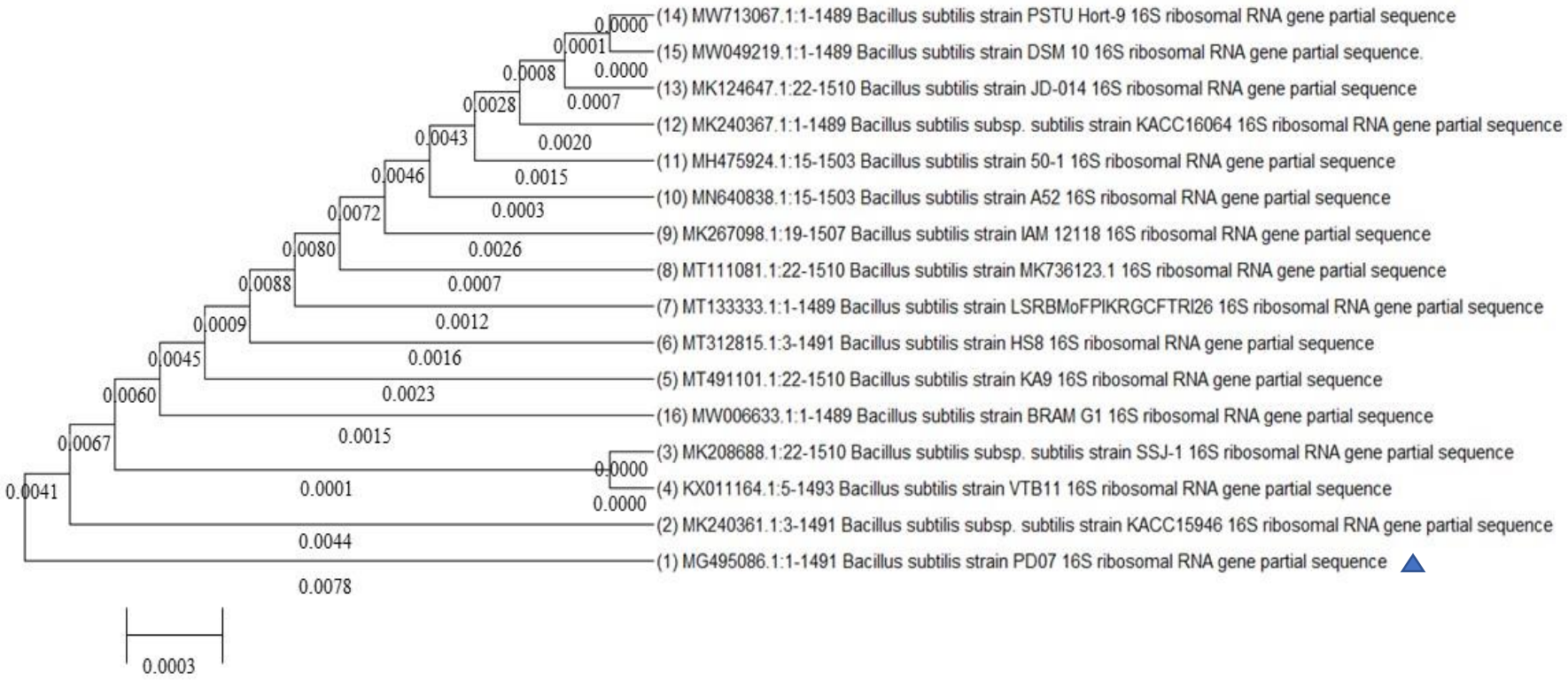


Figure 3.1. Neighbour-joining phylogenetic tree of *Bacillus subtilis* MG 495086. The scale bar corresponds to 0.003 which is the estimated nucleotide substitution per sequence position.

Table 3.4. Summary of the screening parameters with the biosurfactant concentration of biosurfactant producers isolated from the reservoir soil sample

Strain No.	Surface Tension (mN/m) ± S.D.	Oil Displacement Test (cm) ± S.D.	Drop Collapse test	EI ± S.D. (%)	BS Conc. ± S.D. (g/L)
1	43 ± 2	2.9 ± 0.2	No	35 ± 3 %	3.34 ± 0.84
2	40 ± 1	3.8 ± 0.1	No	41 ± 2 %	3.72 ± 0.66
3	**30 ± 2**	**7.1 ± 0.2**	**Yes**	**66 ± 2 %**	**6.87 ± 0.42**
4	38 ± 1	4.3 ± 0.1	Yes	45 ± 3 %	4.23 ± 1.21
5	42 ± 1	3.6 ± 0.1	No	41 ± 2 %	3.51 ± 0.85
6	46 ± 2	2.8 ± 0.1	No	33 ± 3 %	2.59 ± 1.27
7	**32 ± 1**	**6.7 ± 0.1**	**Yes**	**61 ± 2 %**	**6.26 ± 0.81**

Strains 3 and 7 of Table 3.4 showed reasonably better surface-active properties than the other isolates and exhibited reduced ST of 30 ± 2 and 32 ± 1 mN/m and moderate EI of 66 ± 2 and 61 ± 2 %, respectively. Hence the molecular characterization, as well as the strain identification, was performed for both these strains. The nucleotide sequences of strains 3 and 7 were submitted to NCBI and were provided with unique accession numbers of *Bacillus tequilensis* MK 729017 and *Bacillus subtilis* MK 729018, respectively. As *Bacillus tequilensis* MK 729017 (strain 3) exhibited the best surface-active properties, hence further studies were carried out with this strain. The phylogenetic tree of *Bacillus tequilensis* MK 729017 is shown in Figure 3.2. *Bacillus tequilensis* is an anaerobic, gram-positive, spore-forming, rod-shaped species closely related to *Bacillus subtilis* by conventional biochemical analysis (Gatson et al., 2006). Earlier, *Bacillus tequilensis* isolates were found to be positive for *sfp* gene through polymerase chain reaction (PCR) screening, which is responsible for biosurfactant (Surfactin) synthesis (Porob et al., 2013). In fact, these strains have also been explored previously for the production of biosurfactants (Cortés-Camargo et al., 2016; Pathak et al., 2014a).

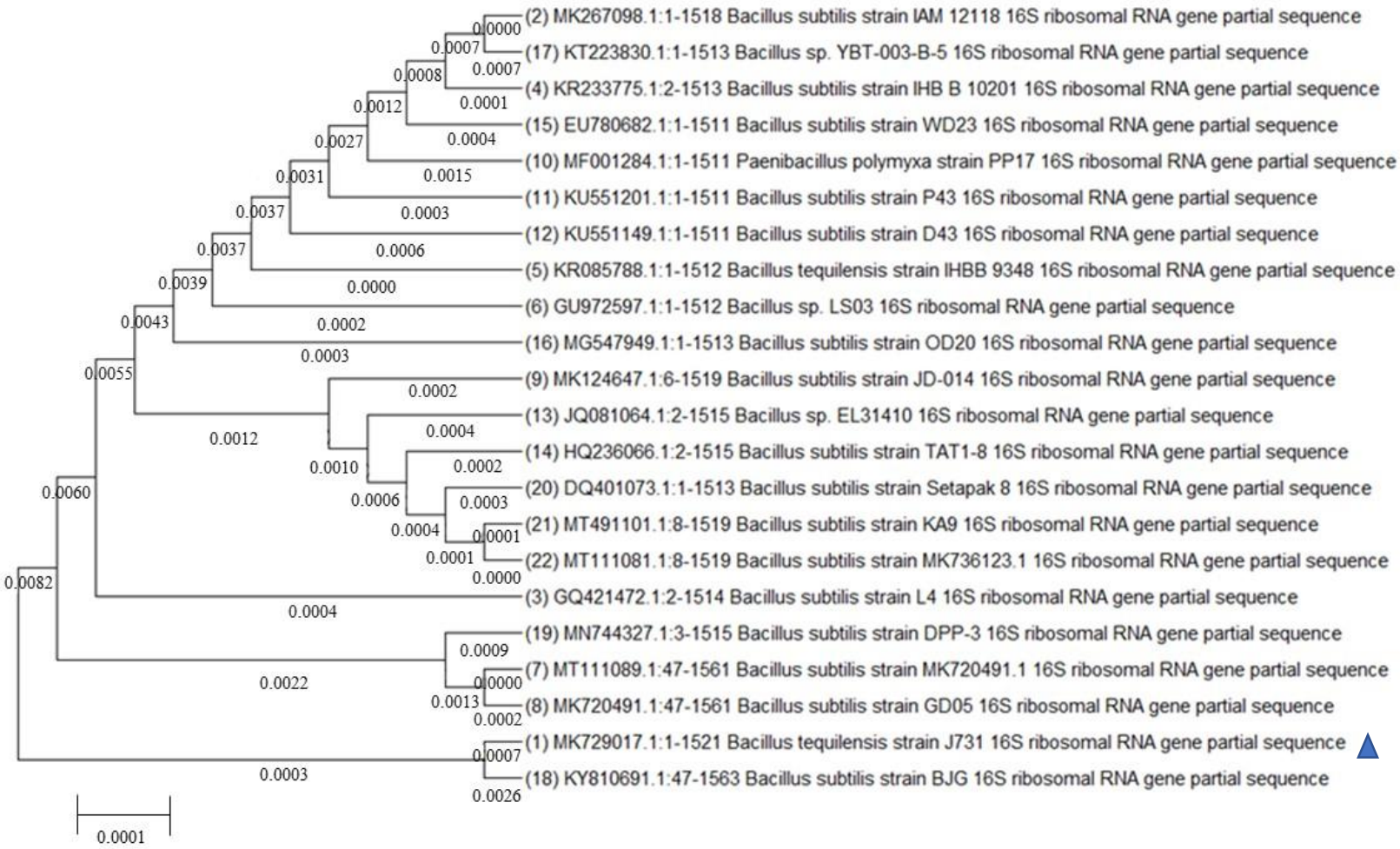


Figure 3.2. Neighbour-joining phylogenetic tree of *Bacillus tequilensis* MK 729017. The scale bar corresponds to 0.001 which is the estimated nucleotide substitution per sequence position.

3.3.2. Bacterial growth profiles

Figure 3.3 (a) shows the bacterial growth profile in three different conditions (aerobic, anaerobic and facultative) at 37 ºC, 180 rpm and it was observed that in the initial hours the bacterial count was increased slightly because of its adaptation to the new environment. The maximum biomass was also found to be 1.5 and 1.3 folds higher in aerobic conditions than anaerobic and facultative conditions, respectively so the rest of the experiments were carried out in the aerobic condition. The strain *Bacillus subtilis* MG 495086 showed an ideal sigmoidal bacterial growth pattern in aerobic conditions (Chandankere et al., 2013). The bacterium grew rapidly in the exponential phase of the aerobic growth curve (16 - 48 hours) and further reached to stationary phase. The specific growth rate ($\mu = (dX/dt)/X$) was found to be 0.08 ± 0.01 hour^{-1}. Figure 3.3 (a) also shows the relationship between bacterial growth in aerobic conditions and the subsequent surface tension measurements. The maximum optical density was found to be 1.78 ± 0.01 in aerobic conditions at 40 hours and its corresponding surface tension value was 32.49 ± 0.05 mN/m.

The growth profile of *Bacillus tequilensis* MK729017 was studied in LB media, which also exhibited a typical sigmoidal growth pattern (Figure 3.3 b). The specific growth rate (μ) was found to be 0.21 ± 0.01 hour^{-1}. The biosurfactant concentration data showed a linear relationship with the cell absorbance data. The biosurfactant production started from the log phase itself and it reached the maximum value of 6.87 g/L at 60 hours (at the end of the stationary phase), indicating a growth-associated production (Purwasena et al., 2019).

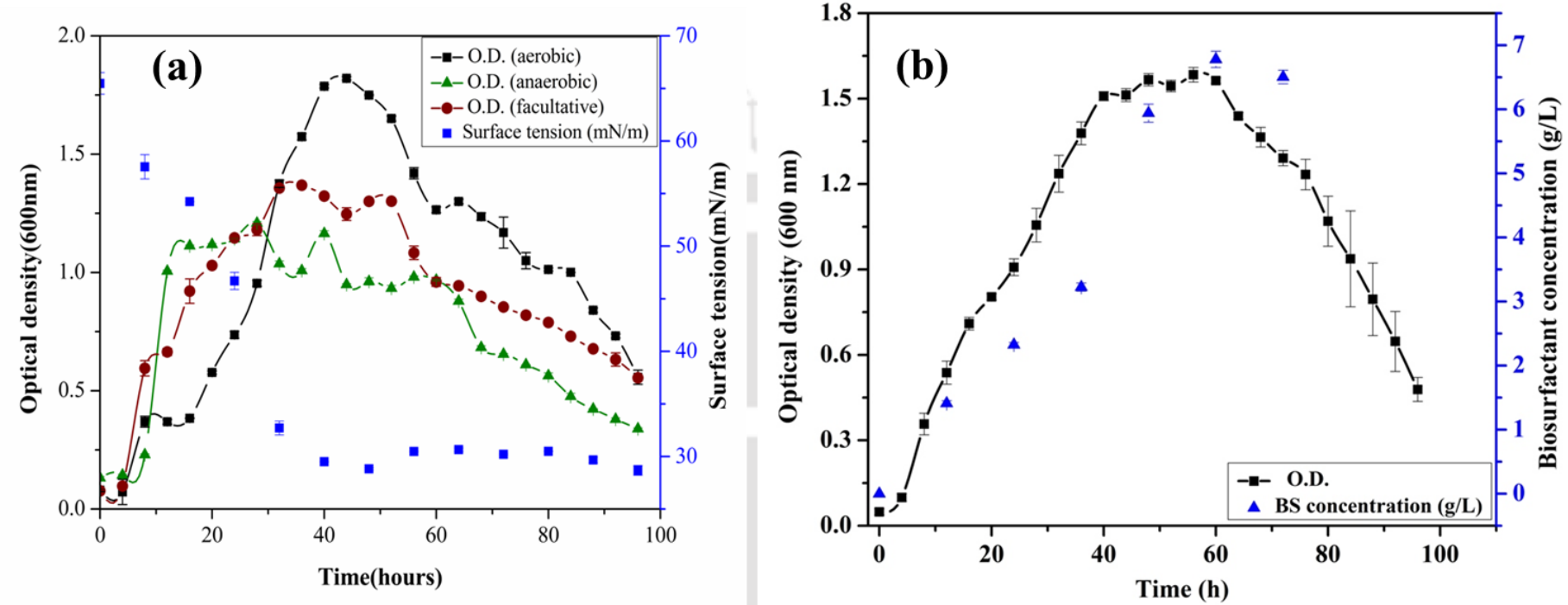


Figure 3.3. (a) Growth kinetics profile of *Bacillus subtilis* MG 495086 in three different conditions and the subsequent surface tension values in the aerobic condition, (b) Profile showing the bacterial growth kinetics of *Bacillus tequilensis* MK 729017 in Luria Bertani, (LB) media at 37 °C at 180 rpm and the subsequent biosurfactant concentration under aerobic condition

3.3.3. Selection of suitable carbon sources

Figure 3.4 (a) depicts the media optimization study by measuring the surface tension reduction supplementing various carbon sources (glucose, heavy paraffin oil, crude oil, hexadecane and light paraffin oil) in the range of 1 – 5 % (v/v) with the MSM media. A common trend was observed that the surface tension got reduced till 3 % of carbon source and again started to increase with 4 % or 5 % carbon source. This is probably due to substrate or product inhibition. The least surface tension was found to be 30.44 ± 0.28 mN/m at pH 7, 37 ºC and 1 % NaCl with 3 % light paraffin oil after 72 hours of incubation. The least value of surface tension with light paraffin oil, compared to other carbon sources considered, may be due to the nature of indigenous strain isolated from the formation water produced with crude oil which contains a significant amount of paraffin.

Figure 3.4 (b) represents the carbon source optimization study to achieve the maximum biosurfactant concentration employing diverse substrates for 96 hours. The production media used for the biosurfactant was MSM supplemented with different C-sources such as glucose (w/v), crude oil (v/v), glycerol (v/v), light and heavy paraffin oils (v/v). The concentrations of the produced biosurfactants (g/L) with different 4 % C-sources were found as follows: glycerol (5.93 ± 0.1) > heavy paraffin oil (5.55 ± 0.1) > glucose (5.49 ± 0.1) > light paraffin oil (5.30 ± 0.1) > crude oil (5.21 ± 0.1). The maximum biosurfactant concentration was obtained with glycerol, which is a cheaper C-source and also a by-product of trans-esterification processes (de Faria et al., 2011; Sharma et al., 2018d). This can also contribute to the cost-effectiveness of the production of biosurfactants.

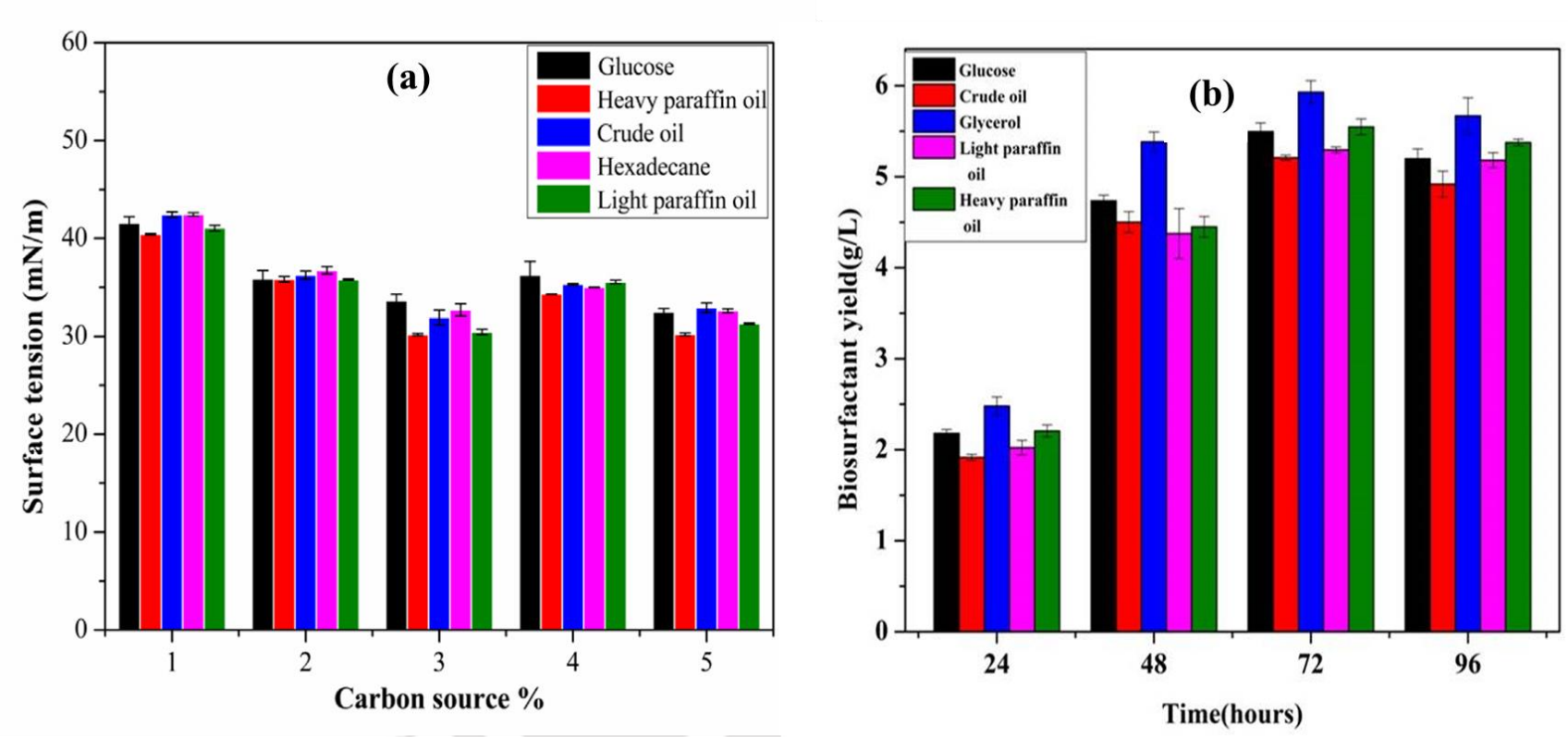


Figure 3.4. The carbon source optimization study of (a) *Bacillus subtilis* MG 495086 by measuring surface tension with glucose, heavy paraffin oil, crude oil, hexadecane, light paraffin oil, (b) *Bacillus tequilensis* MK 729017 by determining biosurfactant concentration with glucose, crude oil, glycerol, light and heavy paraffin oil.

3.3.4. Effect of pH, temperature and salinity

Figure 3.5 shows the effect of environmental conditions such as pH, temperature and salinity on the surface tension for *Bacillus subtilis* MG 495086 in MSM with previously optimized 3 % light paraffin oil. In the pH variation experiment shown in Figure 3.5 (a), the surface tension of the supernatant gradually decreased till pH 9 but as the pH value increased, the corresponding surface tension was also increased. The least surface tension (32.13 ± 0.15 mN/m) was achieved after 72 hours of incubation at pH 9. In the temperature variation experiment as shown in Figure 3.5 (b), the same trend followed till 60 ºC but after that, the surface tension values again started to increase. The lowest surface tension value was found to be 30.19 ± 0.03 mN/m at 60 ºC and pH 9. The influence of salinity on the biosurfactant-producer was examined with a wide range of NaCl concentrations and the surface tension values varied in the range of 29 - 32 mN/m (Figure 3.5 (c)). The minimum surface tension was observed after 72 hours with 6 g/L of NaCl concentration indicating the moderate halophilic nature of the strain. According to the literature, the salinity of the oil reservoirs is about 10 g/L (Yernazarova et al., 2016). So, the salt concentration was maintained at 10 g/L for further experiments.

Similarly, Figure 3.6 shows the impact of environmental conditions such as pH (Figure 3.6 a), temperature (Figure 3.6 b) and salinity (Figure 3.6 c) on the biosurfactant concentration produced by *Bacillus tequilensis* MK 729017 in MSM with previously optimized 4 % glycerol and 3 % potassium nitrate.

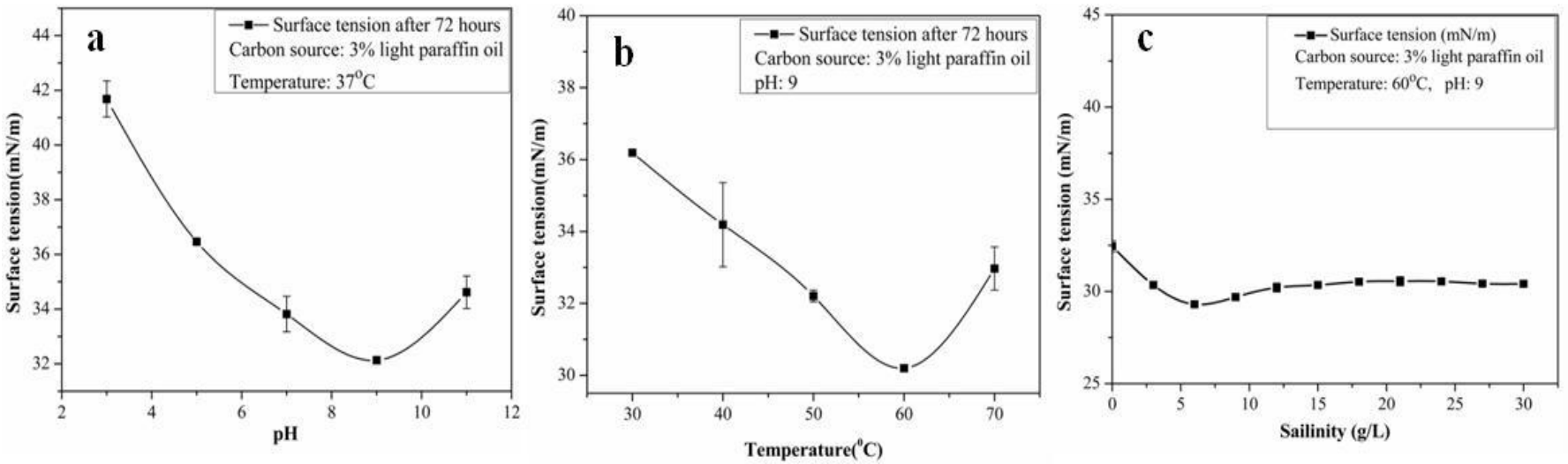


Figure 3.5. Effect of (a) pH, (b) temperature and (c) salinity on the production of biosurfactant produced by *Bacillus subtilis* MG495086.

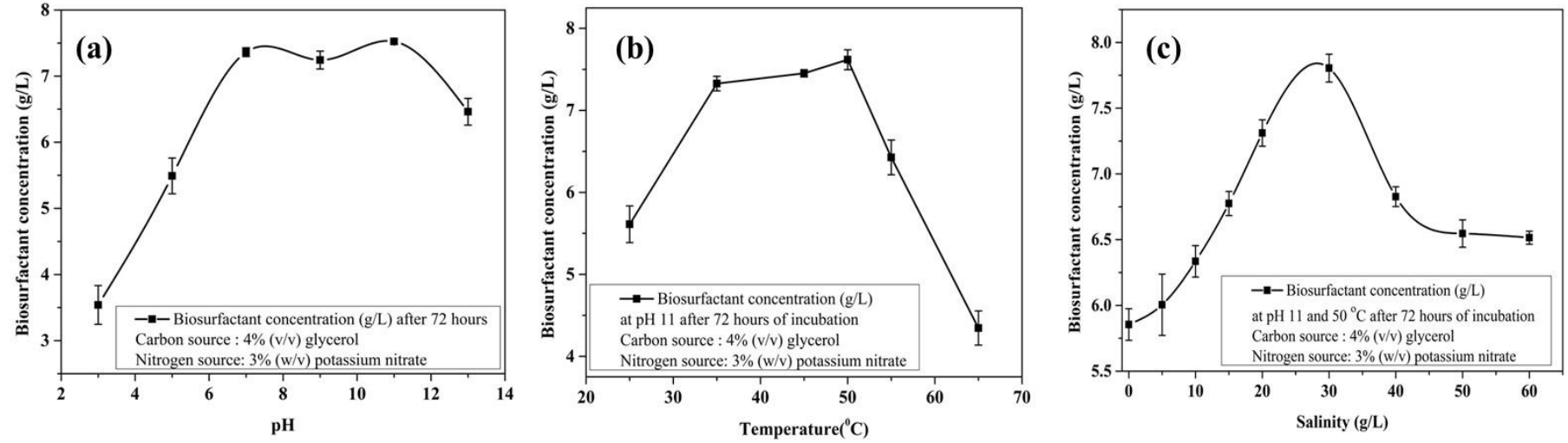


Figure 3.6. Effect of (a) pH, (b) temperature and (c) salinity on the concentration of biosurfactant produced by *Bacillus tequilensis* MK 729017.

Figure (3.6 (a), (b) and (c)) summarizes the influence of pH, temperature and salinity on the biosurfactant concentration after 72 hours which has been produced by *Bacillus tequilensis* MK 729017. Initially, the pH optimization was carried out with the previously optimized carbon source (4 % glycerol) and nitrogen source (3 % potassium nitrate) at 37 ºC with 1 % NaCl and it was observed that at lower pH (3 and 5) the yield was low (3.54 ± 0.3 g/L and 5.5 ± 0.27 g/L), but with a gradual increase in pH from 7 to 11, the yield remained almost the same from 7.36 ± 0.7 to 7.5 ± 0.03 g/L. But at pH 13, the yield became comparatively lower i.e., 6.46 ± 0.2 g/L.

Biosurfactant activity was retained over a wide range of temperatures from 25 ºC to 65 ºC in terms of the quantified yield where all the previously optimized parameters were implemented. At a lower temperature (25 ºC), the biosurfactant yield was found to be 5.6 ± 0.22 g/L but with the increase in temperature, biosurfactant yield got improved till 55 ºC. But when the temperature was raised to 65 ºC, the yield was reduced to 4.35 ± 0.2 g/L due to the heat tolerance constraint of *Bacillus tequilensis* MK 729017. When the strain was grown at 50 ºC, it showed an even better yield of 7.6 ± 0.12 g/L hence, 50 ºC was selected as the optimum temperature for the next experiments.

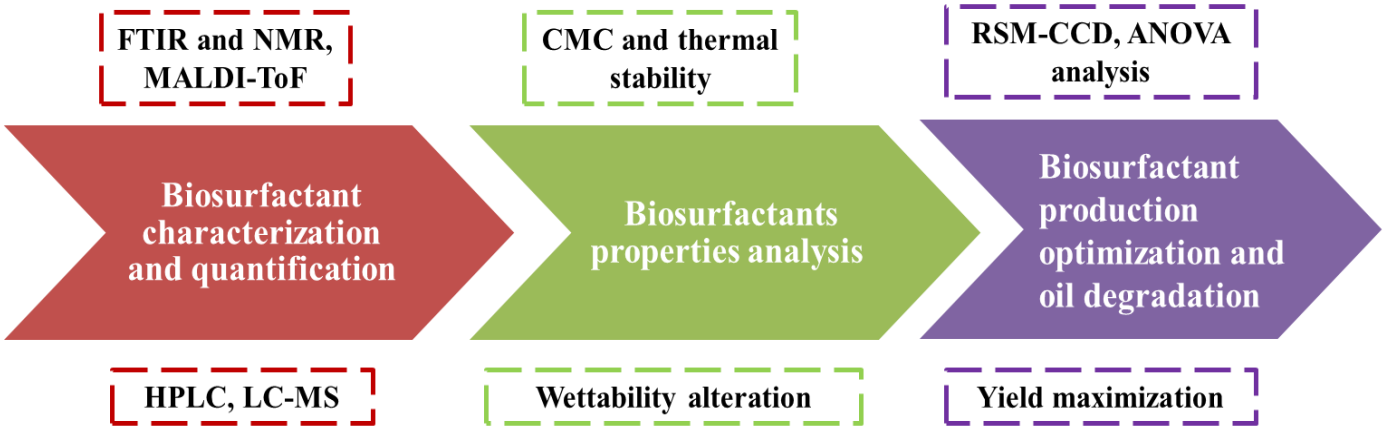
The salinity tolerance of the strain was also investigated by varying the NaCl concentration from 0 to 60 g/L in the culture conditions with the other optimized variables. It followed a sigmoidal trend where the biosurfactant yield got better with the increase in salinity and the maximum yield (7.8 ± 0.1 g/L) was obtained with 30 g/L concentration (v/w). After that when the strain was grown in extreme saline conditions (till 60 g/L), it was able to survive but the biosurfactant concentration reduced a little bit (6.5 ± 0.5 g/L). So, it can be stated that *Bacillus tequilensis* MK 729017 is a very good halophilic strain that could easily survive in the high salinity range of the oil reservoirs.

3.4.Conclusions

This chapter described elaborately the isolation and screening of potential biosurfactant producing and crude oil-utilizing microbial isolates from formation water and the soil samples of the Assam oil reservoir field depending on their ST reduction, oil displacement activity, drop collapse efficiency and emulsification activity. Three strains were identified from formation water, based on the screening test results, such as *Stenotrophomonas* sp. MG 520349, *Bacillus subtilis* MG 520348 and *Bacillus subtilis* MG 495086 and the most potential strain was found to be *Bacillus subtilis* MG 495086. The bacterial growth profile study of *Bacillus subtilis* MG 495086 was performed in three different conditions (aerobic, anaerobic and facultative) and the aerobic condition was found to be the most suitable because the biomass was found to be 1.5 and 1.3 folds higher in aerobic condition than anaerobic and facultative conditions, respectively. The specific growth rate (μ) was found to be 0.08 ± 0.01 $hour^{-1}$. The most favorable carbon source along with the suitable culture conditions for *Bacillus subtilis* MG 495086 were also examined. The least ST was obtained with 3 % light paraffin oil at pH 9, 60 ºC after 72 hours with 6 % salinity. Likewise, seven strains were also isolated from the soil sample, screened and identified considering their crude oil degradation capability and biosurfactant production ability. *Bacillus tequilensis* MK 729017 was chosen based on its better surface-active properties as it reduced the ST to 30 ± 2 mN/m along with a moderate EI of 66 ± 2 %. The media optimization study was also carried out with different hydrocarbon sources. Glycerol was reported to produce the maximum biosurfactant among the other hydrocarbons. The specific growth rate of the isolate was 0.21 ± 0.01 $hour^{-1}$ and Y_{XS} was estimated as 0.1. The effects of process parameters such as wide range of pH (3 - 11), temperature (25 – 65 °C) and salinity (0 – 60 g/L) were experimentally evaluated and the stability of the biosurfactant-producer was validated.

Chapter 4

Characterization and Optimization of the Produced Biosurfactants



(*Bioresource technology* 270, 439-448, 2018 (Datta et al., 2018) and *Journal of Petroleum Science and Engineering* 195, 107612, 2020 (Datta et al., 2020))

The produced biosurfactants from *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017, were identified employing various analytical techniques. The relative abundances of the isoforms were determined using chromatographic techniques and the properties were analyzed by examining surface-active properties. Thereafter, the biosurfactants' production was optimized considering the substrate concentration and environmental parameters in terms of surface tension reduction as well as biosurfactant concentration using a statistical approach. The biosurfactant production along with residual substrate degradation was also measured in order to ensure the value-added product (biosurfactant) formation with subsequent hydrocarbons degradation.

4.1.Introduction

In the previous Chapter 3, the isolation, screening and identification of two *Bacillus* strains from indigenous sources of the Assam oil reservoir have been discussed elaborately. In this Chapter, the characterization and the properties of the produced biosurfactants have been analyzed critically. Glycolipids and lipopeptides are the most commonly utilized biosurfactants, which have already been reported for their effectiveness in MEOR trials at the laboratory and pilot scales (Sen, 2008). Several lipopeptides such as Surfactin, Iturin, Fengycin, Lichenycin and Bacillomycin have also been produced by *Bacillus* sp., and studied for EOR applications (Pathak et al., 2014a). Among them, Surfactin is one of the most potential surface-active biosurfactants, which is a cyclic lipopeptide compound containing seven amino acids in a lactone ring and bonded to a β hydroxyl fatty acid chain of length from C_{12} - C_{16} (IA Haddad et al., 2014). Surfactin has been found to reduce the surface tension (ST) from 72 mN/m to 27 ± 2 mN/m (Al-Wahaibi et al., 2014; Alvarez et al., 2015; Pereira et al., 2013) and possess a lower critical micelle concentration (CMC) value (Datta et al., 2018). Despite better interfacial features of lipopeptides than other biosurfactants, due to low yield and higher production expense, it could not be executed much for successful practical applications. Hence, the solutions lie in utilizing cheaper substrates to reduce the production costs and suitable process optimization strategies to enhance the biosurfactant yield (Dhanarajan et al., 2017).

Following these bottlenecks, economical C-sources (light paraffin oil and glycerol) were selected for the optimal production of the biosurfactants along with a favorable statistical approach to predict the optimum environmental conditions. Response surface methodology (RSM) using central composite design (CCD) was exploited to maximize biosurfactant production in terms of surface tension reduction and biosurfactant concentration. The effects of process parameters such

as pH value, temperature, salinity and amount of carbon source were experimentally evaluated and validated. The produced biosurfactants were chemically characterized to test its suitability for EOR with the help of various analytical techniques; Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance (^{1}H, ^{13}C NMR), matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF), high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), contact angle, tensiometer and thermal stability. Finally, the correlation of the utilizing substrate with the produced biosurfactant along with the surface tension and other factors were also examined for their endorsement for oil recovery applications.

4.2.Materials and Methods

4.2.1. Chemicals and Reagents

The chemicals and reagents used for this study have been mentioned in Chapter 3. The remaining chemicals are trifluoroacetic acid (RM874), acetonitrile (AS029) and methanol (AS061) which are procured from HiMedia Laboratories, India. Rhamnolipid (R90), deuterated chloroform (570699) and Surfactin (S3523) are purchased from Sigma Aldrich.

4.2.2. Physical and chemical characterizations of the produced biosurfactants

The produced biosurfactant was separated from the supernatant by acid precipitation and solvent extraction method using hexane and ethyl acetate as solvents. Initially, the bacterial culture was centrifuged to get the supernatant which was then mixed with *n*-hexane in 1:1 ratio and centrifuged at 13,000 rpm for 10 minutes then the pH of the cell-free supernatant was adjusted to 2 by 1 M HCl. Next, the supernatant was mixed with an equal volume of ethyl acetate and kept for shaking and two layers were formed: bottom aqueous layer and top organic (ethyl acetate) layer. The top organic layer after extraction was separated and transferred to a round bottom flask connected to a rotary evaporator to remove the solvent. After the evaporation, the concentrated liquid was

obtained and referred to as crude biosurfactant (Radzuan et al., 2017). For the quantification of the residual substrate, the cell-free supernatant was extracted with hexane solvent. After solvent extraction, the residual substrate was quantified by the evaporation of the top hexane layer.

4.2.2.1.Functional group analysis of the biosurfactants

The chemical structure of the produced biosurfactant was analyzed using nuclear magnetic resonance (NMR) and Fourier-transform infrared (FTIR) spectroscopy analyses. FT-IR analysis was performed using PerkinElmer Spectrum (Version 10.4.3) to analyze the functional groups of the purified biosurfactants (Elazzazy et al., 2015). The spectrum was obtained in a range of 400 - 4000 cm^{-1} at a resolution of 4 cm^{-1} by averaging 32 scans in the attenuated total reflection (ATR) mode. The chemical structure of the crude biosurfactant was analyzed using 600 MHz proton NMR (^{1}H) and carbon NMR (^{13}C). Almost 20 µL produced biosurfactant was dissolved in 500 µL deuterated chloroform ($CDCl_3$) and the NMR spectra were recorded with 200 scans using a Bruker Ascend 600 spectrometer at 25 °C. Chemical shifts (δ) were expressed in ppm scale relative to tetramethylsilane (Zou et al., 2014).

4.2.2.2.Molecular mass determination of the biosurfactants

MALDI-ToF (BrukerDaltonics flex Analysis) mass spectrometer analysis in the positive ion reflector mode was performed to estimate the molecular mass of the produced biosurfactant from *Bacillus subtilis* MG 495086 (He et al., 2017b; Pereira et al., 2013). The matrix preparation was done through three steps: 1 µl trifluoroacetic acid was dissolved in 999 µl of double distilled water; 50 µl of this mixture was added to 50 µl of acetonitrile (ACN); 3 mg α-cyano-4-hydroxycinnamic acid was dissolved in the previous mixture. The crude biosurfactant was dissolved in a matrix solution of 2 g/L concentration containing 50 % ACN, 50 % water and 0.1 % trifluoroacetic acid (TFA). The sample was mixed with the matrix solution in a different ratio (from 1:1 to 1:6 (v/v)).

An aliquot of 1 µl of each sample/matrix mixture was spotted and slowly dried to allow matrix crystallization.

4.2.2.3.Quantification of produced biosurfactants and residual substrates

HPLC was performed for further identification and quantification of the biosurfactant produced by *Bacillus subtilis* MG495086. An HPLC system (Make: Varian, Model: ProStar. 210) equipped with a UV detector and C18 column (Agilent TC-C18) was operated at 25 ºC. Mobile phase containing acetonitrile (ACN) and 3.8 M trifluoroacetic acid (TFA) in 80:20 ratio was used in isocratic mode with a flow rate of 1 ml/minute. Surfactin (Sigma-aldrich, Catalogue no. S3523) was used as standard (Wei & Chu, 1998). Approximately 30 µl of the sample was injected after filtering through a 0.2 µm syringe filter. The samples from different time intervals i.e., 24, 48, 72 and 96 hours were analyzed and the concentrations of the produced biosurfactant were determined.

The relative abundance of the biosurfactant produced by *Bacillus tequilensis* MK 729017 was quantified using liquid chromatography-mass spectroscopy (LC-MS) and high-performance liquid chromatography (HPLC). The samples for LC-MS were prepared by dissolving the biosurfactant in acetonitrile (ACN) and filtered through a 0.2 µm syringe filter. About 0.5 µL sample was injected and mass spectra were attained using LC-MS (Make: Waters, Model: Q-Tof Premier) in negative ion modes with the following parameters: scan time of 0.5 s; cone voltage of 30 V, the capillary voltage of 3.49 kV; sampling cone 52; extraction cone 20; ion guide 2.1; source temperature 85 ºC; desolvation temperature 350 ºC; cone gas 50 L/h and desolvation gas 450 L/h (Bezza et al., 2015; Bezza & Chirwa, 2015; Radzuan et al., 2017). Then, the spectra were obtained using full scan mode of the total ions with a mass scan of m/z 100 – 1500, which was used for the characterization of the produced biosurfactant based on specific m/z peaks by producing ([M+Na]+, [M−H]− and [M+H]+) ions. LC-MS spectra of a purchased biosurfactant (Surfactin,

98 % purified) from Sigma Aldrich were recorded for comparison. Moreover, the fragmentation pattern by LC-MS analysis of recognized peaks was used to produce the daughter ions of Surfactin isomers for the confirmation of the molecular identity (Bharali et al., 2011; Caldeira et al., 2011; Sarwar et al., 2018).

For HPLC analysis of the biosurfactant from *Bacillus tequilensis* MK 729017, a little different approach was adapted from the previous one. Biosurfactants (produced and procured) were dissolved in methanol, filtered through a 0.2 µm syringe filter and then injected in an HPLC system (Make: Agilent LC system, Agilent technologies; model: 1260 Infinity II) equipped with UV detector and C18 column (Agilent TC- C18 (2)) having dimensions of 4.6 × 250 mm, with a flow rate of 0.7 ml/minute at 26 ºC for 30 minutes in isocratic mode. The mobile phase contained ACN and 3.8 mM TFA in water in the ratio of 90:10. The metabolite (lipopeptide) was detected at 205 and 210 nm with a UV detector.

The residual carbon source (glycerol) was also estimated by HPLC (SHIMADZU LC - 10AD by Shimadzu Co., Kyoto, Japan) equipped with AMINEX HPX 87A column. A standard curve was prepared for the purchased glycerol in different concentrations (1000, 2500, 5000 and 10,000 mg/L) by dissolving them in Mili-Q water. The samples were filtered through a 0.2 µ syringe filter followed by the injection in a liquid chromatography system. 5 mM H_2SO_4 was used as the mobile phase. The runtime was continued for 30 minutes for each sample at a flow rate of 0.6 mL/minute in isocratic mode. The oven temperature was kept at 50 ºC and the spectra were recorded using Refractive Index Detector.

4.2.3. Properties of the produced biosurfactants

4.2.3.1.Determination of critical micelle concentration (CMC) value and thermal stability

Critical micelle concentration (CMC) is one of the primary characteristics of the biosurfactant which corresponds to the specific biosurfactant concentration at which the biosurfactant starts to form micelle. The addition of more biosurfactant beyond CMC value cannot reduce the surface tension (ST) anymore. The ST values of these dilutions were measured at 25 ºC using Tensiometer (Make: Dataphysics, Model: DCAT 11EC). Biosurfactant solutions of different concentrations were prepared and the surface tension values were measured subsequently to determine the CMC values (Liu et al., 2016b). To test the thermal stability, the biosurfactant was exposed to very high temperatures (80 ºC and 90 °C) in a hot air oven for 24 hours and examined if the same STs were obtained as before the thermal exposure.

4.2.3.2.Wettability alteration by the produced biosurfactants

Wettability alteration contributes to MEOR by changing the wetting property of the reservoir (rock) surface. The contact angle of the untreated sample (only media) and produced biosurfactant were measured using the Goniometer, Drop Shape Analysis System (Make: HOLMARC). The measurements were made at 25 ºC and 1 atm pressure on the hydrophobic surface of coverslips and reservoir-like surfaces following the protocol described elsewhere (Al-Sulaimani et al., 2012; Al-Wahaibi et al., 2014) and the average values are reported.

Initially, the wettability alteration by the biosurfactant produced *Bacillus subtilis* MG 495086 was studied by determining the contact angle on a coverslip (Datta et al., 2018). But as a coverslip cannot imitate the proper reservoir surface hence, the surface was modified further to determine the wettability alteration of the biosurfactant produced by *Bacillus tequilensis* MK729017. Therefore, to simulate the reservoir condition, the surface was prepared through several steps: at

first, the soil sample of the reservoir was kept under 100 Bar pressure to make translucent pellets. Then the pellets were kept in brine saturation followed by oil saturation for 7 days. After that these surfaces were chosen as the hydrophobic ones to carry out the wettability alteration experiments.

4.2.4. Optimization of biosurfactant production using RSM and ANOVA analysis

The optimization of the biosurfactant concentration was performed by response surface methodology (RSM). RSM is a statistical tool for optimizing variables that combines experimental design, statistical analysis and model development. It reduces the number of experimental trials and assists to explore the effect of individual independent parameters and their cumulative effect (Selvakumar & Sivashanmugam, 2019). RSM is an empirical technique, used for multiple regression analysis by combining the data obtained from the actual experiments. The experimental design was performed by Minitab (Minitab 17 statistical software).

Central composite design (CCD) was created to understand the effects and interactions among the three independent variables: the percentage of carbon source [A] (1 – 5 %), pH [B] (3 - 11) and temperature [C] (25 – 65 ºC) for the maximum biosurfactant production from *Bacillus subtilis* MG 495086. The surface tension value was considered to be the dependent response variable. Total 20 experiments were suggested to predict the output (surface tension) by studying the effects of interaction among selected variables.

Another face-centered CCD was created to generate 30 experimental runs and the biosurfactant concentration was measured as the response. The regression analysis was performed to determine the response function as a quadratic polynomial equation. The goodness of the model was scrutinized by analysis of variance (ANOVA) (Khademolhosseini et al., 2019). RSM-CCD was used to examine the statistical significance of the variables and their interactions for biosurfactant production (Veshareh et al., 2019). Carbon source concentration and environmental conditions are

considered to have an important impact on bacterial growth. Therefore, four independent variables were chosen namely carbon source concentration (A), pH (B), temperature (C) and salinity (D) (Table 4.1). All the data are expressed as mean ± standard error. Experimental data were analyzed by two-way ANOVA to estimate the statistical parameters to optimize the culture condition (Francis et al., 2003; Khuri & Cornell, 1987; Kiran et al., 2009; Medeiros et al., 2000; Myers et al., 2004; Nampoothiri et al., 2008).

Table 4.1. Parameters used in RSM experimental design for biosurfactant yield from *Bacillus tequilensis* MK729107

Sl. No.	Variables	Codes	Levels		
			-1	**0**	**1**
1.	Carbon source (%)	A	1	3	5
2.	pH	B	3	7	11
3.	Temperature (ºC)	C	25	45	65
4.	Salinity (g/L)	D	10	30	50

4.3.Results and Discussion

4.3.1. Physical and chemical characterization of the purified biosurfactants

4.3.1.1.Functional group analysis by FTIR and NMR

FTIR analysis was carried out to identify the functional groups present in the produced biosurfactants from *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 which is shown in Figure 4.1 (a) and Figure 4.1 (b), respectively. In Figure 4.1 (a), the peaks at 3437 cm^{-1} and 1602 cm^{-1} corresponded to the stretching and bending of the N-H group, respectively. The peaks at 2922 and 2854 cm^{-1} indicated the presence of methyl and methylene respectively. The peaks at 1703, 1455, 1383, 1269 cm^{-1} showed the stretching of C=O, aliphatic chain (-CH_2-),

symmetric nitro group or C-H bend, acyl and phenyl C-O, respectively. Peaks at 1076, 812, 746 cm^{-1} pointed out the presence of alkoxy group, alkene sp^2 C-H bending pattern and aromatic sp^2 C-H bending pattern, respectively.

In Figure 4.1 (b) FTIR spectra, the sharp bands at 2960 and 2927 cm^{-1} signified the presence of C–H stretching mode of CH_3 and CH_2 groups in alkyl chains confirming the existence of aliphatic groups. The peak with the lowest transmittance was observed at 1643 cm^{-1} and indicated the presence of the peptide group (CO-NH) in the produced bio-molecule (Yalaoui-Guellal et al., 2020; Yilmaz et al., 2009). There was another sharp peak at 1532 cm^{-1}, which denoted either the C=O stretching vibrations or the amide group. A weak peak at 1387 cm^{-1} is designated to C-H bending vibrations, which is common in alkyl chain-containing compounds. The broad peak at 1204 cm^{-1} probably corresponded to C-O-C vibration in the ester (Das et al., 2008). Besides, a broad peak was observed in the wave-number range of 3200 to 3700 cm^{-1} with the minima at 3299 cm^{-1} represented hydroxyl and N-H stretching vibrations (Santos et al., 2016). Therefore, both the FTIR results indicated the presence of aliphatic hydrocarbon in combination with peptide group, which is the characteristic feature of a lipopeptide (Surfactin) (Liu et al., 2016b; Pereira et al., 2013).

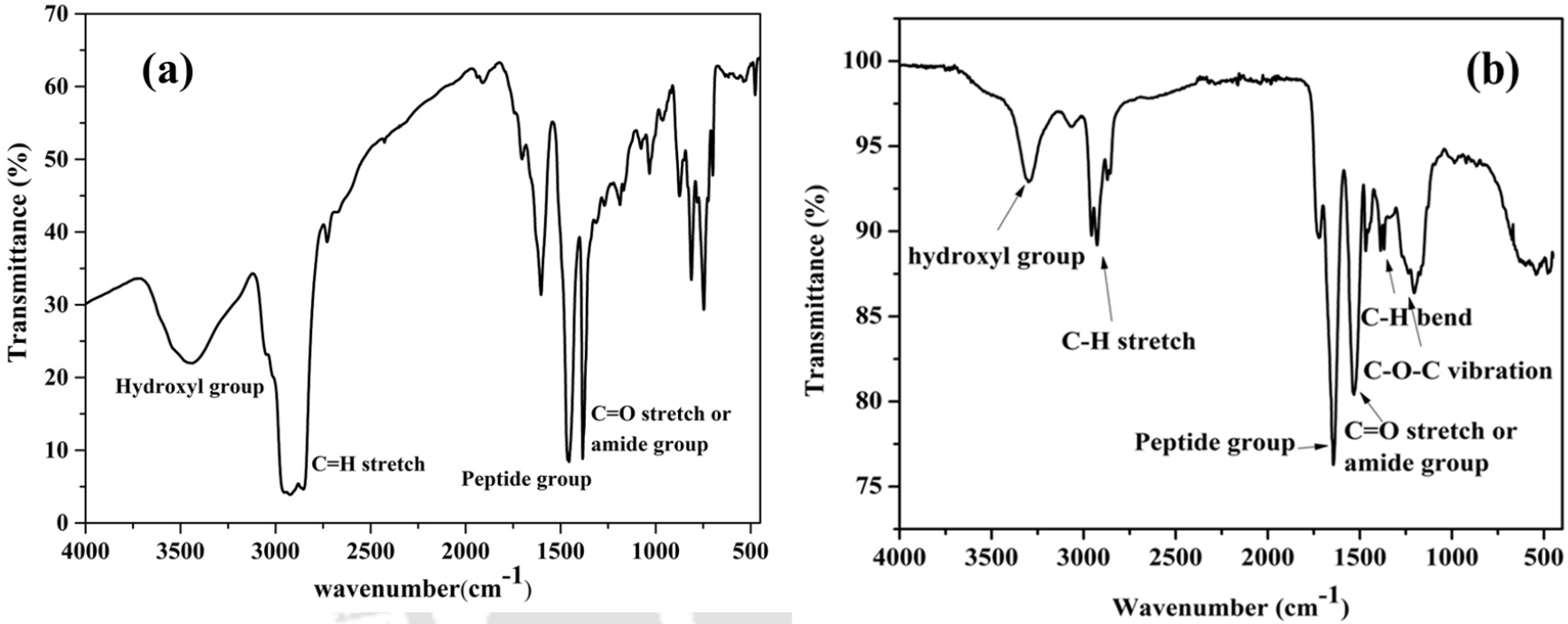


Figure 4.1. FTIR spectra of the biosurfactants produced by (a) *Bacillus subtilis* MG 495086 and **(b)** *Bacillus tequilensis* MK 729017

The NMR spectra of the biosurfactants produced from *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 were analyzed. ^{1}H NMR spectra of the biosurfactant produced by *Bacillus subtilis* MG 495086 (Figure 4.2 a) contained a singlet at 7.26 ppm which corresponded to the peak of $CDCl_3$. Other peaks in the region 1.98 - 2.53 showed the presence of the $-CH_2-C=O$ group. The peaks between 0.75 and 1.34 correspond to sp^3, -CH, $-CH_2$, $-CH_3$ aliphatic protons or it could be due to the presence of amine N-H. The peak at 0.87 and 1.26 ppm indicated the presence of sp^3 C-H and amine N-H, respectively. Very closely related two peaks at 2.05 and 2.1 ppm corresponded to the presence of amide N-H and carbonyl alpha groups. The presence of alkene C-H is confirmed by a 4.12 ppm peak (Bhardwaj et al., 2016). In the ^{13}C NMR spectra, as shown in Figure 4.2 b, three solvent peaks of $CDCl_3$ appeared in the region 77.03, 77.24 and 77.45. In the up-field region the peaks at 14.37 - 29.59 indicated the presence of sp^3, -CH, $-CH_2$, $-CH_3$ aliphatic groups or it could be due to the presence of amides. In the downfield region, the peak at 177.41 pointed to the presence of carboxylic acid or amide derivatives. The presence of aliphatic groups with amide groups helped to predict the structure of the lipopeptide biosurfactant.

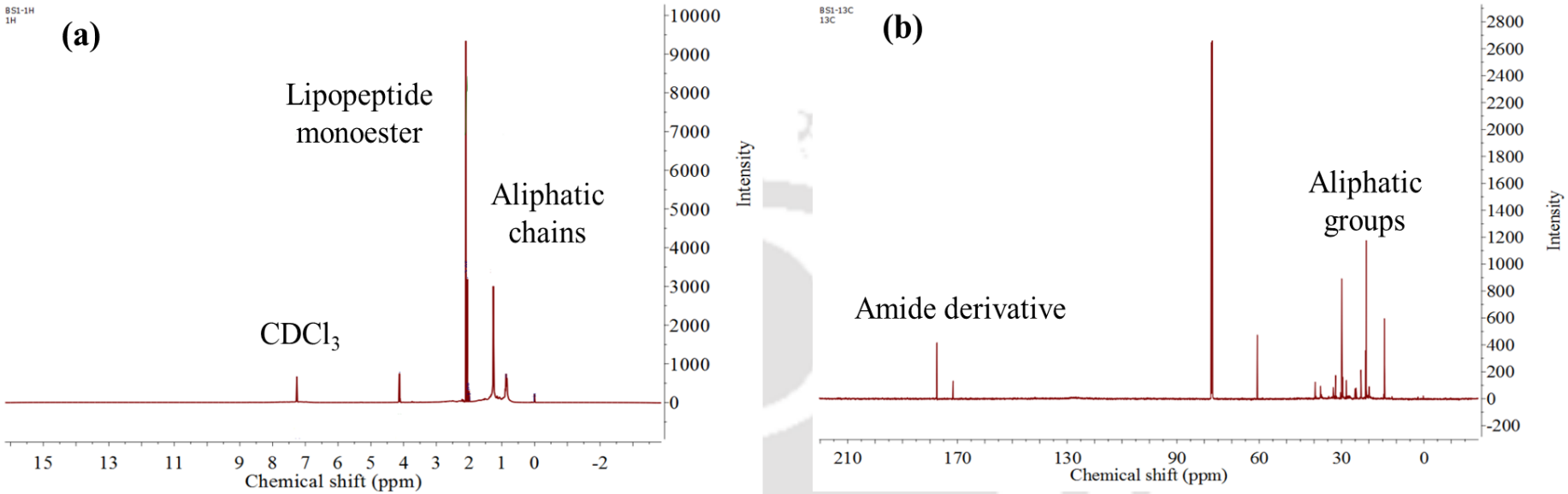


Figure 4.2. (a) ^{1}H and **(b)** ^{13}C 600 MHz NMR spectra of the biosurfactant produced by *Bacillus subtilis* MG 495086

^{1}H NMR spectrum obtained for the produced biosurfactant from *Bacillus tequilensis* MK 729017 is shown in Figure 4.3. The NMR spectra indicated the presence of a long aliphatic chain (CH_2) at 1.5-1.2 ppm, peptide backbone at 7.2 ppm and C-H bond at 5-4 ppm. The peak at 3.67 ppm has already been reported as the lipopeptide monoester spectra, which predicted the presence of Glu and Asp amino acid residues (Tang et al., 2007). An ester carbonyl group was detected at 5.31 ppm, which speculated the existence of a lactone ring in the produced biosurfactant (de Faria et al., 2011). Hence, the lipopeptide nature of the produced biosurfactant is confirmed due to the presence of the aliphatic and peptide moieties. Therefore it is concluded from both FTIR and NMR analyses that the produced biosurfactant was of lipopeptide nature having both aliphatic and peptide groups (Datta et al., 2018; Ismail et al., 2013).

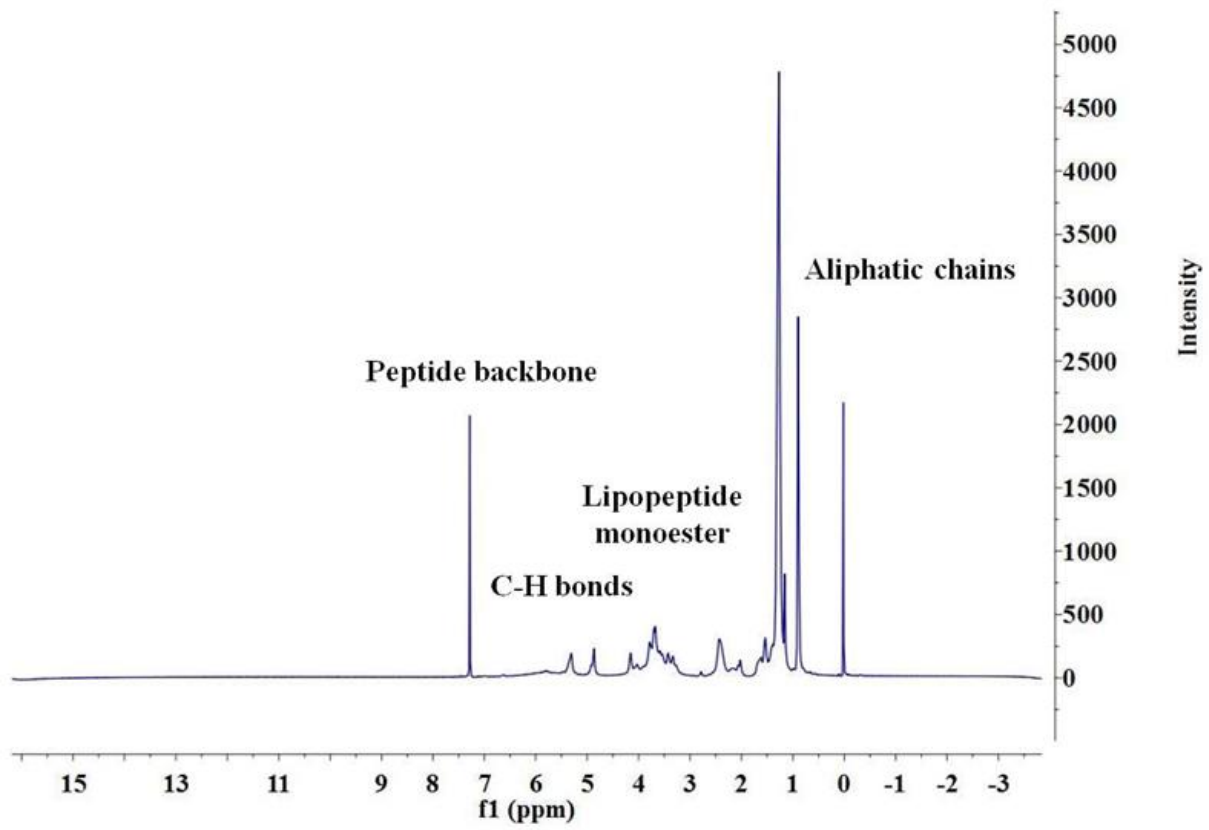


Figure 4.3. 600 MHz ^{1}H NMR spectra of the biosurfactant produced by *Bacillus tequilensis* MK 729017

4.3.1.2.Molecular mass estimation by MALDI-ToF

MALDI-ToF spectra of the biosurfactant obtained from *Bacillus subtilis* MG 495086 showed four major peaks attributed to different isoforms of the produced lipopeptide (Figure 4.4). Each lipopeptide isoform is attributed to the peaks of protonated forms of the sodium adduct. The peaks $[M + Na]^+$ at m/z = 1051, 1067, 1083 and 1100 corresponded to C_{13}, C_{14}, C_{15} and C_{16} hydroxy fatty acid chain of lipopeptide (Surfactin), respectively. The relative abundance of these isoforms of Surfactin, calculated from the respective peak intensities, were found to be 11.06 ± 1.6 % (C_{13}), 20.75 ± 0.3 % (C_{14}), 61.75 ± 1.2 % (C_{15}) and 6.44 ± 0.5 % (C_{16}) indicating the presence of a mixture of different isoforms of Surfactin (Vater et al., 2002).

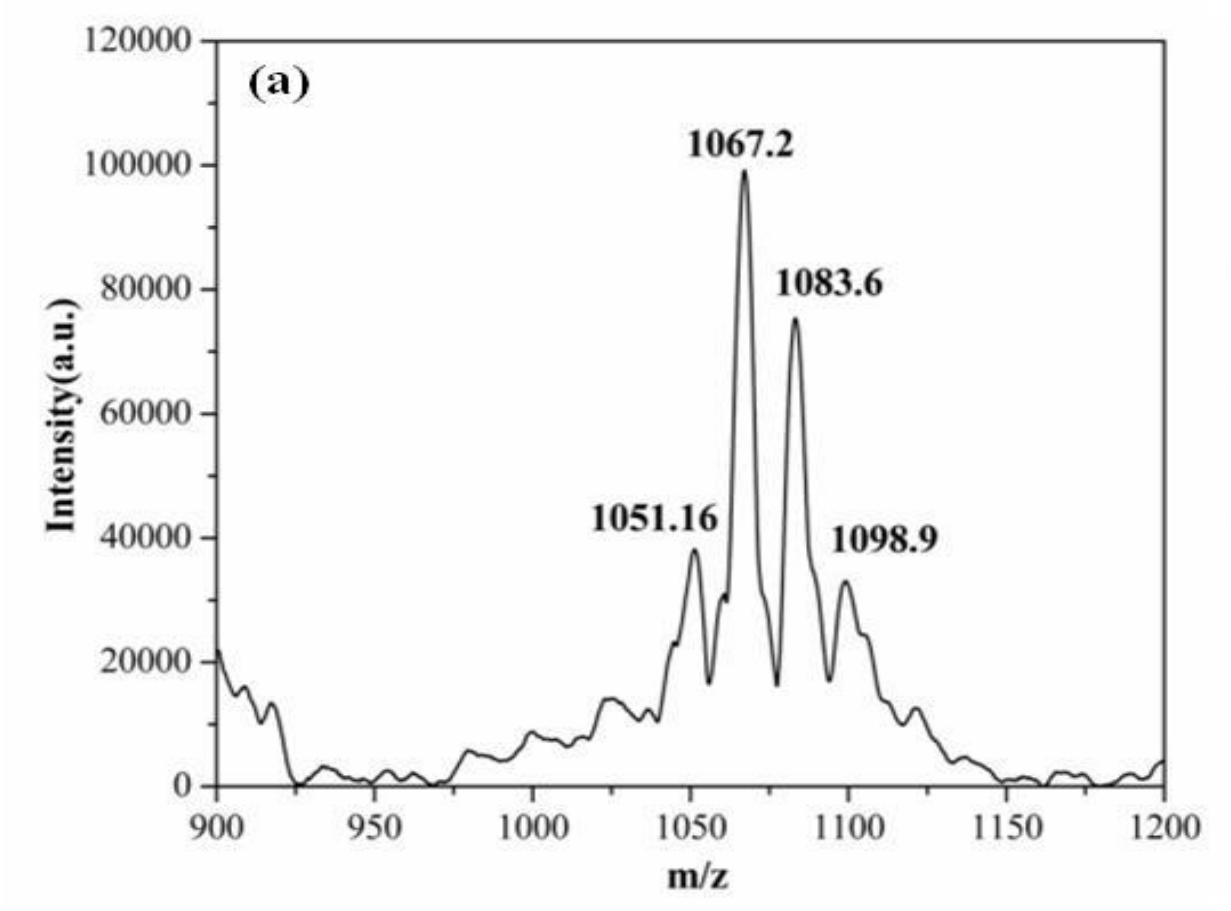


Figure 4.4. MALDI-ToF spectra of the produced biosurfactant by *Bacillus subtilis* MG 495086

4.3.1.3.Quantification of biosurfactant isoforms by chromatography

HPLC analysis was performed to determine the concentrations of produced biosurfactant from *Bacillus subtilis* MG 495086 at 24, 48, 72 and 96 hours. Four major peaks corresponding to C_{13}, C_{14}, C_{15} and C_{16} isoforms of Surfactin have appeared in the standard chromatogram at the retention times of 10.4, 12.6, 17.37 and 21.52 minutes, respectively (Figure 4.5) (Wei & Chu, 1998). The total peak area of a sample at the above retention times was used to determine the concentration of the total biosurfactant present using the standard Surfactin curve. The biosurfactant HPLC data were found to have a good correlation with the biosurfactant extraction data as shown in Figure 4.12.

The relative abundance of the biosurfactant isoforms produced by *Bacillus tequilensis* MK 729017 was quantified by LC-MS and HPLC analyses and was compared with that of the purchased Surfactin (98 % purified). Five distinct peaks were observed for the produced biosurfactant at m/z ratio of 993, 1007, 1021, 1035 and 1049, which are assumed to be corresponding for C_{12}, C_{13}, C_{14}, C_{15} and C_{16} isoforms of the biosurfactant (Figure 4.6 a). These peak positions were compared with the procured Surfactin, wherein the peaks were observed at the same peak positions i.e. at m/z 995, 1009, 1023, 1037 and 1051 (Figure 4.6 b). The detection of the isoforms in the produced biosurfactant differed slightly from the purchased Surfactin due to the ionization of the standard samples in the form of $[M + 2H]^+$ (Das, 2018; Pathak et al., 2014b). The relative abundance of the isoforms was calculated from the peak intensities, which were found to be 5.25 %, 13.81 %, 34.8 %, 41.71 % and 4.4 % for C_{12}, C_{13}, C_{14}, C_{15} and C_{16}, respectively.

The HPLC chromatograms of the purchased Surfactin and the produced lipopeptide are shown in Figure 4.6 (c) and (d), respectively. The compound was resolved in different retention times (minutes) of 10.02 ± 0.18, 11.43 ± 0.24, 13.87 ± 0.33, 14.55 ± 0.35 and 17.03 ± 0.47, which

attributed to C_{13}, C_{14}, C_{15} and C_{16} isoforms of the produced biosurfactant, and C_{15} was found to be the most predominant isoform among them (Shao et al., 2015). These analyses confirmed the produced lipopeptide to be Surfactin.

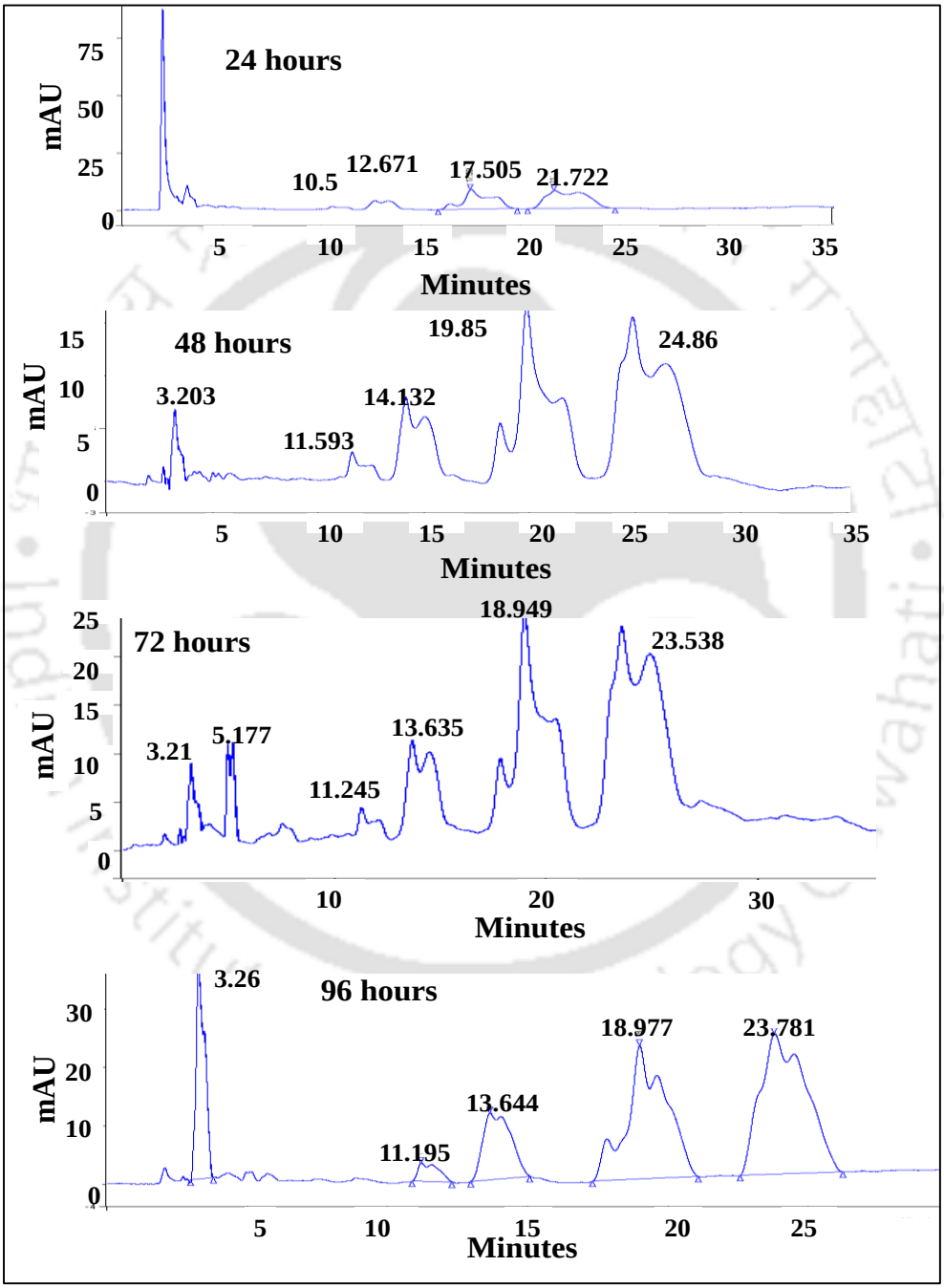


Figure 4.5. HPLC chromatograms of the Surfactin produced by *Bacillus subtilis* MG 495086 after 24 hours, 48 hours, 72 hours and 96 hours

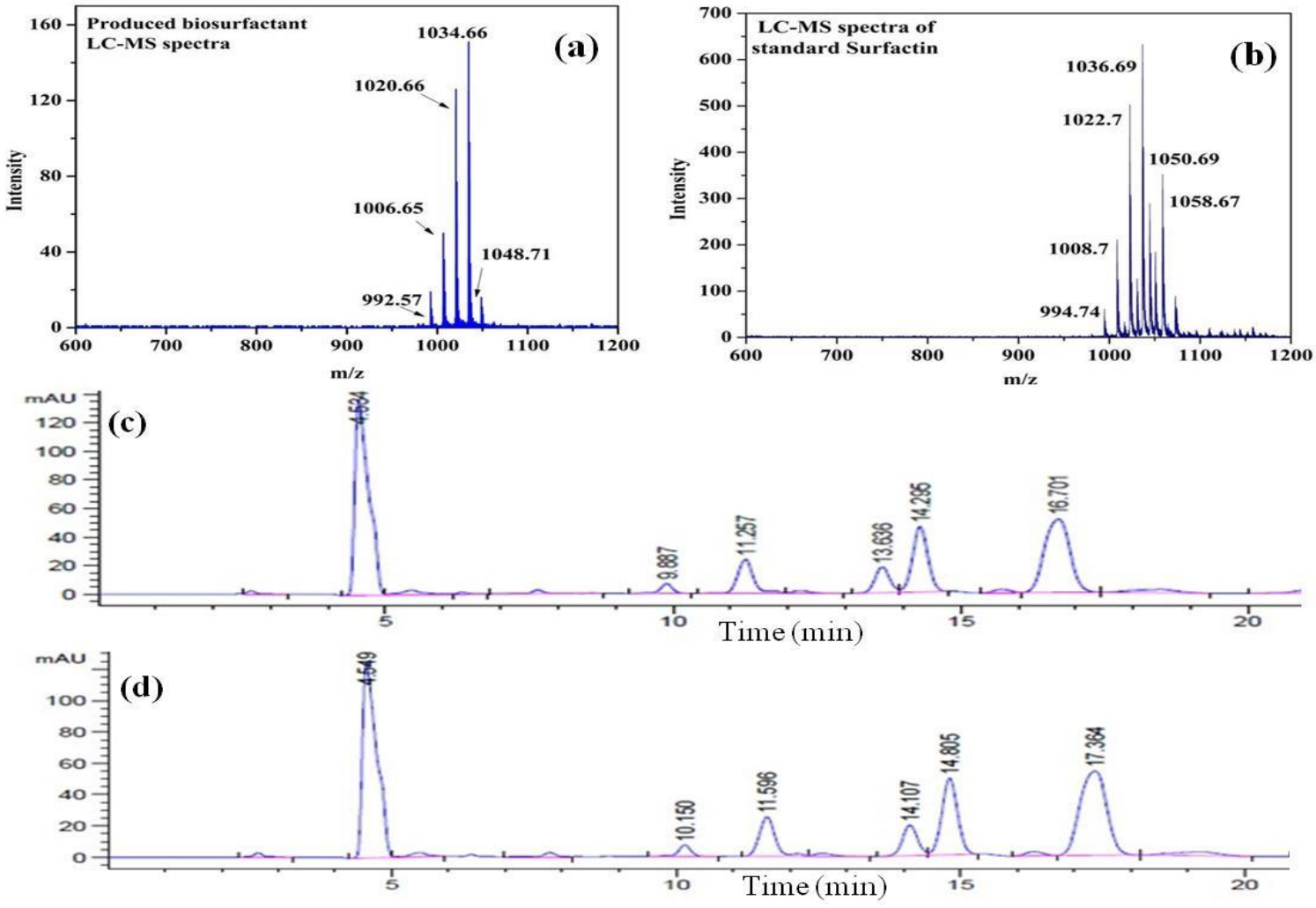


Figure 4.6. LC-MS spectra of the isoforms of the (a) lipopeptide produced by *Bacillus tequilensis* MK 729017, (b) the standard Surfactin purchased from Sigma Aldrich, (c) HPLC chromatograms of the standard Surfactin, (d) HPLC chromatogram of the produced biosurfactant produced by *Bacillus tequilensis* MK 729017

4.3.2. Properties of the produced biosurfactants

4.3.2.1.Determination of CMC value and thermal stability

The ST values were measured for various concentrations of the crude biosurfactant to achieve a specific concentration at which the ST becomes the lowest and micelles form. The ST profiles of the produced biosurfactants from *B. subtilis* MG 495086 and *B. tequilensis* MK 729017 are shown in Figure 4.7 (a) and Figure 4.7 (b), respectively. In Figure 4.7 (a) the ST values were recorded for a wide range of biosurfactant solutions (0 – 81 mg/L) produced by *B. subtilis* MG495086. With the increasing biosurfactant concentration, the value of ST was reduced from 62.95 ± 0.58 to 32.36 ± 0.04 mN/m. At 40 mg/L of biosurfactant concentration, the ST was 32.53 ± 0.16 mN/m and thereafter no significant change in the ST was observed with the addition of biosurfactant solution. Hence the CMC value of the produced biosurfactant was found to be 40 mg/L which is relatively lower than Tween 20 (60 mg/L) and CTAB (335 mg/L) and other biosurfactants (Bhardwaj et al., 2016; Liu et al., 2016b; Zou et al., 2014).

As shown in Figure 4.7 (b), the crude biosurfactant decreased the ST of water (72 ± 0.5 mN/m) to a minimum with the gradual increase in biosurfactant concentration. The CMC value of the biosurfactant produced by *B. tequilensis* MK 729017 was estimated to be 90 mg/L as the ST value remained constant even at higher concentrations. The obtained CMC value was found to be quite lower than the reported CMC value of the rhamnolipid which lies in the range of 200 to 500 mg/L (Pornsunthorntawee et al., 2009) and that of the chemical surfactants (CMC_{CTAB} 335 mg/L and CMC_{SDS} 2347 mg/L) (Shi et al., 2011). That is one of the reasons for preferring biosurfactants over chemical surfactants in EOR applications because a very small amount of biosurfactant becomes adequate to achieve the desired IFT. Moreover, even after ageing i.e., exposing the produced biosurfactants at 80 °C and 90 °C, they could exhibit almost the same CMC values as the native ones hence, they could be easily employed in the high-temperature reservoirs without affecting their effectiveness.

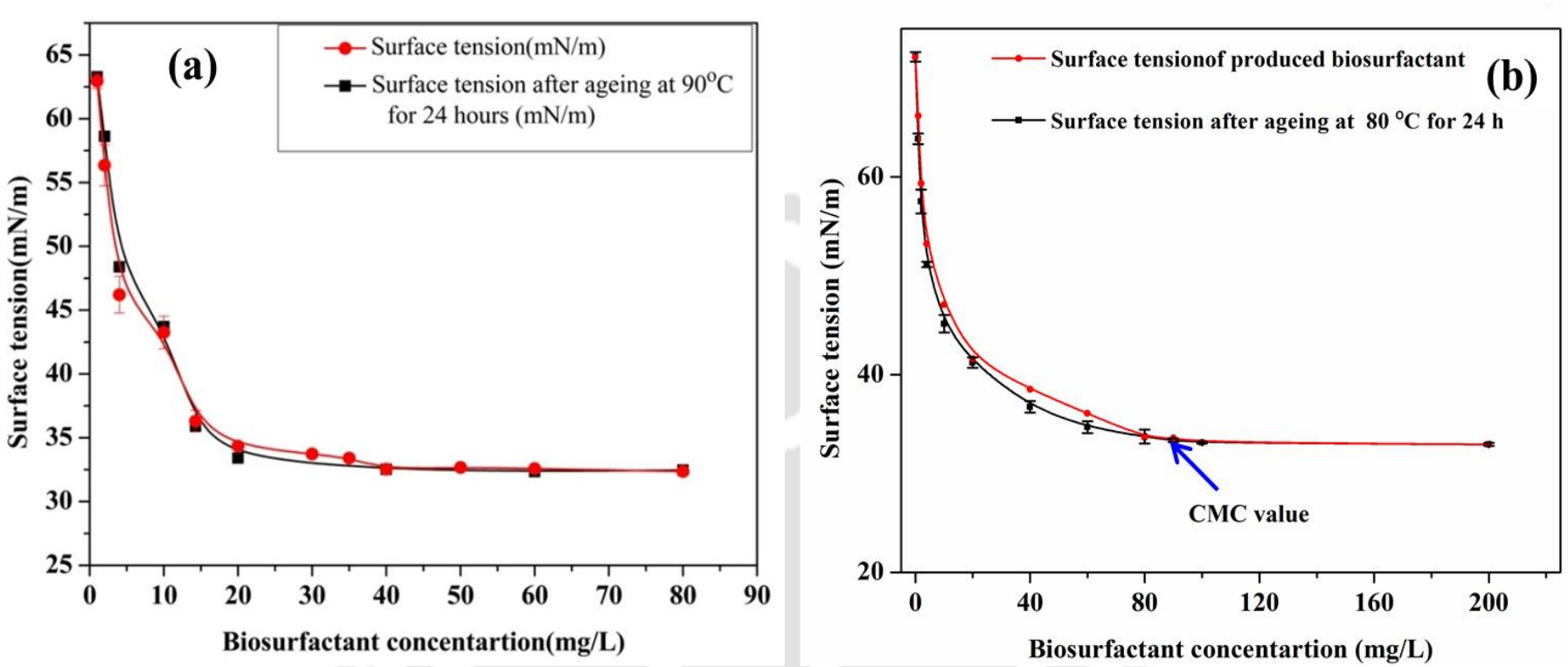


Figure 4.7. CMC profiles of the crude and heat-treated (aged for 24 hours at 90 ºC and 80 °C) biosurfactants produced by (a) *Bacillus subtilis* MG 495086 and (b) *Bacillus tequilensis* MK 729017

4.3.2.2. Wettability alteration by the produced biosurfactants

Wettability alteration has been reported to be one of the significant mechanisms for the successful MEOR process because it has a correlation with IFT and it can determine the wetting property of biosurfactant. Therefore, the wettability alteration due to biosurfactant produced by *Bacillus subtilis* MG 495086 was studied by determining the contact angle with Goniometer on a coverslip as revealed in Figure 4.8. The contact angle of the un-inoculated media and the biosurfactant solution (40 mg/L) was found to be 70 ± 1º and 28 ± 1º respectively. Al-Sulaimani *et al*., reported the contact angle alteration from 70.6 ± 0.3º to 25.32 ± 0.06º by biosurfactant solution at 25 °C under 1 atm pressure (Al-Sulaimani et al., 2012). Another similar study reported the reduction of contact angle from 58.7 ± 0.85º to 28.4 ± 1.03º and 27.2 ± 0.72º for B30 biosurfactant in CG medium (carbon source: glucose) and MDM (carbon source: date molasses) media, respectively (Al-Wahaibi et al., 2014).

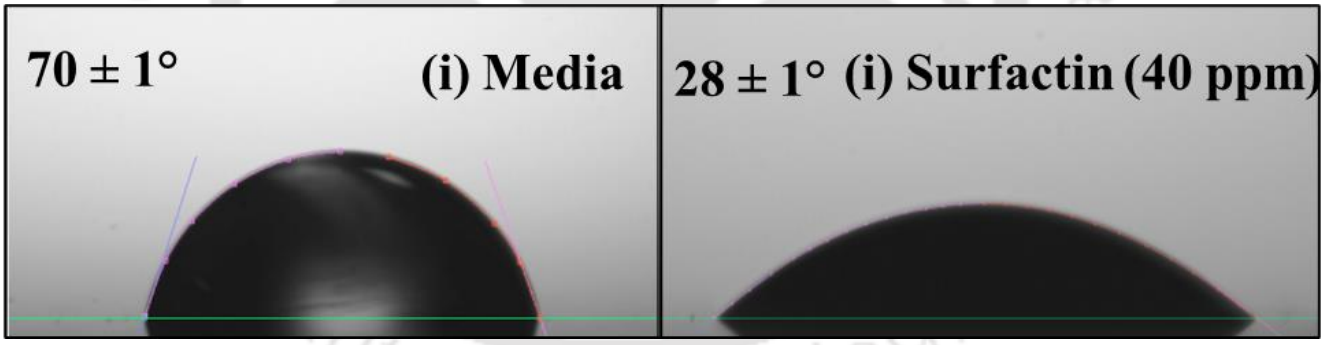


Figure 4.8. Wettability alteration by Surfactin produced by *Bacillus subtilis* MG495086 on coverslip surface

Instead of using coverslip as the surface, the contact angles of the other biosurfactant solutions were determined on the freshly prepared hydrophobic oil-brine-saturated soil surfaces are presented in Figure 4.9. The contact angle of the un-inoculated media was performed as the control experiment and measured to be 90 ± 1º due to the hydrophobic nature of crude oil, which agreed

to the reported data (Alghamdi et al., 2019). The biosurfactant solutions produced by *Bacillus tequilensis* MK 729017 enhanced wettability to 37 ± 1º and 26 ± 1º at 90 mg/L and 200 mg/L concentrations, respectively. This indicated wetting properties of the produced biosurfactant i.e. more contact area with the hydrophobic oil-saturated surface, which in turn is reported to enhance the oil recovery (Ayirala et al., 2006). Similar observations were reported where contact angle decreased from 70.6º to 25.32º with the treatment of 0.25 % (w/v) biosurfactant produced by *Bacillus subtilis* W19, isolated from Omani oil field (Al-Sulaimani et al., 2012). In another study, *Bacillus subtilis* resulted in wettability alteration from 49º to 23º and further the produced biosurfactant was explored for MEOR applications (Park et al., 2019). The contact angle of the produced lipopeptide was also compared with another biosurfactant, rhamnolipid solution at its CMC (200 mg/L), which was measured to be 51 ± 2º. It indicated better surface wetting properties of the Surfactin than Rhamnolipid for a hydrophobic surface saturated with crude oil.

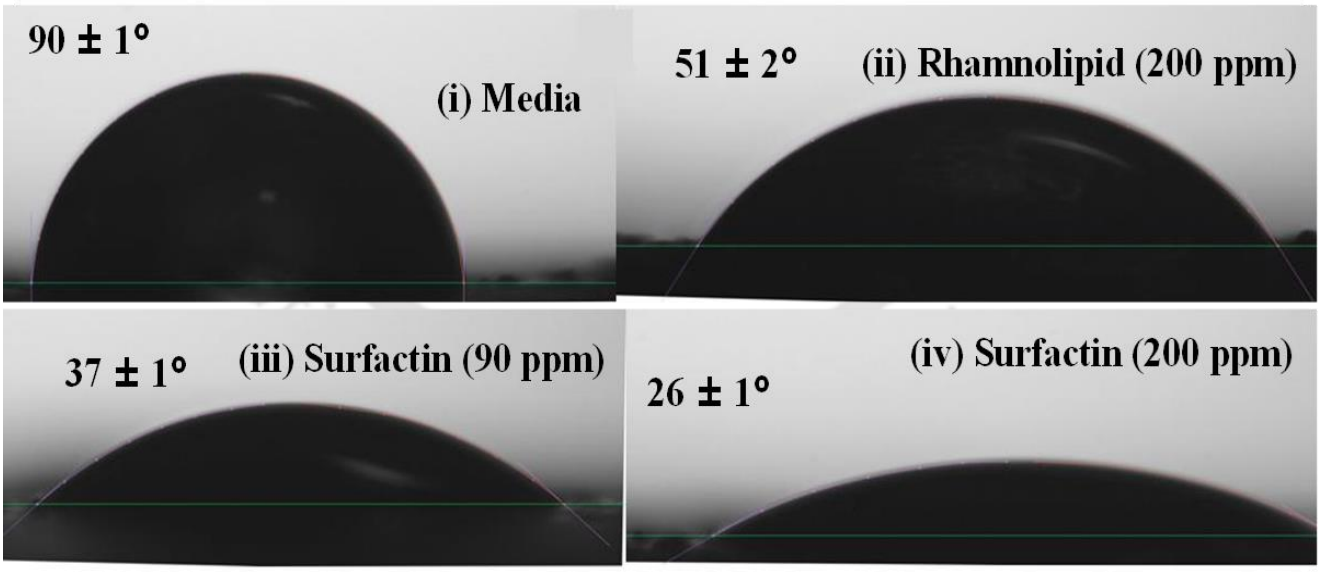


Figure 4.9. Wettability alteration of the reservoir-imitating surface by (i) Media, (ii) Rhamnolipid, (iii) Surfactin (90 mg/L) and (iv) Surfactin (200 mg/L) produced from *Bacillus tequilensis* MK 729017

4.3.3. Model prediction and verification for the optimization of biosurfactant production

The percentage of light paraffin oil (A; 1 – 5 %), pH (B; 3 - 11) and temperature (C; 25 – 65 °C) were selected as the parameters to optimize the biosurfactant production from *Bacillus subtilis* MG 495086 using RSM-CCD. Table 4.2 (a) summarizes software-suggested 20 experimental runs with the actual surface tension values as the investigational output. The isolated strain was able to survive upto 78 ºC temperature indicating the thermophilic nature of the strain and it helped to reduce the surface tension to 33 mN/m at 78 ºC, which endorses its suitability for EOR applications.

After performing the RSM experiments, a quadratic equation was obtained explaining the relationship among the process variables. Equation (4.1) shows a quadratic model to determine surface tension values of Surfactin as a function of the dependent variables (A, B, C) in coded units.

$$\text{Biosurfactant surface tension (actual)} = 58.11 - 2.988\text{A} - 2.346\text{B} - 0.4908\text{C} + 0.3592\,\text{A}^2 + 0.1138B^2 + 0.004032C^2 + 0.0333AB - 0.0011AC + 0.00745BC \quad (4.1)$$

Table 4.2 (a). RSM-CCD predicted runs with experimental and predicted surface tension values

Run No.	Carbon source conc. % (A)	pH (B)	Temperature (°C) (C)	Surface tension (mN/m) (experimental)	Surface tension (mN/m) (predicted)
1	1	3	25	38.87	39.69
2	5	3	25	37.23	36.64
3	1	11	25	34.21	35.42
4	5	11	25	33.82	33.44
5	1	3	65	34.67	35.42
6	5	3	65	33.04	32.21
7	1	11	65	32.58	33.54
8	5	11	65	31.83	31.39
9	3	7	45	30.14	29.82
10	3	7	45	30.21	29.85
11	3	7	45	30.33	29.85
12	3	7	45	30.27	29.85
13	0	7	45	39.25	37.13
14	6.3	7	45	31.35	32.89
15	3	0.5	45	38.03	38.11
16	3	13.5	45	34.62	33.96
17	3	7	12	38.54	38.06
18	3	7	78	33	32.9
19	3	7	45	30.35	31.18
20	3	7	45	30.28	31.18

The predicted surface tension values were determined using the model Equation (4.1), which are listed in Table 4.2 (a). The response surface plots for obtaining the minimum surface tension due to the biosurfactant from *Bacillus subtilis* MG 495086 considering the above parameters are depicted in Figure 4.10.

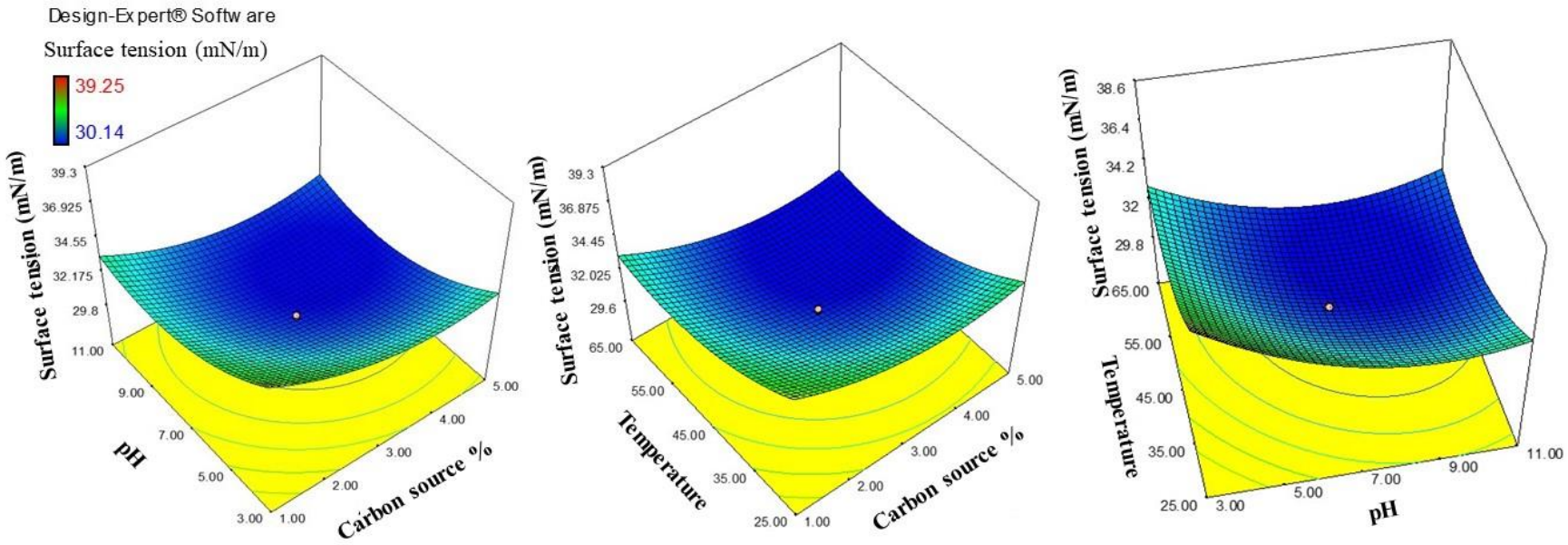


Figure 4.10. Response surface 3-D plots for the minimum surface tension of *Bacillus subtilis* MG495086 considering carbon source concentration, pH and temperature as the parameters.

A very high correlation coefficient ($R > 0.96$) indicated a very good connotation between predicted values and the actual surface tension results. The result of ANOVA is presented in Table 4.2 (b), from which it could be predicted that the values of A, B and C are significant as the P-value was less than 0.05 and F value was comparatively higher for linear and square interactions (A^2, B^2 and C^2). The model F-value 11.14 proved that the model is significant. However, two-way interactions were found to be insignificant. In this model square regression statistics, the R^2 value (93 %) was also substantially good. The adjusted R^2 value of 84.23 % suggested the implication of this model. The "lack of fit" value 524.62 also supported the significance of the model. The T values for linear and square interactions were found in negative.

The optimal conditions for the biosurfactant production were found to be carbon source concentration 3.89 %, pH 7.73, temperature 62.5 ºC. The predicted value of the response by the above-mentioned quadratic model (Equation 4.1) was found to be 30 mN/m under the optimized conditions whereas the experimental value was found to be 29.85 mN/m under similar conditions. The effect of key process independent variables (Datta et al., 2018) i.e. carbon source concentrations (% v/v) (A), pH (B), temperature (°C) (C) and salinity (g/L) (D) on biosurfactant production from *Bacillus tequilensis* MK 729017 were also investigated using RSM-CCD. The lower and upper limits of the variables have been listed in Table 4.1. To obtain the optimum value of the process variables for the maximum biosurfactant concentration, the experiments were performed as per the experimental data set designed by CCD. A total of 30 experiments with different combinations of variables were performed. The actual and predicted values of the response (biosurfactant concentration) are reported in Table 4.3 (a).

Table 4.2 (b). Result of analysis of variance (ANOVA) for the produced quadratic model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	10	181.57	18.16	11.14	0.001
Blocks	1	8.46	8.46	5.19	0.049
Linear	3	77.25	25.75	15.81	0.001
A	1	22.47	22.47	13.8	0.005
B	1	21.52	21.52	13.21	0.005
C	1	33.25	33.25	20.41	0.001
Square	3	92.43	30.81	18.91	0.000
A^2	1	27.26	27.26	16.73	0.003
B^2	1	43.8	43.8	26.88	0.001
C^2	1	34.36	34.36	21.09	0.001
2-Way Interaction	3	3.43	1.14	0.7	0.575
AB	1	0.57	0.57	0.35	0.57
AC	1	0.015	0.02	0.01	0.925
BC	1	2.84	2.84	1.75	0.219
Error	9	14.66	1.63		
Lack-of-Fit	5	14.64	2.93	524.62	
Pure Error	4	0.022	0.0056		
Total	19	196.23			

Table 4.3 (a). RSM-CCD predicted runs with experimental and predicted values of biosurfactant concentration from *Bacillus tequilensis* MK 729017

Run no.	Carbon source conc. % (A)	pH (B)	Temperature (°C) (C)	Salinity (g/L) (D)	Biosurfactant concentration (g/L) (experimental)	Biosurfactant concentration (g/L) (predicted)
1	1	3	25	10	4.83	4.11
2	5	3	25	10	5.32	5.23
3	1	11	25	10	5.46	4.75
4	5	11	25	10	5.82	5.78
5	1	3	65	10	5.27	4.49
6	5	3	65	10	5.64	5.55
7	1	11	65	10	5.85	4.99
8	5	11	65	10	6.17	5.95
9	1	3	25	50	4.94	4.36
10	5	3	25	50	5.36	5.36
11	1	11	25	50	5.69	4.91
12	5	11	25	50	5.84	5.81
13	1	3	65	50	5.76	4.93
14	5	3	65	50	5.97	5.87
15	1	11	65	50	6.06	5.34
16	5	11	65	50	6.32	6.18
17	3	7	45	30	7.41	7.28
18	3	7	45	30	7.37	7.28
19	3	7	45	30	7.24	7.28
20	3	7	45	30	7.28	7.28
21	-1	7	45	30	1.87	4.02
22	7	7	45	30	6.46	5.98
23	3	-1	45	30	2.84	3.6
24	3	15	45	30	3.65	4.56
25	3	7	5	30	4.01	4.65
26	3	7	85	30	4.35	5.39
27	3	7	45	-10	5.63	6.55
28	3	7	45	70	6.28	7.03
29	3	7	45	30	7.44	7.28
30	3	7	45	30	7.12	7.28

The quadratic regression equation obtained from the RSM-CCD is presented as Equation (4.2). This regression Equation (4.2) can be used to predict the biosurfactant concentration from the above four experimental parameters in the mentioned range.

$$Biosurfactant\ concentration\ (g/L) = -1.56 + 1.161\ A + 0.797\ B + 0.1372\ C + 0.023\ D - 0.1421\ A^2 - 0.0499\ B^2 - 0.001411\ C^2 - 0.0003\ D^2 - 0.0031\ AB - 0.00041\ AC - 0.00078\ AD - 0.00047\ BC - 0.00028\ BD + 0.000122\ CD \quad (4.2)$$

The quality of the developed model could be proved by the regression (R^2) value, which was statistically found to be 93.16 % and adjusted R^2 was 85.82 %. A higher value of R^2 proved the statistical correlation between actual and predicted data, which confirmed the importance of the model. The ANOVA was calculated to evaluate the significance of the model coefficients to intensify the response which has been listed in Table 4.3 (b). From the ANOVA analysis, it can be speculated that the P-values were 0.004, 0.014, 0.021 and 0.108 for carbon source concentration (A), pH (B), temperature (C) and salinity (D), respectively. This validated the significance of the model as the P-values were less than 0.05 for A, B and C (except D). The F-value is a measurement of the variance of data about the mean based on the ratio of the mean square (MS) of group variance due to error. The F value was considerably higher for linear (11.41) and square interactions (34.27). The P-value of the entire model was found to be significant (0.029). The quadratic effects of A^2, B^2, C^2 and D^2 were also found to be less than 0.05, which could also be considered as significant terms to influence the response. The response surface images of *B. tequilensis* MK 729017 are shown in Figure 4.11. The optimum conditions for the maximum response (biosurfactant concentration) were predicted to be 3.85 % (v/v) of carbon source i.e., glycerol, pH of 7.6, 48.6 ºC temperature with 39.3 g/L salinity. The projected maximum biosurfactant concentration was 7.44 g/L.When the experiment was performed at the above optimum conditions, the actual biosurfactant concentration was found to be 7.46 ± 0.39 g/L, which validated the model predictions.

Table 4.3 (b). Analysis of variance (ANOVA) for the quadratic model

Source	DF	Adj SS	Adj MS	F Value	P Value
Model	15	48.62	3.24	12.71	0.029
Blocks	1	1.89	1.89	7.4	0.017
Linear	4	11.65	2.91	11.41	0
C %	1	7.16	7.16	28.07	0.004
pH	1	2.01	2.01	7.89	0.014
Temp	1	1.72	1.72	6.75	0.021
Salinity	1	0.75	0.75	2.95	0.108
Square	4	34.97	8.74	34.27	0
C% × C%	1	13.76	13.76	53.92	0
pH × pH	1	22.63	22.63	88.68	0
Temp × Temp	1	4.91	4.91	19.26	0.001
Salinity × Salinity	1	0.0018	0.0018	0.01	0.935
2-way interaction	6	0.116	0.19	0.08	0.998
C % × pH	1	0.059	0.59	0.23	0.639
C % × Temp	1	0.0002	0.0002	0	0.981
C % × Salinity	1	0.03	0.03	0.12	0.738
pH × Temp	1	0.0053	0.0053	0.02	0.888
pH × Salinity	1	0.02	0.02	0.09	0.775
Temp × Salinity	1	0.0003	0.0003	0	0.973
Error	14	3.57	0.26		
Lack-0f-fit	10	3.53	0.35	33.2	0.002
Pure error	4	0.04	0.01		
Total	29	52.2			

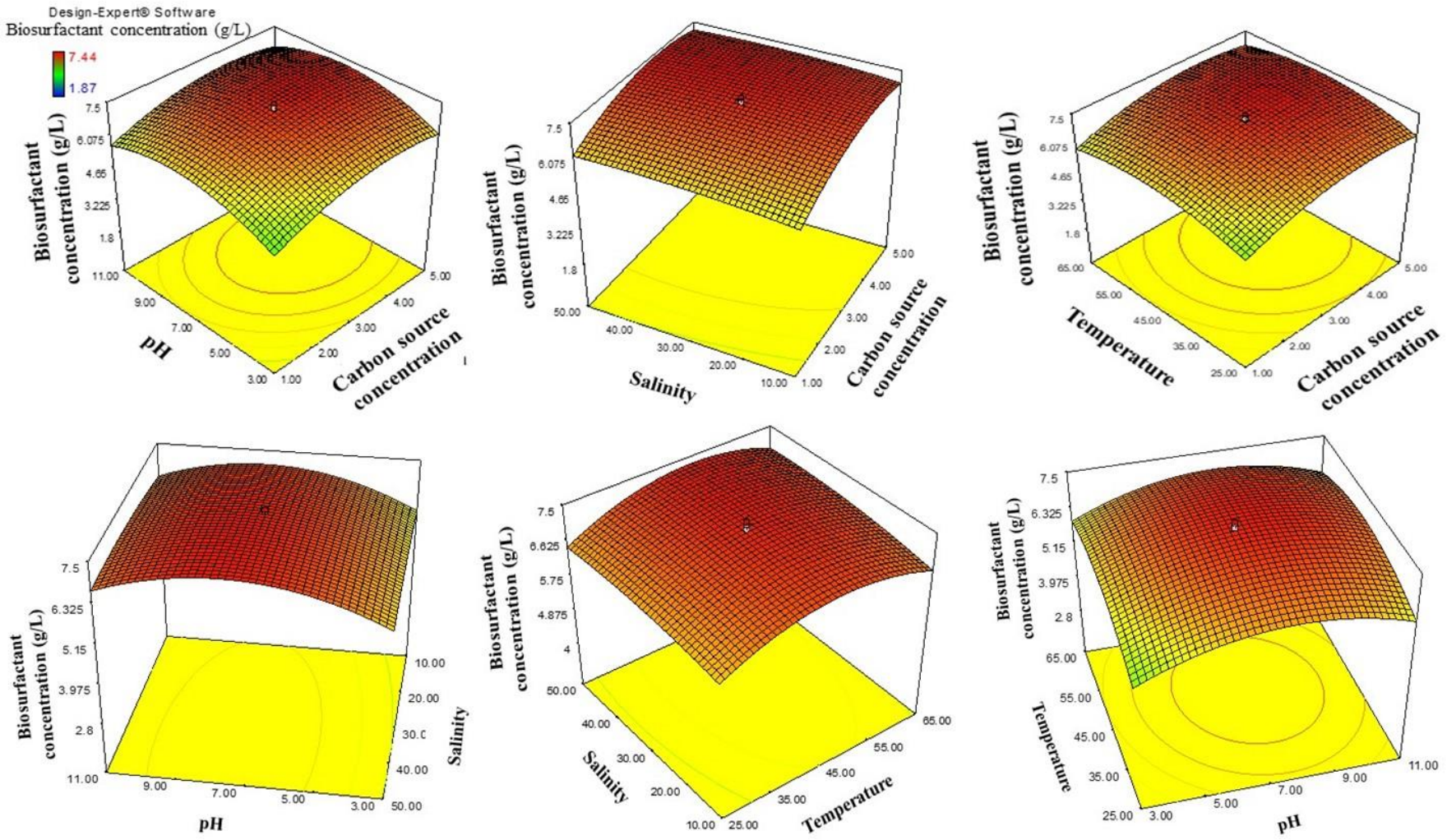


Figure 4.11. Response surface 3-D plots for the maximum biosurfactant concentration from *Bacillus tequilensis* MK729017 considering carbon source concentration, pH, temperature and salinity as the process variables.

4.3.4. Biosurfactant production and subsequent hydrocarbon substrate degradation

The bacterial culture showed very good frothing property indicating the presence of biosurfactant and the appearance of viscous concentrated biosurfactant after acid precipitation and solvent extraction methods. The kinetic growth curve of *Bacillus subtilis* MG 495086 in MSM media and subsequent biosurfactant yield followed a similar trend as observed and reported (Chandankere et al., 2013) (Figure 4.12). The maximum dry biomass was 2.16 g/L and the corresponding biosurfactant concentration was 5.84 g/L at the end of the exponential phase (90 hours). The maximum biosurfactant yield (6.3 ± 0.1 g/L) was achieved during the exponential phase (at 70 hours) when the biomass concentration was 1.54 g/L and the corresponding surface tension was found to be 29.85 mN/m. The HPLC data were observed to be in good coordination with the biosurfactant extraction data as shown in Figure 4.12.

It can be concluded from Figure 4.12 that the biosurfactant production started from the initiation of the growth phase itself but the maximum yield was achieved during the exponential phase so the product (biosurfactant) can be referred to as primary metabolite i.e., growth-associated product (Chandankere et al., 2013; Elazzazy et al., 2015; Fontanille et al., 2012; Liu et al., 2013; Ruiz et al., 2010). But after that, the biosurfactant concentration started to decrease, presumably due to the fact that the bacterial cells started to consume the biosurfactant as their secondary carbon source because of nutritional insufficiency. In fact, the residual substrates were determined at every 10 hours intervals to determine the rate of substrate degradation along with biosurfactant production. Two different hydrocarbon degradation rates were observed as shown in Figure 4.12. Slope upto 70 hours was found to be about three folds higher (0.0244) as compared to that during 80 - 120 hours. Hence the decrease in oil consumption rate and simultaneously decrease in biosurfactant

concentration indicated the possible utilization of biosurfactant as a secondary carbon source. After 120 hours, the light paraffin oil degradation was found to be about 91.3 ± 5 %.

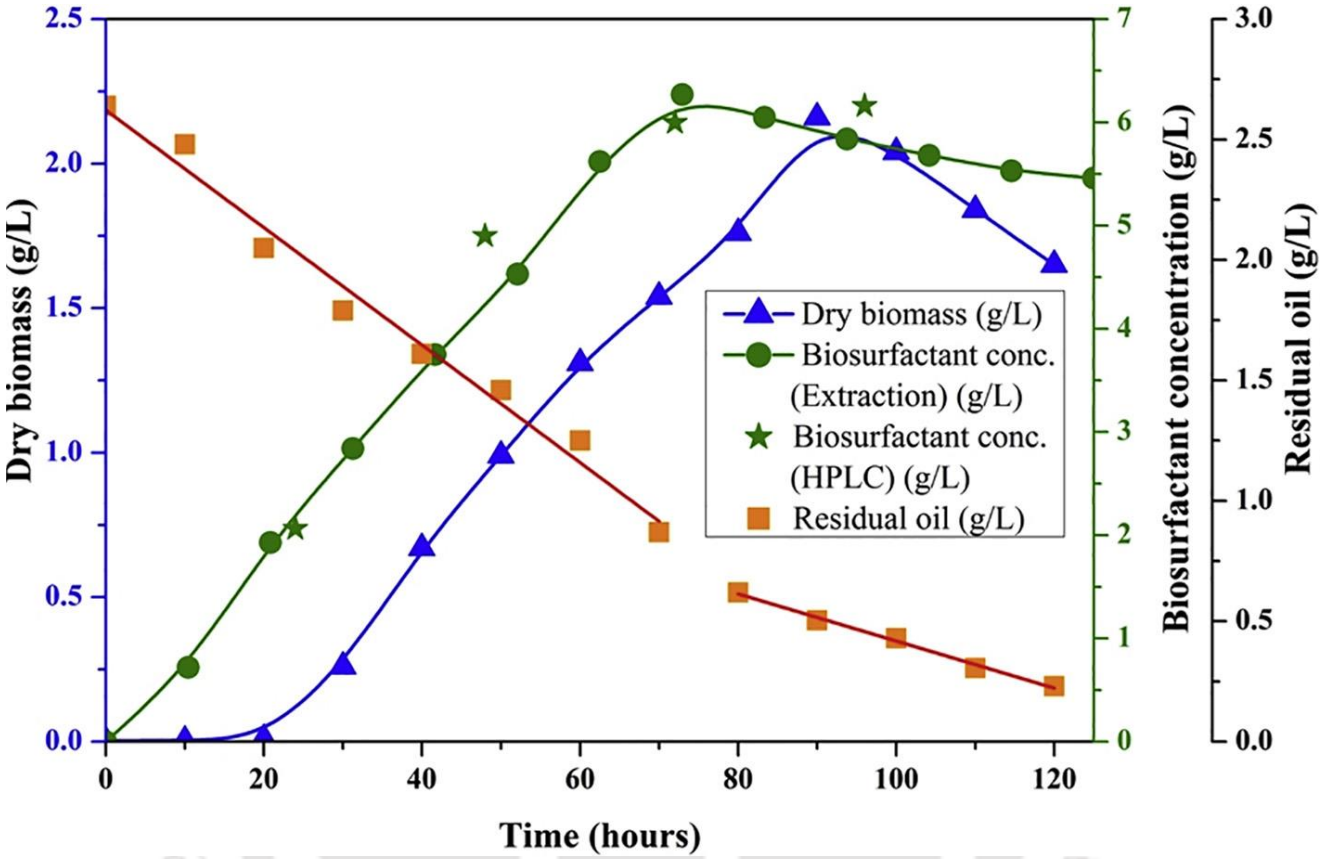


Figure 4.12. Profile of average biosurfactant concentration and dry biomass of *Bacillus subtilis* MG 495086 along with residual oil.

Another similar batch kinetic study was performed at the optimized conditions for the biosurfactant production from *Bacillus tequilensis* MK729017. The samples were taken at every 12 hours intervals to measure dry biomass, ST, biosurfactant concentration as well as residual glycerol concentration. The maximum dry biomass was 1.5 ± 0.02 g/L at 72 hours when the biosurfactant production was also found to be the maximum (7.46 ± 0.39 g/L). ST reached its minima much earlier (48 hours) because of concentration higher than CMC. The residual glycerol concentration was gradually decreased with time and was almost exhausted after 96 hours (Figure 4.13). Besides,

the cell surface hydrophobicity (CSH) of *Bacillus tequilensis* MK 729017 was also studied. The CSH was found to be 43 ± 2 % at 48 hours. The maximum hydrophobicity was obtained as 52 ± 0.5 % at 72 hours when the biosurfactant concentration was the highest. This indicated a good correlation between hydrocarbon degradation and biosurfactant production. The result indicated that the biosurfactant improved the bioavailability of the hydrocarbon in the liquid culture so that it could be easily consumed by the bacteria (Chandankere et al., 2014). A similar pattern was also observed during the course of octadecane degradation and simultaneous rhamnolipid production by *Pseudomonas* sp. DG 17 (Hua & Wang, 2012). To analyze the growth kinetics systematically, the related parameters should be stoichiometrically defined. The kinetic parameters were estimated using the following Equation 4.3 and Equation 4.4. (Kargi & Shuler, 1992).

$$q_P = \mu \frac{Y_{PS}}{Y_{XS}} \tag{4.3}$$

$$q_S = \frac{\mu}{Y_{XS}} + \frac{q_P}{Y_{PS}} + m_S \tag{4.4}$$

Where μ is the specific growth rate of *Bacillus tequilensis* MK 729017, Y_{PS} is the yield coefficient of product formation ($\Delta P/\Delta S$), $Y_{XS}$is the biomass yield ($\Delta X/\Delta S$), q_P is the specific product (biosurfactant) formation rate, q_S is the specific substrate (glycerol) utilization rate and m_S is the maintenance coefficient used to describe the specific rate of substrate consumption for cellular maintenance, which has been assumed as zero during the growth phase. μ value was found to be 0.08 ± 0.01 hour^{-1}. Y_{PS} and Y_{XS}were determined as 0.45 ± 0.01 g Surfactin/g glycerol and 0.1 ± 0.001 g cells/g glycerol, respectively during the growth phase. The values of q_P and q_S were estimated as 0.68 g Surfactin/g cells-h and 3.07 g glycerol/g-h using Equation (4.3) and Equation (4.4), respectively.

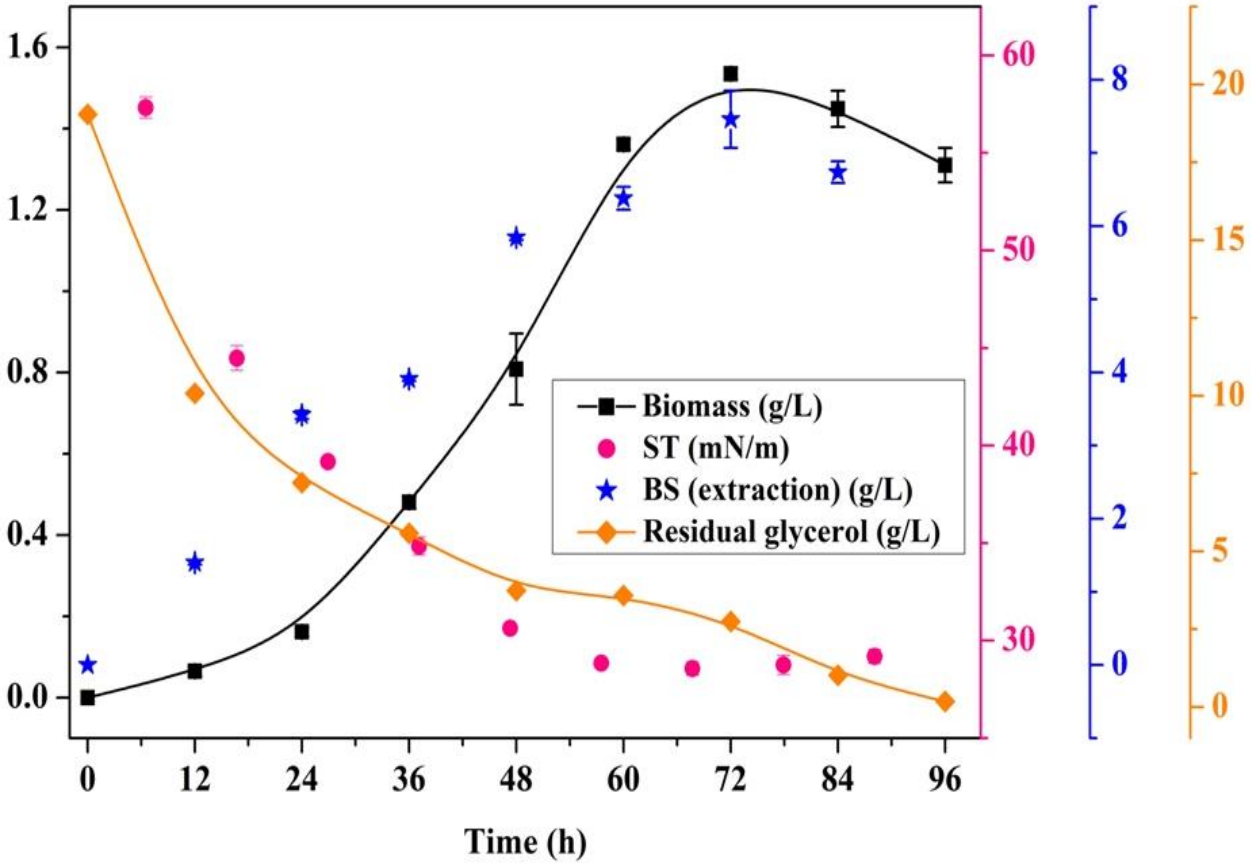


Figure 4.13. Profile of produced biosurfactant concentration and dry biomass of *Bacillus tequilensis* MK 729017 along with residual glycerol. The surface tension (ST) is gradually decreased along with the production of the biosurfactant

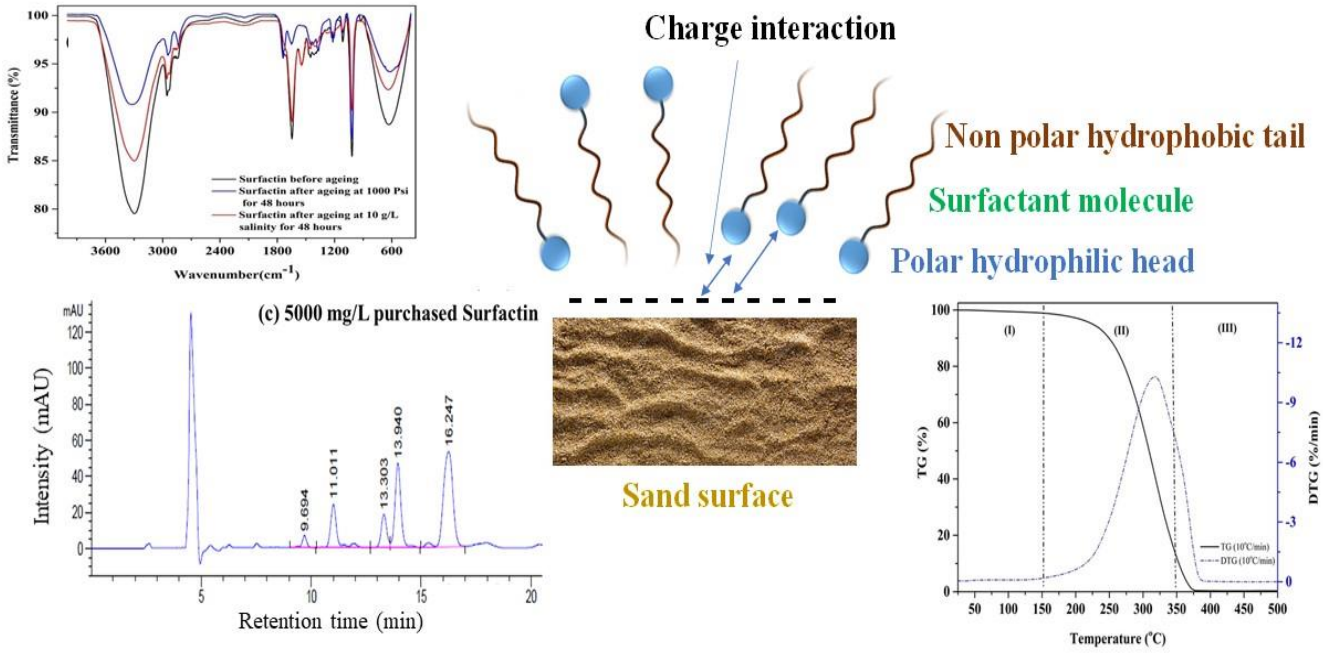
4.4.Conclusions

The produced biosurfactants from *Bacillus* sp. were chemically characterized with FTIR and NMR analyses which revealed the presence of both aliphatic and peptide groups which are a signature feature of the lipopeptide biosurfactant. Additionally, the presence of five isoforms (C_{12}, C_{13}, C_{14}, C_{15} and C_{16}) utilizing LC-MS and HPLC analyses identified and confirmed the lipopeptide to be Surfactin at the lower CMC values of 40 mg/L and 90 mg/L produced by *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017, respectively. Statistical analysis with RSM-CCD and

ANOVA also validated the optimized condition (carbon source and environmental parameters) by determining the surface tension reduction by *Bacillus subtilis* MG495086 and the maximum production of Surfactin by *Bacillus tequilensis* MK 729017, respectively. The optimum biosurfactant production (6.3 ± 0.1 g/L) and the minimum surface tension 29.85 mN/m were obtained after 96 hours of incubation of *Bacillus subtilis* MG 495086 under optimal conditions i.e., 3.8 % (v/v) of light-paraffin oil as a sole carbon source at 62.4 ºC and pH 7.7 with the maximum oil degradation capability of 91.3 ± 5 %. Similarly, the maximum biosurfactant concentration of 7.46 ± 0.39 g/L was achieved from *Bacillus tequilensis* MK 729017 after 72 hours incubation with 3.85 % (v/v) glycerol, pH of 7.6, 48.6 °C temperature with 39.3 g/L salinity. The biosurfactant solutions of *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 altered the wettability of hydrophobic rock surface from 70 ± 1º to 28 ± 1º and 90 ± 1º to 26 ± 1º, respectively indicating an excellent interfacial interaction. The IFT of the produced biosurfactants was found to be 0.32 ± 0.02 mN/m. Batch kinetic studies were performed to measure the correlation of dry biomass, ST, biosurfactant concentrations, as well as the concentrations of the residual substrates at the optimized conditions by withdrawing samples at certain time intervals. The residual light paraffin oil and glycerol concentrations gradually decreased with time and were almost exhausted after 96 hours indicating the significance of the process in hydrocarbon degradation along with value-added (biosurfactant) product formation which established their suitability for microbial enhanced oil recovery.

Chapter 5

Adsorption Behaviour of the Produced Biosurfactants



(*Journal of Environmental Chemical Engineering* 10, 107083, 2021 (Datta et al., 2021))

This chapter describes the adsorption phenomena of Surfactin onto model silica (sand) surfaces. The mineralogy/morphology of the model core surface was studied. Different adsorption isotherms and kinetics models were fitted with the experimental data to represent the concerned surfactant adsorption characteristics. The adsorption behavior with diverse adsorbate doses at various temperatures was also conducted to mimic reservoir conditions so as to understand the feasibility and spontaneity of the process. The findings provide newer insights into real-time biosurfactant adsorption characteristics, which are often ignored in conventional approaches and methodologies.

5.1.Introduction

Petroleum reservoir essentially comprises of a substantial pool of hydrocarbons in the porous rock formations that can be extracted by employing different oil recovery techniques (Pal et al., 2018b). In enhanced oil recovery (EOR) chemical slug or bio slug is injected into the reservoir to effectively displace the entrapped oil. The alkali-surfactant-polymer (ASP) flooding is one of the main subsets of EOR. Different EOR processes require different strategies to optimize the surfactant selection, and the choice of surfactants highly depends on the conditions of oil reservoirs and the behavior of the surfactant inside the reservoir such as interaction with the rock sample. Sometimes, the acid present in the crude oil reacts with the alkaline solution to produce *in situ* surfactant (ionized acid) (Liu et al., 2004).

Oil reservoirs are classified into two main categories depending on the type of formation rocks: sandstone and carbonate. The adsorption of surfactants is influenced by the surface charge of the rock surface and the fluid interface. Typical sandstone contains a large amount of quartz (silica, SiO_2) and a small amount of carbonate and silicate materials (Mannhardt et al., 1992). Since silica bears a negative charge, the adsorption of anionic surfactant is mostly inhibited due to the electric repulsion between the formation rock and the surfactant (Thibaut et al., 2000). It has been found that the adsorption of cationic surfactant on the sand surface is more than anionic surfactants whereas the non-ionic surfactants show intermediate behavior (Bera et al., 2013; Curbelo et al., 2007).

Surfactant flooding is quite promising to recover higher residual oil by increasing the oil displacement efficiency by lowering the IFT oil-aqueous interface (Ma et al., 2013; Pal et al., 2018a), which results in the mobilization of the residual oil. However, the adsorption of surfactants onto reservoir rock could leave a negative impact on the EOR process because unwarranted

adsorption of surfactant on rock surface causes the loss and reduction in the surfactant concentration and negatively influences the process economics of EOR. Hence, the fundamentals of adsorption behavior need to be studied well, in order to decide the surfactant concentration range and to optimize the bio-slug design for better recovery efficiency and improve process economics (Tackie-Otoo et al., 2020) by conducting successful EOR procedure. Several parameters such as the charge of fluid and sand, rock surface area, salinity, temperature and internal pH have been reported to critically influence the surfactant adsorption characteristics (Bera et al., 2013; ShamsiJazeyi et al., 2014). Although the adsorption behavior of chemically synthesized surfactants has been previously studied by many researchers, but investigation on the interpretation of adsorption data of biosurfactants has not been well understood. Thus, there is a need to identify and study the physicochemical characteristics of biosurfactants which is a potentially good candidate for application in the petroleum industry (Arabloo et al., 2015).

This Chapter discusses the adsorption behavior of a lipopeptide biosurfactant Surfactin onto sandstone silica samples by applying the real reservoir scenarios in the experimental setup with temperature variation (25, 37 and 55 °C) utilizing synthetic formation water. High-performance liquid chromatography (HPLC) is used to quantify the amount of surfactant. The rock mineralogy along with the impact of aqueous media salinity and experimental temperature are the major variables. Both the kinetics and equilibrium adsorption data were obtained from the batch mode studies to identify the best-fit model for Surfactin adsorption characteristics. The thermodynamics feasibility of the adsorption process was also examined to verify the spontaneity of the process.

5.2.Materials and Methods

5.2.1. Chemicals and Reagents

The chemicals used for this study and their sources have been mentioned in previous chapters. The remaining chemicals such as sodium dodecyl sulfate (SDS) (GRM6218) and cetrimonium bromide (CTAB) (RM4867) are procured from HiMedia Laboratories, India.

5.2.1.1.Preparation of synthetic formation water

Synthetic formation water was prepared in the laboratory to carry out the adsorption experiments in order to see the difference in the adsorption pattern for water and formation water. The formation water was prepared by following the literature previously reported from our group (Saha et al., 2017a). The chemicals used to prepare the synthetic formation water were $NaHCO_3$ (99 % purity, Sigma Aldrich Pvt Ltd.), NaOH (GRM467), NaCl (GRM031), KCl (GRM698), $MgSO_4.7H_2O$ (GRM684) and $CaCl_2$ (GRM710). Except for $NaHCO_3$, all chemicals were purchased from HiMedia Laboratories, India.

5.2.2. Adsorbent (sand) sample preparation and characterization

The sand samples for adsorption experiment were collected from the nearby construction sites of IIT Guwahati. Then they were screened through the sand-sieves possessing 90 µm and 150 µm mesh size. The normal distribution of sand grain particles of the Assam oil reservoir has been reported to be in the range of 4 to 2 phi scale which is equivalent to the particle size of 63 µ to 250 µ (fine sand) (Gogoi et al., 2015). Hence the sand particles with sizes 90 µm and 150 µm or less were mixed in equal amounts to prepare a heterogeneous adsorbent surface (mimicking core surface) to study the adsorption phenomena of the biosurfactant. The sand mixture was cleaned properly with Mili-Q water and kept for drying in the hot air oven at 70 °C. The washed dried sand

samples were then employed for experimental purposes as the adsorbent. Detailed characterization of the sand sample was accomplished utilizing several analytical instruments (EDX, XRD and BET) to assess the nature of the charge, mineral composition, surface properties and pore sizes. Electron dispersive X-ray (EDX) analysis of the sand sample was carried out in a Field emission scanning electron microscope (FESEM; Make: Zeiss, Model: Sigma 300). The EDX detector was attached with FESEM to find out the probable elemental composition of the sand sample. X-ray diffractogram (XRD) analysis provides structural information about crystallinity and the possible chemical composition of the material. XRD of the prepared sample was documented in a wide range of Bragg angle 2θ ($10° \leq 2\theta \leq 90°$) by Rigaku Smartlab 9KW. For the XRD, the powder form of the sample was used. Finally, the BET (Brunauer–Emmett–Teller) analysis determined the surface area of the sample via a Quantachrome, Autosorb - IQ MP BET surface analyzer. In BET, liquid nitrogen was used as the adsorbate gas where the degassing temperature is kept 110 °C.

5.2.3. Surfactin production, quantification and standard solutions preparation

The biosurfactant which was thoroughly utilized in this study was Surfactin, produced from *Bacillus tequilensis* MK 729017. Surfactin is a well-studied lipopeptide type of biosurfactant whose extraction procedure has already been elaborated in Chapter 3 and Chapter 4 (Datta et al., 2018). In brief, Surfactin is a cyclic lipopeptide that contains beta-hydroxy fatty acids as hydrophobic moiety along with seven amino acids and is investigated to be one of the influential biosurfactants for its exceptional surface properties to mobilize the entrapped oil (Hentati et al., 2019; Liu et al., 2015). The fatty acid alkyl chain of Surfactin varies in 13 to 15 carbon atoms length. The sequentially arranged amino acids is usually Glu-Leu-Leu-Val-Asp-Leu-Leu. Surfactin contains its isoform mixture which is generated due to carbon chain length variations

and fatty acid composition (Hentati et al., 2019; Wang et al., 2020). It is an interesting aspect to observe that how positively or negatively Surfactin responds in high thermal and salinity conditions (similar to internal oil reservoir conditions) because it has not yet been much explored in such extreme conditions (Alvarez et al., 2020).

The biosurfactant samples (produced, procured and after adsorption) were analyzed by HPLC analysis (Jian et al., 2018). Initially, biosurfactants were dissolved in methanol, filtered through a 0.2 µm syringe filter and then injected in an HPLC system (Make: Agilent LC system, Agilent technologies; model: 1260 Infinity II) equipped with a C18 column (Agilent TC- C18 (2)) having dimensions of 4.6 × 250 mm, with a flow rate of 0.7 ml/minute at 26 ºC for 30 minutes in isocratic mode. The mobile phase contained ACN and 3.8 mM trifluoroacetic acid (TFA) in water in the ratio of 90:10. The metabolite (lipopeptide) was quantified by detecting at the dual-wavelength of 205 and 210 nm with a UV detector (Datta et al., 2018). Specific concentration (5000 mg/L) of Surfactin standard solution was prepared by dissolving the purified Surfactin in Mili-Q water in the volumetric flasks. Then the prepared solutions were sonicated for 30 minutes in a water-bath-type sonicator for smooth mixing to perform further adsorption studies (Arabloo et al., 2015).

5.2.4. Adsorption experiments of Surfactin

Adsorption experiments were conducted primarily to assess the influence of water and synthetic formation water on the adsorption behavior of the Surfactin onto the prepared silica samples. Approximately 50 g of pre-treated sand sample was combined with 100 ml of 5000 mg/L biosurfactant solution. The flasks were kept for shaking at 37 °C in a shaker incubator at 180 rpm. The experimental samples of surfactant solutions were withdrawn from the container at different time intervals of adsorption (15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours and 8 hours) to quantify the Surfactin concentration with HPLC till it

reached to a constant minimum. Batch experiments were performed at three diverse temperatures i.e., 25 °C, 37 °C and 55 °C. Changes in the biosurfactant concentration during the course of the adsorption process were then determined by HPLC. The concentration of the biosurfactant adsorbed onto the silica surfaces was calculated by the difference in the initial biosurfactant concentration and the concentration after the adsorption process. The adsorption capacity is measured using the following Equation 5.1:

$$q_t = \frac{(C_0 - C_t) \times V}{W} \tag{5.1}$$

Where, q_t (mg/g) was the biosurfactant adsorption rate i.e., the amount of Surfactin adsorbed by adsorbents at time 't', C_0 (mg/L) was initial Surfactin concentration and C_t (mg/L) was the Surfactin concentration at the time 't'. W and V were the weight of the adsorbent (g) and volume of the solution (L), respectively. All the experiments were performed in triplicate for calculating the average and standard deviation (Sharma et al., 2018c; Tiwari et al., 2017).

5.2.4.1.Adsorption equilibrium isotherms

An adsorption isotherm model is necessary for any adsorption system to foresee the capacity of the adsorption matrix for a specific adsorbate concentration. Adsorption isotherms could be achieved by plotting the amount of adsorption (mol/g adsorbent) vs. the equilibrium concentration of the biosurfactant. There are various equilibrium adsorption models, such as Langmuir, Freundlich, Temkin, Jovanovic, Halsey, Hill, Redlich-Peterson, Sips, Toth, Brouers - Sotolongo and Khan model (Barati-Harooni et al., 2016). The adsorption kinetics investigations have been conducted in triplicate (at 37 °C) and the average values are reported.

5.2.4.1.1. Langmuir isotherm

The Langmuir isotherm is grounded on the fundamental assumptions that adsorption only occurs at a definite homogenous site and adsorption cannot occur at the site which has been already occupied by the solute. The adsorption rate is proportionate to the solute concentration gradient and the amount of bare surface. This model is appropriate for monolayer adsorption due to the homogeneous surface containing a finite number of identical sites (Altintig et al., 2018). When the adsorption surface is supposed to be completely homogenous, the Langmuir isotherm model is used to anticipate the equilibrium adsorption rate (q_e) that was proposed by Irving Langmuir (1916) (Langmuir, 1916). The non-linear expression of Langmuir isotherm is depicted as followed in Equation (5.2):

$$q_e = \frac{q_0 K_L C_e}{1 + K_L C_e} \tag{5.2}$$

$$R_L = \frac{1}{1 + K_L C_0} \tag{5.3}$$

Where, q_e (mg/g) is the amount of biosurfactant adsorbed at the equilibrium, C_e (g/L) is the equilibrium concentration. Similarly, q_0 (mg/g) and K_L(L/mg) symbolize the adsorption capacity of the Langmuir model and adsorption equilibrium constant, respectively. R_L is the dimensionless separation factor and C_0 (g/L) is the initial surfactant concentration. Hence, a graph of C_e vs. C_e/q_e forms a straight line if the Langmuir isotherm is followed. q_0 and K_L can be calculated using the slope and intercept of the line, respectively (Ahmadi & Shadizadeh, 2015; Tiwari et al., 2017). R_L is used to predict the viability of the adsorption phenomena and calculated using Equation (5.3). The value of R_L predicts the nature of isotherm that could be irreversible ($R_L = 0$), favourable ($0 < R_L < 1$), linear ($R_L = 1$) or reversible ($R_L > 1$) (dos Santos Bispo et al., 2021; Hall et al., 1966).

5.2.4.1.2. Freundlich isotherm

When the adsorbent is assumed to have a heterogeneous surface containing various types of adsorption sites, the Freundlich model is used, but this isotherm model cannot guesstimate the adsorbate amount necessary to saturate the adsorbent (Freundlich, 1907). The Freundlich isotherm model quantitatively assumes that the amount of adsorption is proportionate to the solute equilibrium concentration in the heterogeneous surface by multilayer sorption. The non-linear expression of Freundlich isotherm is presented by Equation (5.4) (Park et al., 2015):

$$q_e = K_F C_e^{\,1/n} \tag{5.4}$$

Where, K_F (mg/g) (L g^{-1})$^{1/n}$ are the Freundlich constants. K_F shows the amount of the adsorbed biosurfactants for unit equilibrium concentration and 1/n is the dimensionless adsorption intensity factor. Previous studies suggested that the value of 1/n less than one means it was a favorable heterogeneous adsorption process and the value of 1/n more than one shows to be a multilayer cooperative adsorption process (Ahmadi & Shadizadeh, 2015; Fytianos et al., 2000; Sharma et al., 2018c). At a constant temperature, the Freundlich constants K_F and 1/n are associated with adsorption capacity and adsorption intensity, a smaller 1/n value implies more heat of adsorption resulting in higher adsorption intensity (Park et al., 2015).

5.2.4.1.3. Temkin isotherm

The passive interaction of adsorbate – adsorbate on adsorption isotherms is considered in this model. The Temkin isotherm is usually represented by the subsequent Equation (5.5) (Temkin & Pyzhev, 1940):

$$q_e = B\,(\ln K_T + \ln C_e) \tag{5.5}$$

Where B and K_T (L/g) are Temkin constant and equilibrium binding constant, respectively. A graph of q_e vs. ln C_e helps to estimate the values of B and K_T from the slope and the intercept of the straight line, respectively.

5.2.4.2.Adsorption kinetic models

The second significant component for an adsorption system is the adsorption kinetics that is dependent upon the interaction of adsorbate, adsorbent and the process conditions. In this study, the role of contact time has been scrutinized on the adsorption capacity (q_t) employing 5000 mg/L Surfactin. Four common kinetic models viz pseudo-first-order, pseudo-second-order, intra-particle diffusion and Elovich kinetic rates were examined to find out the best-fit kinetic model for Surfactin adsorption onto the silica surface. The adsorption kinetics experiments have been conducted in triplicate (at 37 °C) and the average values are reported.

5.2.4.2.1. Pseudo first-order kinetic model

The kinetics of the liquid-solid phase adsorption process is modeled as the pseudo first-order which is depicted as follows in Equation (5.6) (Sharma et al., 2018c):

$$q_t = q_e\,[1 - \exp(-K_1 t)] \qquad (5.6)$$

Where q_t (mg/g) and q_e (mg/g) are the amount of adsorbate adsorbed onto a unit mass of adsorbent at time t and equilibrium condition, respectively. K_1 (min^{-1}) is the rate constant of the pseudo-first-order adsorption reaction (Ho & McKay, 1999; Lagrergen, 1898; Ruthven, 1984), where the intercept and slope of the plot of ln ($q_e - q_t$) against t used to determine the values of $K_1$and q_e.

5.2.4.2.2. Pseudo second-order kinetics model

The pseudo-second-order kinetic model is associated with chemisorption and physisorption, where it anticipates the physiochemical interaction of adsorbent with an adsorbate. This is a rate-limited step which is exhibited in non-linear form as shown in Equation (5.7) (Pandey, 2019):

$$q_t = \frac{q_e^2 K_2 t}{1 + K_2 q_e t} \tag{5.7}$$

Where, K_2 (g/mg.min) is the rate constant of pseudo-second-order adsorption. The definitions and units of the other variables are the same as those of the pseudo-first-order kinetic Equation. By plotting $\frac{t}{q_t}$ vs. t, the equilibrium rate (q_e) and the rate constant (K_2) are calculated from the slope and intercept of the linear curve. This type of model has been reported to foresee the kinetic behavior of the liquid-solid systems within the specified process variables range. The pseudo-second-order Equation has mainly been used in describing the adsorption kinetics of metal ions, oils, dyes and organic compounds from aqueous solutions. The initial adsorption rate (h) (mg g^{-1} min^{-1}) and half adsorption time ($t^{1/2}$) (g mg^{-1} min^{-1}) could be estimated using the following Equation (5.8) (Ho & McKay, 1999; Lagrergen, 1898; Ruthven, 1984):

$$h = K_2 q_e^2 = 1/t^{1/2} \tag{5.8}$$

5.2.4.2.3. Intra-particle diffusion (IPD) model

First-order and second-order kinetic models are mainly employed to determine the reaction type and adsorption rate. In general, the adsorption process in a homogeneous system has two steps. Initially, the adsorbate is adsorbed on the outer surface of the particle which is followed by slow

diffusion of adsorbate in the internal structure of the adsorbent. Therefore, the adsorption process can be classified as a multi-event process that takes place sequentially starting with boundary layer diffusion, adsorption of biosurfactants on active binding sites, and finally intra-particle diffusion (Tiwari et al., 2017). To understand to diffusion mechanism in the adsorption process, it is important to validate some diffusion models. One of such proposed models is the intra-particle diffusion model which is expressed as follow:

$$q_t = K_i\, t^{1/2} + \mathrm{C} \tag{5.9}$$

According to the above-mentioned Equation (5.9), the slope of the equation ($q_t\ vs.\ t^{1/2}$) is the rate constant (K_i) of the intra-particle diffusion whose unit is $mg.g^{-1}.min^{-1/2}$ and C (mg/g) is a constant that relates to boundary layer thickness (McKay, 1983).

Initially, the biosurfactant molecules are transported to the surface of the adsorbent, which is termed film diffusion (boundary layer diffusion), then the biosurfactant is diffused at specific adsorption sites, which is called surface diffusion. After that, the adsorption of biosurfactant occurs on the internal/external surface of mineral particles, which is a comparatively fast process so it cannot be considered as the rate-controlling mechanism. It is well known that the adsorption rate is controlled by the slowest step, usually, either intraparticle or film diffusion or a combination of both steps is considered as the rate-controlling mechanism. The first steep step is related to the diffusion or transport of surfactant molecules from the bulk solution to the mineral surface. While the second step describes the intraparticle diffusion step. A linear graph that passes through the origin, is specified as pure intraparticle diffusion (IPD). However, if the graph was multilinear, then it was indicated that there was an involvement of the other diffusion process than IPD that endorsed the initial rapid adsorption of the surfactant molecule followed by a slower IPD. Similar

multi-linearity trends have also been observed in the pieces of literature by following Equation (5.9) (Arabloo et al., 2015; Bera et al., 2013; Sharma et al., 2018c).

5.2.4.2.4. Elovich's equation

The Elovich model describes the chemical nature of adsorption and assumes that adsorption sites increase exponentially with adsorption indicating multilayer adsorption. The differential form of Elovich model is expressed in the form of Equation (5.10) (Ho & McKay, 1999; Lagrergen, 1898; Low, 1960):

$$\frac{dq_t}{dt} = \alpha e^{-\beta q_t} \tag{5.10}$$

Where q_t (mg/g) is the adsorption capacity at time t. α (mg/g.min) and β (g/mg) are the initial adsorption rate and adsorption constant for a specific study, respectively. Assuming $\alpha\,\beta\,t \gg 1$, then integrating the above equation over the boundary conditions (q_t = 0, at t = 0; $q_t = q_t$ at t = t), gives the following Equation (5.11):

$$q_t = \frac{\ln(\alpha\beta)}{\beta} + \frac{\ln(t)}{\beta} \tag{5.11}$$

Hence, α *and* β can be calculated from the slope and intercept of q_t vs. $\ln(t)$ the linear plot, respectively. This model defines well the adsorption process for the metal ions and α and β are related to the chemisorption rate and surface coverage, respectively.

5.2.5. Evaluation of thermodynamic parameters

Gibbs free energy change (ΔG) is the main factor that determines the spontaneity of any reaction. If the ΔG (J/mol) value becomes negative, this indicates the reaction to be spontaneous. Despite

the temperature variation, a reaction would be called spontaneous if the enthalpy (ΔH) (J/mol) and entropy (ΔS) (J/mol.K) becomes negative and positive, respectively (i.e. ΔH < 0; ΔS > 0) (Schramm, 2000). Gibbs free energy change (ΔG) can be estimated using the subsequent Equation (5.12):

$$\Delta G = -RTlnK_C \quad (5.12)$$

Where R is the universal gas constant (8.314 $Jmol^{-1}K^{-1}$), T is the experimental temperature (K) and K_C is the standard thermodynamic equilibrium constant (q_e/C_e). Besides that, other thermodynamic parameters like enthalpy and entropy change can be determined by Equation (5.13) and Equation (5.14):

$$\Delta G = \Delta H - T\Delta S \quad (5.13)$$

$$\ln K_C = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \quad (5.14)$$

The enthalpy and entropy values were estimated using the slope and intercept of a plot of ln K_C vs. 1/T, respectively (Barati et al., 2016; Saha et al., 2017a; Sharma et al., 2018c).

5.3. Results and Discussion

5.3.1. Adsorbent (sand) characterization for adsorption experiments

The pre-treated sandstone sample was characterized with various analytical methods so as to know the information about the mineralogy, porosity and surface properties. Figure 5.1 (a) shows the EDX elemental peaks of the sand (silica) sample containing majorly Oxygen and Silica with some traces of Aluminium, Iron, Sodium, Calcium, Potassium and Magnesium.

The data analysis was carried out following JCPDS files (Bera et al., 2013). Figure 5.1 (b) represents the XRD diffraction trend that shows the presence of two to three main peaks along with several other minor peaks. The characteristic peaks were obtained at 20.88°, 26.66°, 28°, 50.14° and 60°. The main single-headed peak at 26.66° indicates that only one phase is present in the sample without any impurity. The XRD result was found to be similar to the XRD pattern of the study by (Hazarika & Gogoi, 2020) which was conducted in the Upper Assam sandstone reservoir. JCPDS (file no. 861630) data denoted the occurrence of silica in the sand and the same was also supported by the EDX images. The other peaks showed the presence of quartz in a small amount (Bahoria et al., 2018; Bera et al., 2013). The BET analysis revealed the surface area of the silica sample to be 1.169 m^2/g (Ma et al., 2013).

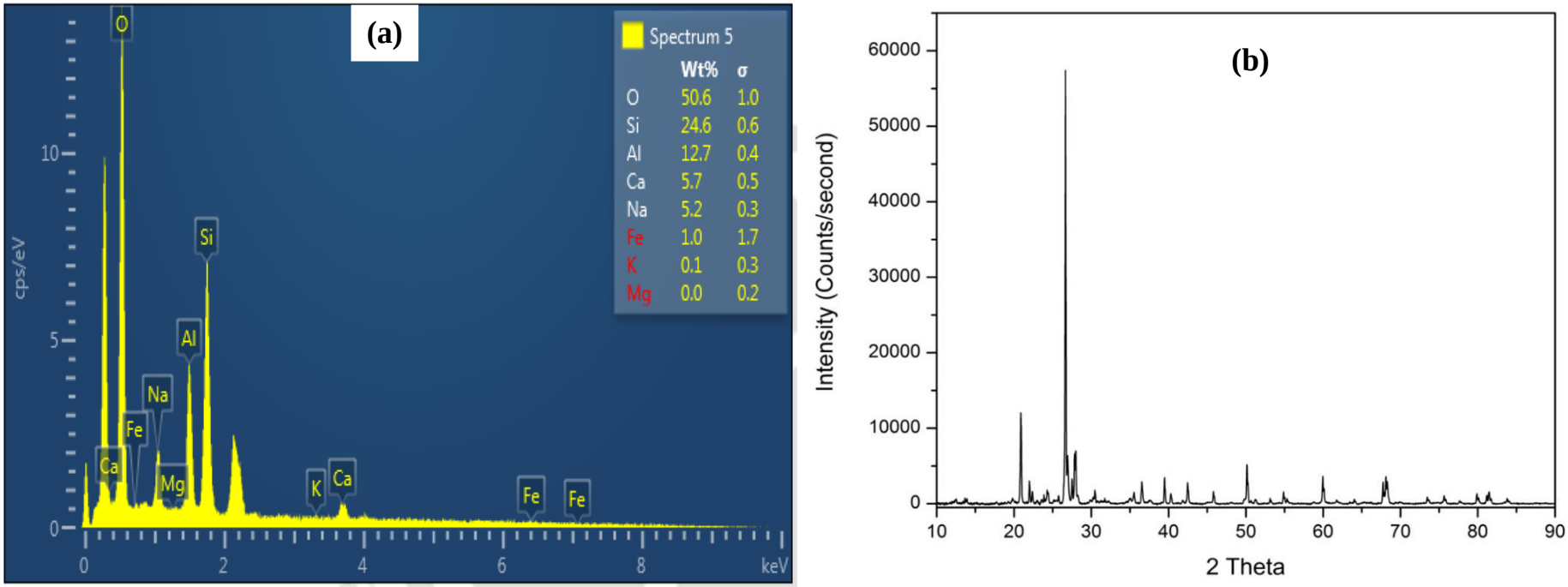


Figure 5.1. Characterization of the model sand surface by (a) EDX and (b) XRD

5.3.2. Quantitative analysis of Surfactin by HPLC

Initially, HPLC analysis was performed for 1250, 2500, 5000 and 10000 mg/L of commercial Surfactin from Sigma - aldrich, to prepare the standard curve. The Surfactin biomolecules were resolved at consecutive retention times of 9, 10, 12 and 13, 14 or 16 minutes which attributed to C_{13}, C_{14}, C_{15} and C_{16} isoforms of Surfactin (Figure 5.2). Among them isoforms, C_{15} was the major one contributing to the highest relative abundance. The standard curves from the HPLC are reported below in the form of Equation 5.15. and Equation 5.16.:

$$y = 0.649x + 88.93 \ (R^2 = 0.999) \text{ at 205 nm} \quad (5.15)$$

and $y = 0.347x + 41.66 \ (R^2 = 1)$ at 210 nm. (5.16)

The concentrations of Surfactin in the solutions before and after the adsorption process were calculated using Equations 5.15. and 5.16 where x denotes the final Surfactin concentration and y indicates the corresponding peak area. The standard curve of Surfactin is shown in Appendix 5A by Figure 5A-1.

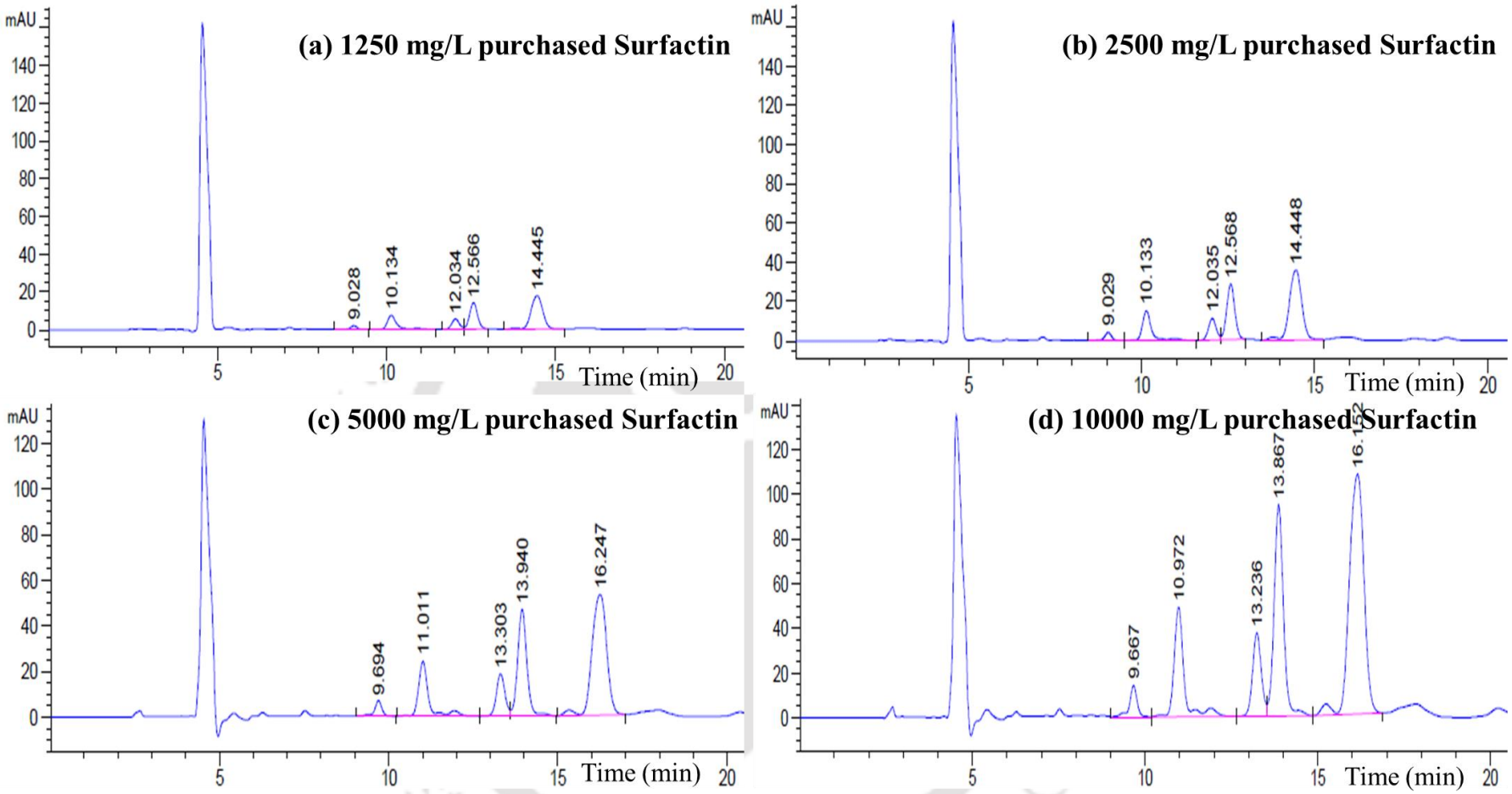


Figure 5.2. HPLC chromatograms of the purchased (standard) Surfactin having concentrations of (a) 1250 mg/L, (b) 2500 mg/L, (c) 5000 mg/L and (d) 10,000 mg/L

5.3.3. Equilibrium Adsorption models

Three familiar adsorption isotherms such as Langmuir, Freundlich and Temkin (Ahmadi & Shadizadeh, 2015; Lagrergen, 1898; Ruthven, 1984) have been mainly used to elucidate the equilibrium adsorption behavior of Surfactin in water and formation water which has been represented by Figure 5.3 (a) and (b), respectively. The corresponding synopsis of correlations, coefficient of determination (R^2) and relevant parameters for all equilibrium adsorption isotherms have been represented in Table 5.1. The adsorption capacities were calculated using Equation (5.1) and estimated to be 1.1 mg/g and 0.98 mg/g at 5000 ppm Surfactin which was increased to 2.4 mg/g and 1.7 mg/g at 20,000 ppm Surfactin dissolved in water and synthetic formation water, respectively. HPLC chromatograms of some of the data points (Surfactin concentrations of 500 mg/L, 2000 mg/L, 5000 mg/L, 10,000 mg/L, 20,000 mg/L and 25,000 mg/L in water and formation water) have been presented in the Appendix section 5A with the Figure 5A-2 and Figure 5A-3.

For Langmuir isotherm, the experimental data were fitted in non-linear Equation (5.2), which has been illustrated in Figure 5.3 (a) and (b) and provided the coefficient of determination (R^2) of 0.96 and 0.87 for Surfactin adsorption in water and formation water, respectively. The relevant parameters and root mean square error (RMSE) are displayed in Table 5.1. The non-dimensional separation factors R_L were also estimated using Equation (5.3) that ranges between 0.9 - 0.96 indicating the favourability of the adsorption process. Amongst all the isotherm models considered, Freundlich model fitness plot was obtained by fitted in Equation (5.4) and resulted in the maximum coefficient of determination (R^2) of 0.98 and 0.89 for 5000 mg/L Surfactin adsorption in water and formation water, respectively. The isotherms have been shown in Figure 5.3 (a) and (b) along with the correlations and other obtained parameters in Table 5.1. The error analysis results have also been mentioned in Table 5.1. The inverse adsorption intensity factor

(1/n) was calculated to be 0.59 and 0.44 for aqueous and synthetic formation water media, respectively, indicating the favourability of the heterogeneous adsorption phenomena. The obtained data were fitted in non-linear expression of Temkin isotherm in Equation (5.5). The isotherm and the resultant parameters are also depicted in Figure 5.3 (a), 5.3 (b) and Table 5.1, respectively.

Following the observations, it could be concluded that the experimental data were in good consistency with the non-linear Freundlich model fitting. Freundlich isotherm model is the best fit model to describe the concerned Surfactin adsorption process with R^2, K_F (L/mg) $(L\ g^{-1})^{1/n}$ and n values are 0.98, 0.402 and 1.69 in Surfactin aqueous solution and 0.89, 0.404 and 2.29 for Surfactin adsorption in formation water, respectively. The outcomes revealed the adsorbent surface to be heterogeneous due to the presence of different sizes of sand particles which lead to multilayer adsorption. This model also states that the binding affinity decreases exponentially when the binding sites of the adsorbent gets occupied gradually. Similar behavior was observed by Bispo *et al*., (dos Santos Bispo et al., 2021) while studying the adsorption behavior of activated carbon from *Moringa oleifera Lam* for the removal of oil and greases.

Table 5.1. Parameters related to various adsorption isotherm models fitting along with the coefficient of determination and error values

Isotherm model	Parameters	R^2	RMSE
Langmuir (W)	q_0 (mg g^{-1}) = 3.76 K_L (L g^{-1}) = 7.66 × 10^{-2} $R_L = 0.96$	0.96	0.134
(FW)	$q_0 = 1.65$ $K_L = 22.08 \times 10^{-2}$ $R_L = 0.9$	0.87	0.132
Freundlich (W)	n = 1.69 K_F (mg g^{-1}) (L g^{-1})$^{1/n}$ = 0.402	0.98	0.086
(FW)	n = 2.29 K_F (mg g^{-1}) (L g^{-1})$^{1/n}$ = 0.404	0.89	0.117
Temkin (W)	B (kJ mol^{-1}) = 0.549 K_T (L g^{-1}) = 1.81	0.89	0.199
(FW)	B (kJ mol^{-1}) = 0.329 K_T (L g^{-1}) = 2.9	0.88	0.123

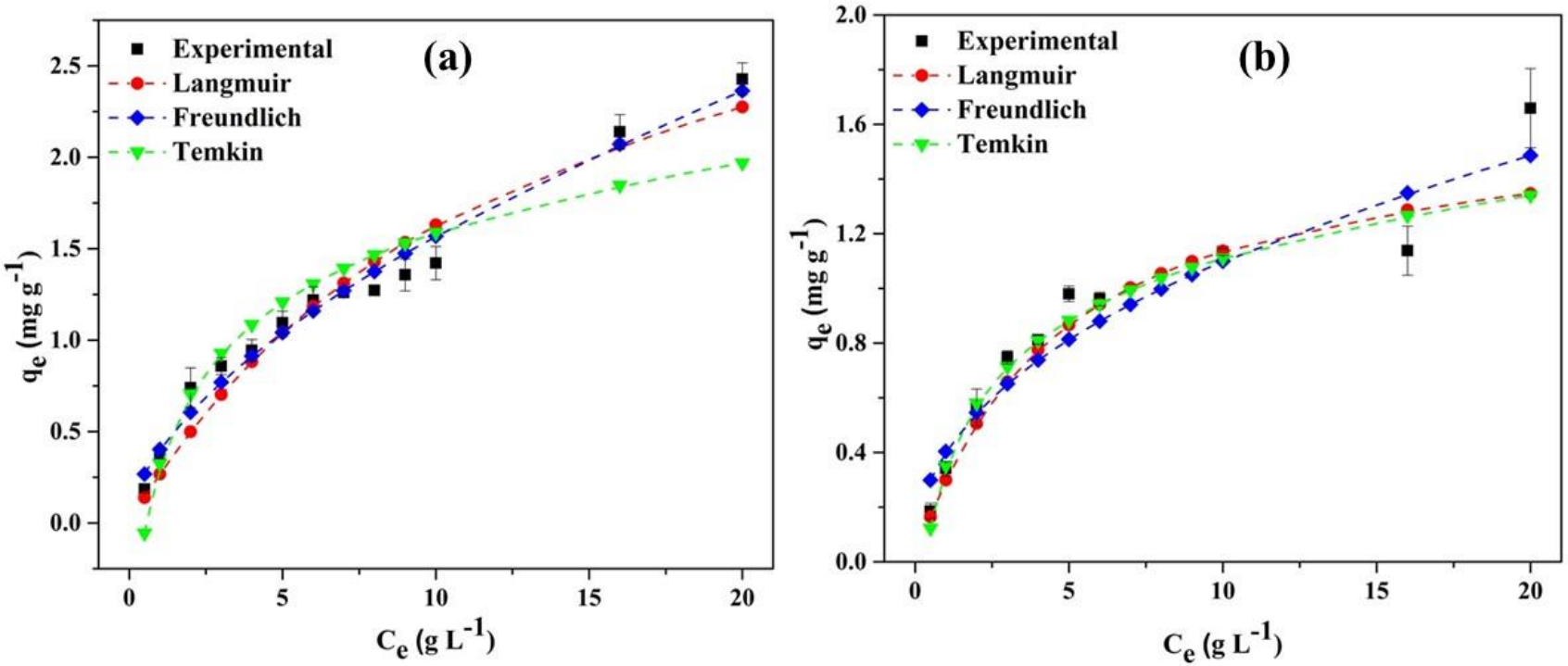


Figure 5.3. Adsorption isothermal non-linear model fitting of (a) Surfactin solution in water and (b) Surfactin in synthetic formation water onto silica sand sample; and comparison using Langmuir (red circle with dash-line), Freundlich (blue diamond with dash-line) and Temkin (green triangle with dash-line) isotherm models with experimental data (black square) at 37 °C.

5.3.4. Adsorption kinetic models

The role of contact time and on the adsorption capacity (q_t) of biosurfactant has been scrutinized employing 5000 mg/L Surfactin in water and synthetic formation water. In the kinetics experiment, the highest adsorption capacity of 5000 mg/L Surfactin after 8 hours was observed to be 0.8 mg/g, 0.54 mg/g in water and synthetic formation water, respectively. During the early biosorption stage, biosurfactant molecules moved from bulk solution, swiftly reached the boundary layer of highly porous adsorbent by mass transfer. In the next stage, once all the exterior sites were occupied, diffusion took place within the inner porous structure of the adsorbent from the outer boundary layer (Sharma et al., 2018c). HPLC chromatograms of some of the data points have been displayed in the Appendix section 5A with Figure 5A-4 and Figure 5A-5.

The experimental data were plotted following Equation (5.6) for pseudo first-order kinetics which has been exhibited in Figure 5.4 (a) and (b). The model parameters, the corresponding R^2 values and root mean square error (RMSE) values at 5000 mg/L Surfactin are calculated and stated in Table 5.2. The values for the coefficient of determination (R^2) were obtained to be 0.95. The pseudo-second-order plots for Surfactin adsorption have been obtained from Equation (5.7) and Equation (5.8) are also presented in Figure 5.4 (a) and (b) with the obtained parameters listed in Table 5.2 in which R^2 was found to be 0.97.

The model parameters and corresponding values for intra-particle diffusion kinetics at 5000 mg/L Surfactin concentration in water and formation water were obtained using Equation (5.9) and are shown in Figure 5.5 (a) and (b) and summarized in Table 5.2. In this study, the initial phase of rapid adsorption was carried out till 1 hour and then, the next phase of diffusion was continued till 8 hours and the rate constants are denoted by $K_{initial}$ and K_{intra}, respectively (Table 5.2). The

contribution of both initial surface adsorption (SA) and IPD could be calculated using Equation (5.17) as follows (Verma et al., 2021):

$$\%\ contribution = \frac{(q_t - q_i)}{q_t} \times 100 \quad (5.17)$$

Where, i = 0 and t = 60 minutes, respectively. Contributions of SA were determined to be 60.63 % and 42.14 % for 5000 mg/L Surfactin in water and formation water, respectively. Similarly, contributions of IPD were calculated to be 39.37 % and 57.86 % for 5000 mg/L Surfactin in water and formation water, respectively. Contributions of rate-limited IPD were found to be quite significant that indicated the presence of SA and IPD during the adsorption process of Surfactin. Intercept value indicated the thickness of film and boundary layer effect. $K_{initial}$ values were found to increase with the increase in Surfactin concentration; however, K_{intra} values were found to be rate-limiting. Summarily, initial adsorption was kinetic controlled due to higher bulk concentration followed by intraparticle diffusion, which was found to be rate-limiting (Sharma et al., 2018c).

In Elovich kinetics, α and β are calculated using Equation (5.11) from the slope and intercept of q_t vs. $\ln(t)$, respectively and the Elovich model fittings for 5000 mg/L Surfactin adsorption in water and formation water are exhibited in Figure 5.4 (a) and (b) along with the relevant parameter tabulation in Table 5.2. The R^2 values were reported to be noticeably the best which lies between 0.97 to 0.99. Elovich model also signifies multilayer adsorption which supports the Freundlich isotherm data as well. Hence, among all models, it could be concluded that the Elovich model fits the best to represent the measured adsorption kinetics data. Utilizing this kinetics analysis, the adsorption of Surfactin onto model silica surfaces could be predicted within this concentration range and time duration.

Table 5.2. Kinetics model parameters of the Pseudo-first-order, Pseudo-second-order, Intraparticle diffusion and Elovich models obtained after fitting to experimental data for adsorption of 5000 mg/L Surfactin solution in water and synthetic formation water

Kinetic model name	Surfactant Concentration (mg/L)	q_e (mg/g)	K		R^2	RMSE
			K_1 (min^{-1})			
Pseudo-first order	W 5000	0.709	0.786		0.95	0.061
	FW 5000	0.497	0.626		0.95	0.043
			K_2 ($g.mg^{-1}.min^{-1}$)	h (mg $g^{-1}min^{-1}$)		
Pseudo-second order	W 5000	0.842	1.062	0.753	0.97	0.044
	FW 5000	0.599	1.172	0.421	0.97	0.032
			$K_{initial}$, ($mg.g^{-1}.min^{-1/2}$),	$C_{initial}$ (mg g^{-1})		
Intra particle diffusion	W 5000		0.41	0	0.99	0.031
			K_{intra} ($mg.g^{-1}.min^{-1/2}$)	C_{intra} (mg g^{-1})		
			0.21	0.18	0.99	0.004
			$K_{initial}$, ($mg.g^{-1}.min^{-1/2}$),	$C_{initial}$ (mg g^{-1})		
	FW 5000		0.26	0	0.97	0.015
			K_{intra} ($mg.g^{-1}.min^{-1/2}$)	C_{intra} (mg g^{-1})		
			0.17	0.07	0.99	0.009
			α (mg.g^{-1}.min^{-1})	β ($g.mg^{-1}$.)		
Elovich model	W 5000		1.815	5.845	0.99	0.022
	FW 5000		1.055	8.228	0.97	0.022

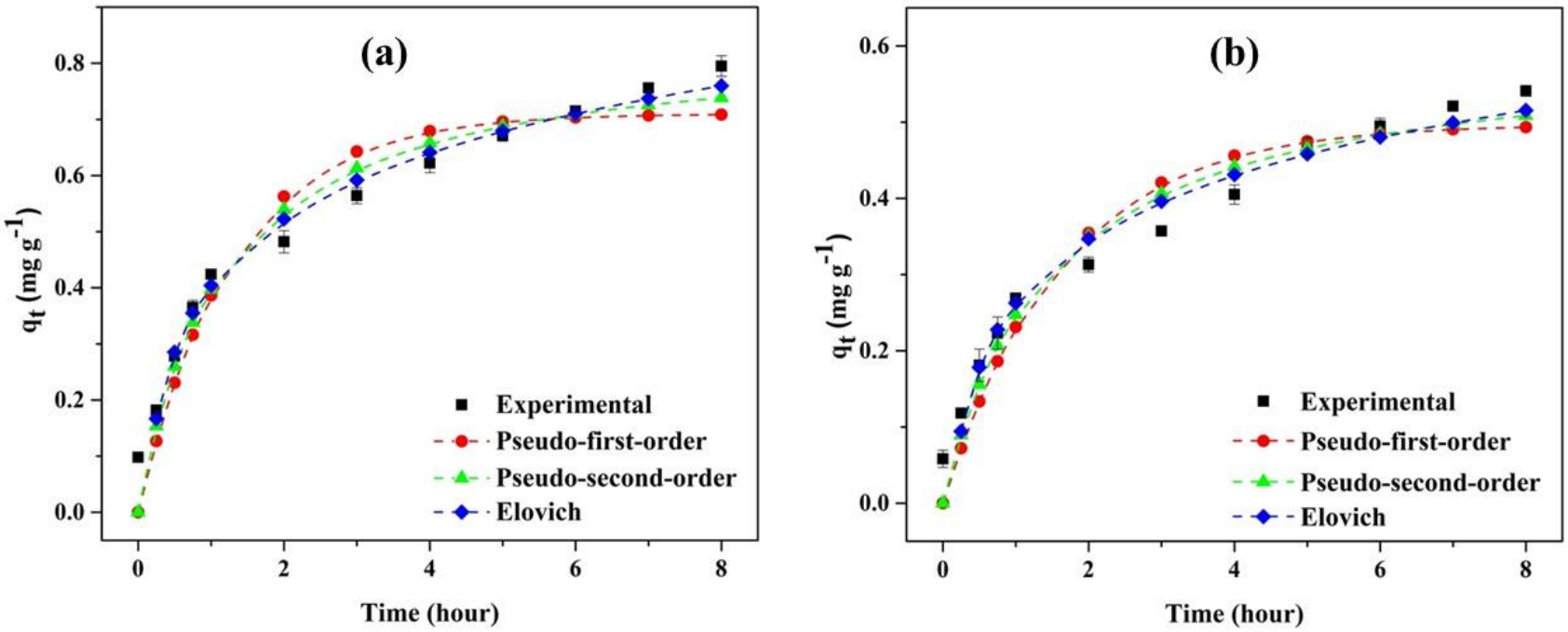


Figure 5.4. Fitting of kinetic models such as Pseudo-first-order (red circle with dash-line), Pseudo-second order (green triangle with dash-line) and Elovich (blue diamond with dash-line) to experimental data (black square) for adsorption of 5000 mg/L Surfactin onto silica sand sample at 37 °C in (a) water and (b) synthetic formation water, respectively.

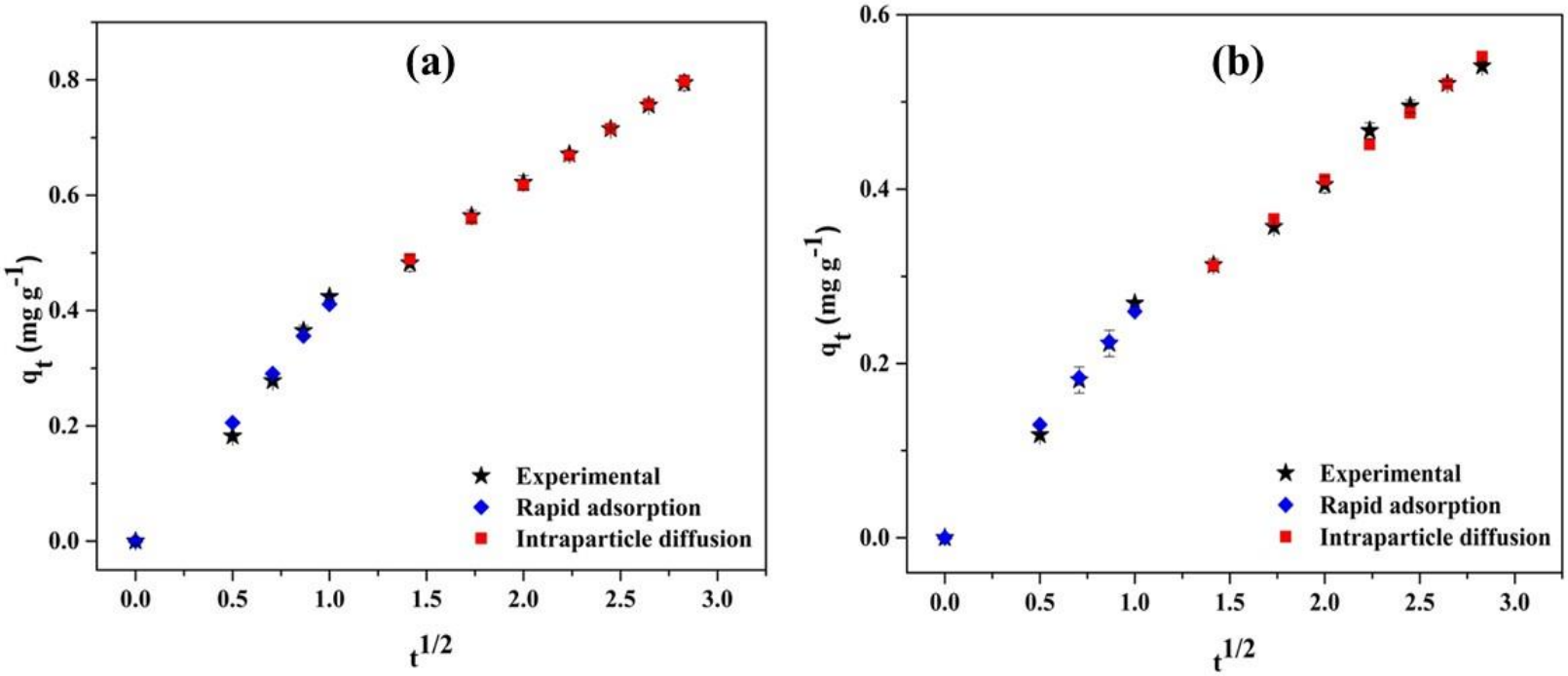


Figure 5.5. Fitting of intraparticle diffusion kinetic model to experimental data (black star) for adsorption of 5000 mg/L Surfactin onto silica sand sample at 37 °C in (a) water and (b) synthetic formation water, respectively indicating two processes: the rapid adsorption (blue diamond) and intraparticle diffusion (red square)

5.3.5. Effect of temperature and formation water on adsorption characteristics

The temperature dependency of the adsorption characteristics of Surfactin in synthetic formation water onto silica sandstone surface was examined. The maximum adsorption capacity of 5000 mg/L Surfactin was found to be 0.64 and 0.45 mg/g at 25 and 55 °C, respectively. The reduction in adsorption with the increase in temperature was observed because, at high temperatures, the adsorption rate of adsorbate within the surface and interior pores of adsorbent are assumed to decrease due to reduction in viscosity (Bera et al., 2013; Saha et al., 2017a). A further variation of adsorption with temperature can be explained with the help of thermodynamic parameters as Gibbs free energy, enthalpy and entropy. The HPLC chromatograms of some of the sample data points have been exhibited in the Appendix section 5A with Figure 5A-6 and Figure 5A-7.

5.3.6. Thermodynamic studies of Surfactin adsorption

Thermodynamic parameters of some biosurfactants (Rhamnolipid and Surfactin) and their adsorption at the water-air interface have been reported in the literature (Mańko et al., 2014; Rekiel et al., 2020; Zdziennicka et al., 2018). Knowledge of the standard Gibbs free energy, enthalpy and entropy of adsorption is very important to explain what happens during the transfer of biosurfactant molecules from the bulk phase to the surface layer at the water-air interface. The standard Gibbs free energy of adsorption indicates whether the adsorption occurs spontaneously or not. The standard enthalpy depends on the number of bond creations or disruptions during the adsorption process. The standard entropy results from the changes of water molecules' structure and orientation of surfactant molecules in the surface layer as well as changes in their free motion (Zdziennicka & Jańczuk, 2017). The basic thermodynamic parameters like Gibbs free energy, enthalpy and entropy were estimated using equilibrium constant whose value depends upon the

temperature of the system and they have been determined using Equation (5.12), Equation (5.13) and Equation (5.14), respectively. The calculated thermodynamic parameters were determined by plotting to ln K_c vs. 1/T (Figure 5.6) and tabulated in Table 5.3. The positive value of Gibb's free energy indicated the process to be unspontaneous presumably due to a decrease in entropy of biosurfactants with the increase in concentration. A negative enthalpy value indicates the exothermic nature of the process and hence lower adsorption capacity is observed at a higher temperature. The negative value of entropy expresses the reduction in randomness at the interface.

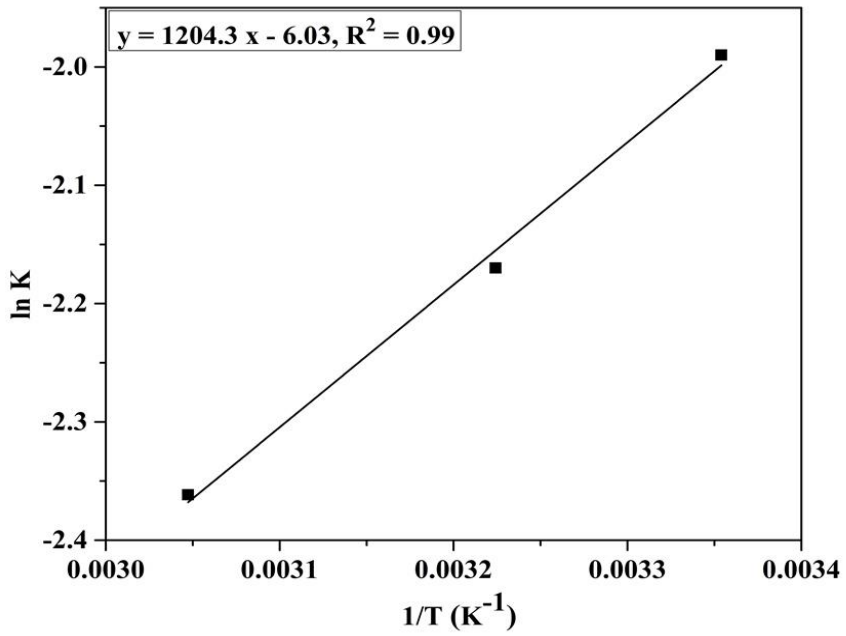


Figure 5.6. Thermodynamic analysis of the Surfactin adsorption at 25, 37 and 55 °C

Table 5.3. Values of thermodynamic parameters

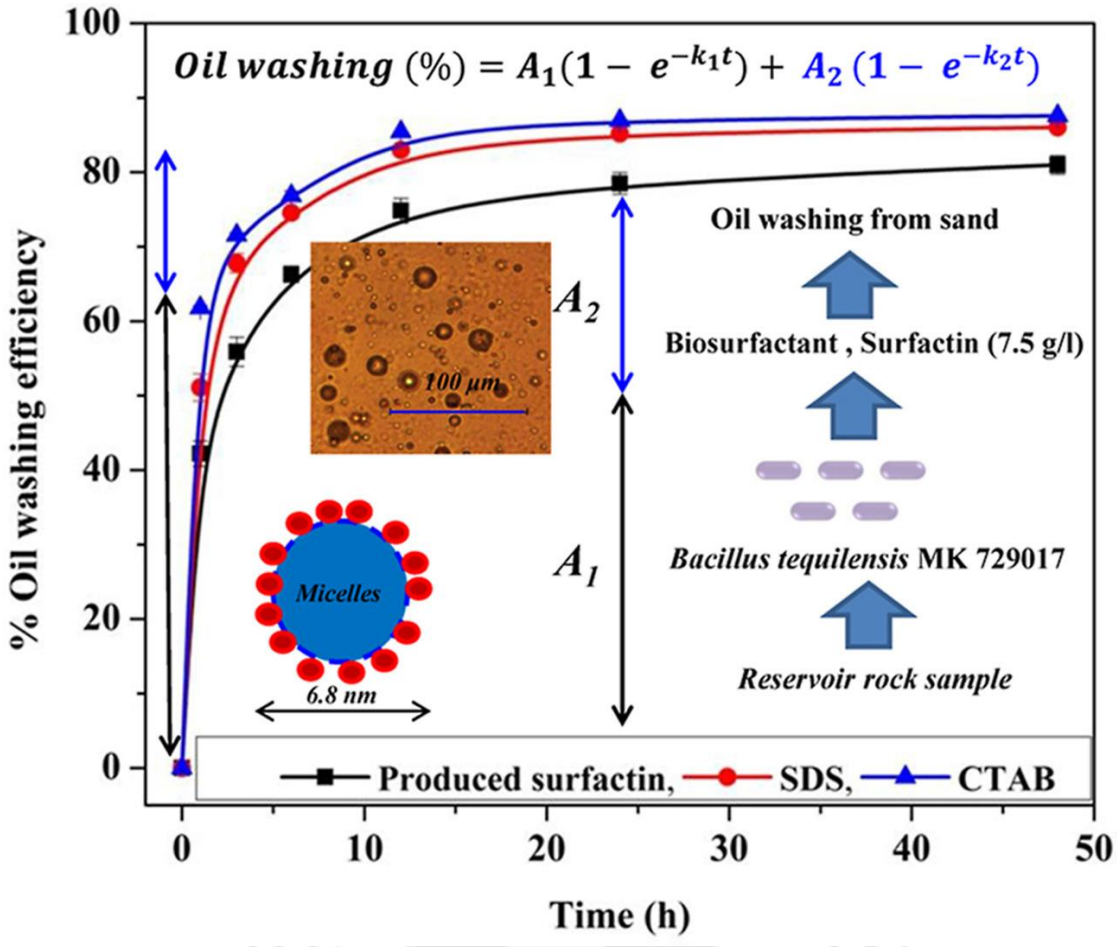
Temperature	ln K	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J/mol.K)	R^2
298.15	-1.99001	4.93			
310.15	-2.17001	5.59	-10.01	-50.2	0.99
328.15	-2.36159	6.44			

5.4. Conclusions

The adsorption characteristics of Surfactin onto model sand (silica) surface have been systematically elaborated by employing the real reservoir scenarios in the experimental procedure with temperature variation (25, 37 and 55 °C) and synthetic formation water. The mineralogical composition of the sand sample was obtained from elemental analyses via EDX and XRD showed the presence of silica which acts as the active site for the adsorption process of biosurfactants. Experiments were performed to study the adsorption equilibrium isotherms, kinetic behavior and thermodynamic properties of Surfactin adsorption. Among the adsorption equilibrium models such as Langmuir, Freundlich and Temkin isotherms, the Freundlich model was the best fit to describe the equilibrium adsorption process. Therefore, the process was assumed to be multilayer adsorption on a heterogenous surface. Analysis of kinetic data was performed following Pseudo-first-order, Pseudo-second-order, Intra particle diffusion (IPD) model and Elovich model in which Elovich model represented well the experimental data which also supported the trend of multilayer adsorption. The intra-particle diffusion models described that the process with high coefficients of determination value which occurred via two steps: initial rapid adsorption followed by slower intraparticle diffusion. Subsequently this model also showed that the intra-particle diffusion mechanism is not the only rate-controlling process and other processes such as boundary layer diffusion also influence the adsorption to some extent. The effect of contact time and temperature on adsorption capacity was also examined. Thermodynamic properties of the adsorption process determined the decrease in the randomness of the system.

Chapter 6

Suitability Assessment of the Potential Biosurfactants for Promising MEOR Applications



(*Journal of Petroleum Science and Engineering* 195, 107612, 2020) (Datta et al., 2020), (*Journal of Environmental Chemical Engineering* 10, 107083, 2021) (Datta et al., 2021)

This chapter discusses the suitability of the biosurfactants produced from isolated, characterized and optimized strains *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 for their applicability for EOR applications in terms of stability studies, oil washing, IFT and other wetting properties. Their potential uses for *in-situ* and *ex-situ* MEOR are analyzed by investigating their sustainability at the higher thermal condition and through oil

washing efficiency, respectively. The oil washing proficiency of the produced biosurfactants is also compared with other chemical surfactants along with procured biosurfactant (Rhamnolipid). Steps involved in the washing process are scrutinized critically to understand the major controlling factors. The interfacial properties are also examined during the screening of the optimized flooding slug depending on the lowest IFT. The toxic effects of the produced Surfactin are also evaluated by the phytotoxicity assay as well as antimicrobial activity.

6.1. Introduction

Biosurfactant production is known to be a vital microbial strategy that influences the bioavailability of hydrophobic compounds (Xia et al., 2014). The biosurfactant-synthesizing strains consume various hydrocarbons (petroleum, paraffin and other oil types) and subsequently transform them into smaller units. There are several approaches for biosurfactants to achieve better surface properties such as reducing surface tension (ST) and interfacial tension (IFT), mobility improvement by declining viscosity, increasing emulsifying capability, lowering critical micelle concentration (CMC) and wettability alterations are the major strategies required for the process (Saxena et al., 2017). Biosurfactants have also been found to be quite compatible with reservoir brine. Another advantage of biosurfactants is their comparatively lower adsorption rate on the reservoir formation rock (Pal et al., 2018a).

The performance stability of the biosurfactant in extreme environmental conditions assists to ensure their sustainability in harsh reservoir conditions suggesting the suitability for *in-situ* MEOR. The improved oil biodegradation and washing efficiency are two key parameters that interpret the feasibility of the biosurfactants for *ex-situ* MEOR applications (Fanaei et al., 2020). *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017, showed excellent surface properties and their biosurfactant concentrations were reasonably higher utilizing hydrocarbons which simultaneously enhanced the biodegradation process as discussed in Chapter 4. The mode of action of biosurfactant to extract the entrapped oil from the reservoir

rock pores consists of various strategies. Biodegradation of crude oil is also one of the significant approaches of MEOR in which the complex constituents are transformed into simpler ones which change the traits of the crude oil, the viscosity is reduced and as a result, fluidity and recovery of crude oil are improved (Aitken et al., 2004).

Different combinations of surfactant and alkali have been analyzed depending on their capability to reduce the IFT with the reservoir crude oil. The antimicrobial activity of the biosurfactant against the pathogenic strain (*E.coli*) was also investigated and compared to kanamycin. The obtained results are expected to establish a future scope for the application of the biosurfactant in microbial enhanced oil recovery (MEOR) and bioremediation.

6.2. Materials and Methods

6.2.1. Chemicals and Reagents

The chemicals required for this study have been mentioned in the earlier chapters along with their procuring details. The remaining chemicals i.e., silicone oil (GRM705) and acetone (AS025) are procured from HiMedia Laboratories, India.

6.2.2. Thermal Stability analysis of the produced biosurfactants

6.2.2.1. Determination of CMC values

Surface tension measurement is very significant for determining the CMC values of the biosurfactants. It is very useful when a very small amount of biosurfactant is employed for the experiment. In the present study surface tensions of different concentrations of the Surfactin solutions were measured by a tensiometer (Make: Dataphysics, Model: DCAT 11EC) under atmospheric pressure by the Du Noüy ring method. During the measurement, the experimental temperature was maintained at 298 K and the surface tension of water was measured at the very first as the control experiment. The platinum ring was thoroughly cleaned with acetone and flame dried before each measurement. The CMC values correspond to the point where the biosurfactant first shows the lowest surface tension which remains relatively constant after this

point. In all cases, the standard deviation does not exceed ± 0.1 mN/m (Bera et al., 2013). The biosurfactants were exposed to a higher temperature (similar to internal reservoir temperature) such as 90 °C for 10 days to observe the effect of temperature on the surface property (surface tension) of the biosurfactants after ageing.

6.2.2.2. Thermal consistency analysis by FTIR, NMR and HPLC

The thermal stability of the biosurfactant was examined using FTIR, NMR and HPLC following the methods as mentioned in Chapter 4 employing the crude and thermally aged Surfactin. The thermal ageing was carried out by exposing the Surfactin at 80 °C and 90 °C for 10 days.

6.2.2.3. Thermal gravimetric analysis (TGA)

The thermal behavior of the biosurfactants produced by *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 was examined using a thermal gravimetric (TG) system (NETZSCH STA 449F3). Approximately 10 mg biosurfactant was loaded in an aluminum crucible and heated under an argon atmosphere at a heating rate of 10 ºC/minute. The TG thermogram was obtained by measuring weight loss in the temperature range of 0 – 500 ºC (Chandankere et al., 2014; Lan et al., 2015a; Liu et al., 2016b). The thermal stability of the biosurfactants was investigated by measuring their surface tension to ensure their sustainability of native property even after keeping it at higher temperature exposure.

6.2.3. Pressure stability investigation of the produced biosurfactants

The Surfactin solutions (100 ml) from *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 are kept at a very high pressure of almost 1000 Psi for 48 hours to mimic the pressure condition of the internal reservoir (Lea Jr & Rowlan, 2019). Then their surface properties were compared with the crude Surfactin solutions by FTIR, NMR, surface tension and HPLC to observe the effect of pressure on the pressurized Surfactin. Figure 6.1. shows the schematic of Floating Piston Accumulator (FPA) where the Surfactin solutions are injected to

examine pressure stability. It consists of a cylinder with two end closures and a floating piston that can move freely inside the cylinder. The piston separates the cylinder into two chambers: one for the driving fluid (silicone oil), another for the process fluid (Surfactin solution). The piston moves following the pressure gradient between the two chambers.

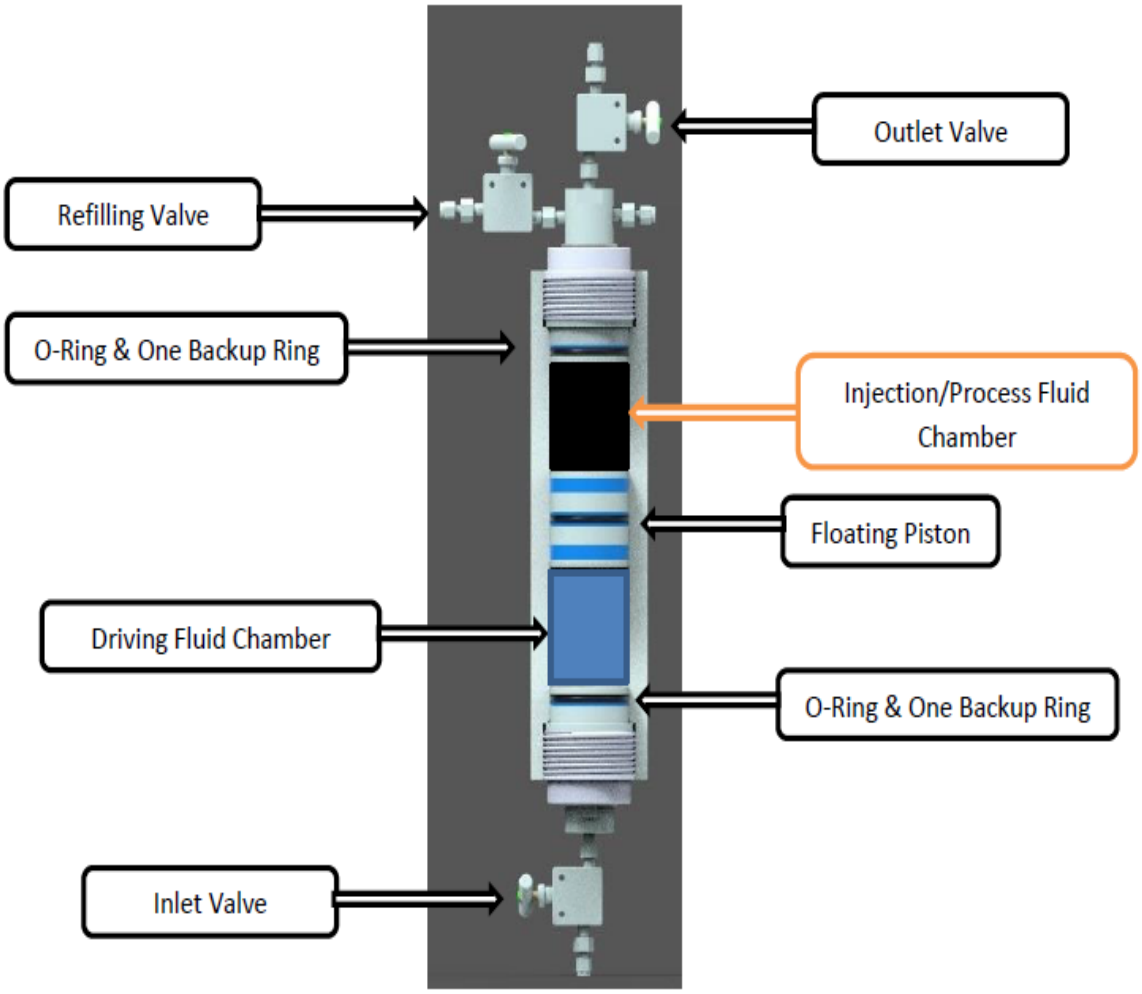


Figure 6.1. Floating piston accumulator to inspect pressure stability of biosurfactants

6.2.4. Salinity stability assessment of the produced biosurfactants

The produced Surfactins from *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 were exposed to 10 g/L NaCl solutions in sealed serum vials to imitate the anaerobic halophilic conditions of the interior oil reservoir. After that, the FTIR, NMR and HPLC spectra

and CMC values of the aged samples were compared with the crude ones to find out the changes in the produced Surfactin occurred due to salinity.

6.2.5. Alkaline stability evaluation by particle size and zeta potential analyses

The particle size of the produced biosurfactant in water was measured using dynamic light scattering (DLS) with a particle size analyzer (Make: Anton Paar, model: Litesizer 500) equipped with a He-Ne laser as a light source (wave-length: 658 nm; scattering angle at 90°) at 25 °C. Samples were equilibrated at room temperature for 30 minutes and then transferred into a 3 mL glass cuvette. The hydrodynamic sizes were calculated from the relative frequency intensity and relative frequency number obtained from software Kalliope version 2.0.1 assuming the refractive index of water to be 1.33 for the samples. The zeta potential of the samples was measured by loading the samples at different pH in omega cuvette at 25 ºC in 200 V voltage using Helmholtz–Smoluchowski equation (Fan et al., 2014).

6.2.6. Oil washing efficiency of the produced biosurfactants

The application of the produced biosurfactants in oil recovery was evaluated using artificially oil-saturated sand samples. The sand samples were collected from the nearby construction sites of IIT Guwahati and screened by sand-sieve with the mesh size of 90 µm and 150 µm. After that, the sand particles having sizes of 90 µm and 150 µm were mixed in a 1:1 ratio (Saha et al., 2017b). The screened sand mixture was washed several times with Mili-Q water and dried overnight in the hot air oven. Initially, 50 g pre-treated sand was mixed with 5 ml of crude oil and kept for mixing in a shaker incubator for 24 hours at 37 ºC at 150 rpm. Then 30 ml biosurfactant solutions of different concentrations (Surfactin from *Bacillus subtilis* MG495086 – 40 mg/L, 100 mg/L and 200 mg/L; Surfactin from *Bacillus tequilensis* MK729017 - 90 mg/L, 200 mg/L and 500 mg/L; and Rhamnolipid – 200 mg/L) and synthetic surfactant solutions (SDS – 2350 mg/L, CTAB - 335 mg/L) were added to previously prepared each flask following an incubation at 37 ºC for 1, 3, 6, 12, 24 and 48 hours. After the incubation, the mixture was

centrifuged at 6000 rpm to separate the laundering solution from the sand. The residual oil was extracted from the sand by adding hexane. Control experiments were performed using Mili-Q water at the above-stated conditions (Balan et al., 2017; Bezza & Chirwa, 2015). Oil washing efficiencies were calculated using the following Equation (6.1) (Liu et al., 2015).

$$\text{Oil washing efficiency} = \left[1 - \left(\frac{Residual\ oil\ in\ sand}{Total\ oil\ in\ sand}\right)\right] \times 100 \quad (6.1)$$

Water-oil emulsions were also prepared using the same (1:6 v/v) ratio of oil and water (surfactant solutions). The size of these emulsions was estimated by capturing microscopic images of their thin layers on glass slides using Nikon inverted fluorescent microscope (Nikon Eclipse Ti−S). All the experiments in the present study were carried out in triplicates and the results are expressed as the average ± standard deviation.

6.2.7. Interfacial tension measurement

When biosurfactant interacts with the oil, the interfacial tension (IFT) between oleic and aqueous phases gets reduced due to the accumulation of biosurfactant molecules at the interface. The IFT between Assam crude oil and the produced biosurfactant solutions was measured by spinning drop tensiometer (Make: Kruss, Model: Site100). Initially, the IFT experiments were performed with individual surfactant solutions (heavy bulk phase) and Assam crude oil (light phase) (Saha et al., 2017a). Then the combinations of biosurfactant solution - chemical surfactant solution, biosurfactant solution – alkali solution, biosurfactant solution - chemical surfactant solution - alkali solutions were examined in order to achieve the minimum IFT value. The IFT values were calculated using the following Equation (6.2) (Saha et al., 2017b):

$$\boldsymbol{\sigma = \frac{r^3 \omega^2\ (\rho_H - \rho_L)}{4}} \quad (6.2)$$

Where σ (mN/m) is the IFT, ρ_H (g/cm^3) and ρ_L (g/cm^3) are the density of the heavy water phase (1.05 g/cm^3) and density of the oil phase (0.89 g/cm^3), respectively, ω is the rotational velocity

which was maintained at 6000 rpm so that L/D remains ≥ 4. D (mm) is the measured drop width (2r) and L (mm) is the length of the oil drop. The IFT reported in the study was measured at equilibrium.

6.2.8. Phytotoxicity study of the produced biosurfactant

The biological activity or the toxic effect of the biosurfactant produced by *Bacillus subtilis* MG 495086 was examined by performing the phytotoxicity assay via pot experiments (Marecik et al., 2012). Two types of seeds, *Cicer arietinum* (Chickpea) and *Vigna radiate* (Mung bean) were selected for the studies. Initially, 10 mL of biosurfactant solutions (0.04 g/L, 1g/L) were prepared and dispensed into freshly prepared pots. Ten seeds were added to each pot and incubated in sunlight. Three main parameters such as relative seed germination, relative shoot length and the germination index were determined after incubating for certain intervals. In these pot experiments, tap water was used as the control.

6.2.9. Antimicrobial activity of the biosurfactant

The antimicrobial activity of the crude biosurfactant was evaluated by the agar diffusion method. Mueller Hinton agar (final pH 7.3 ± 0.2) was poured into the petri-dish. Aliquots (50 µl) of freshly grown pathogenic strain; *Escherichia coli* were spread uniformly on the solidified Muller-Hinton agar. Then wells were made in the agar plates using a sterile cork-borer. 50 µl crude biosurfactant (0.8 mg/ml) was added to the wells. Kanamycin (0.5 mg/ml) (Fopase et al., 2020) and distilled water were added to other wells of the plate as the control experiments. The plates were incubated at 37 ºC for 48 hours. The appearance of the clear zone of inhibition confirms the antibacterial property of the biosurfactant (Kiran et al., 2009; Mahalingam et al., 2011; Mouafi et al., 2016).

6.3. Results and Discussion

6.3.1. Thermal Stability analysis of the produced biosurfactants

6.3.1.1. Determination of CMC values

The biosurfactants reduce the surface tension by getting adsorbed on the liquid-gas interface. Surface tensions of the Surfactin solutions of different concentrations were measured and plotted as a function of concentration as shown in Figure 6.2. The CMC values of the biosurfactants were determined from the plot of surface tension vs. biosurfactant concentration. The CMC values of the biosurfactants produced by *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 were calculated to be 40 and 90 mg/L before ageing, respectively. After exposing the produced Surfactin at 90 °C for 48 hours and 10 days, it was observed that the CMC values did not differ much from the CMC values of the untreated produced biosurfactant. Before ageing, the lowest surface tension achieved by Surfactin produced by *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 was reported to be 33 ± 1 and 31 ± 1 mN/m, respectively, whereas, after ageing for 10 days at 90 °C, obtained surface tension values were 34 ± 1 and 32 ± 1 mN/m which proved the excellent thermotolerant property of the produced Surfactin.

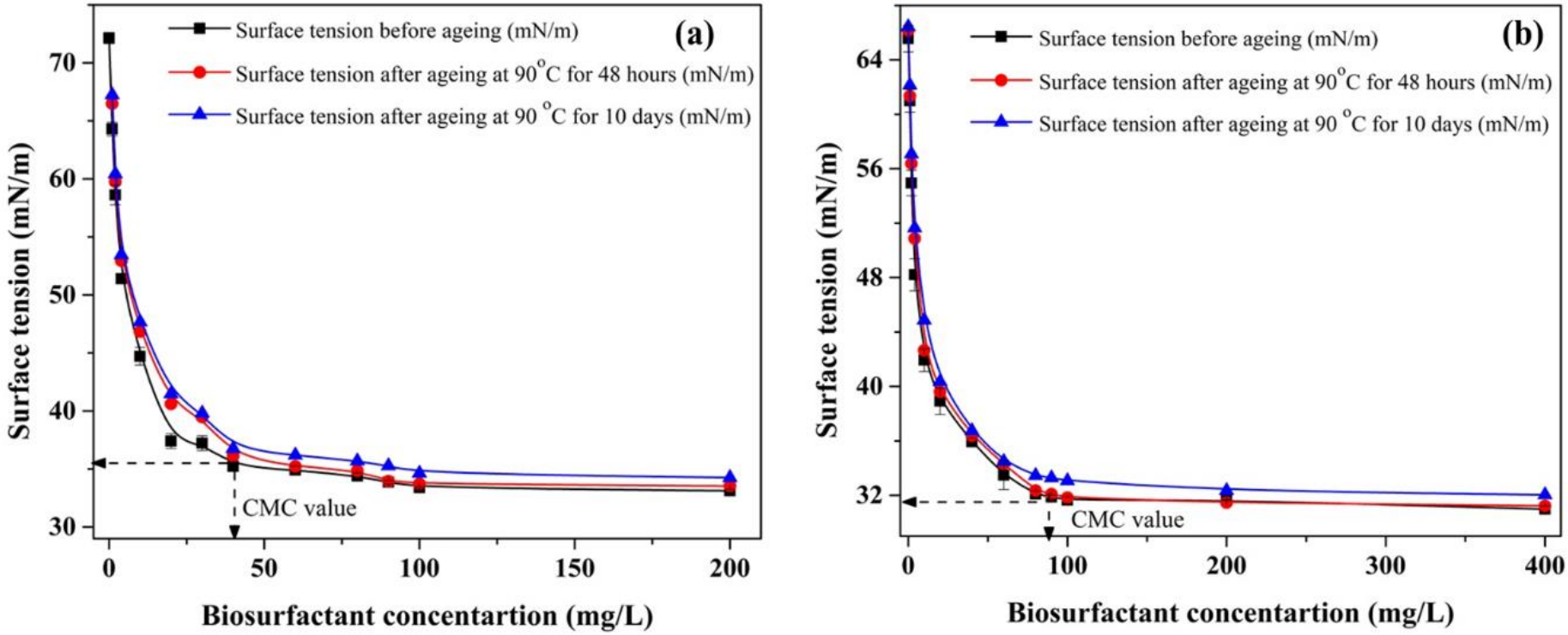


Figure 6.2. The surface tension profiles of the crude and thermally treated Surfactin produced by (a) *Bacillus subtilis* MG495086 and (b) *Bacillus tequilensis* MK729017, respectively

6.3.1.2. Thermal consistency analysis by HPLC, FTIR and NMR

The stability of the biosurfactant was examined using HPLC, FTIR and NMR analyses. In India, the temperature of most of the mature oil reservoirs is as high as 70 - 80 °C (Patel et al., 2015), therefore, Surfactin produced by *Bacillus tequilensis* MK 729017, was exposed for ageing at the maximum 90 °C (approx. reservoir temperature) for 2 - 10 days and their functional retentivity was investigated in terms of peak positions and quantification. Figure 6.3 (a) depicts the HPLC chromatogram of Surfactin before ageing, in which the peak area was estimated to be 43282 mAU.s. Gradually with the increase in temperature, the peak areas were reduced to 41331, 41076, 40065 and 39391 mAU.s at 60, 70, 80 and 90 °C, respectively as shown in Figure 6.3 (b), (c), (d) and (e) indicating a slight decrease in the Surfactin concentrations.

Similarly, the Surfactin produced from *Bacillus subtilis* MG 595086 was also exposed upto 90 °C for 48 hours and then their peak areas in HPLC chromatograms were measured. Figure 6.4. (a) indicates the peak area of the crude Surfactin to be 14306 mAU.s whereas, it was observed to slowly decreasing to 14114, 13484 and 11801 mAU.s at 70, 80 and 90 °C, respectively (Figure 6.4 b, c and d). These minor changes in peak area might be due to the mass loss in such high-temperature exposure.

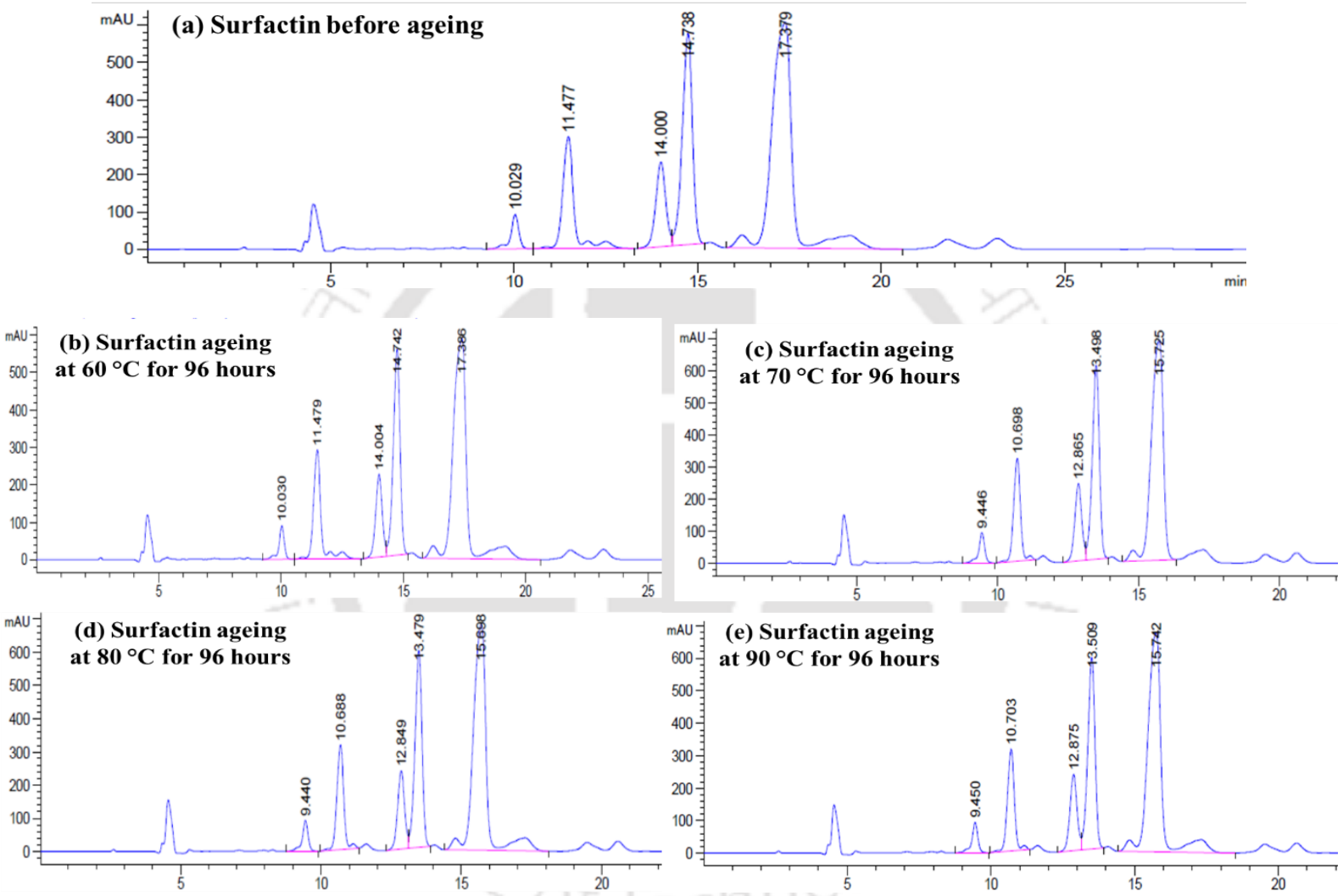


Figure 6.3. HPLC chromatograms of Surfactin produced by *Bacillus tequilensis* MK 729017 (a) before ageing, and after ageing at (b) 60 °C, (c) 70 °C, (d) 80 °C and (e) 90 °C for 96 hours.

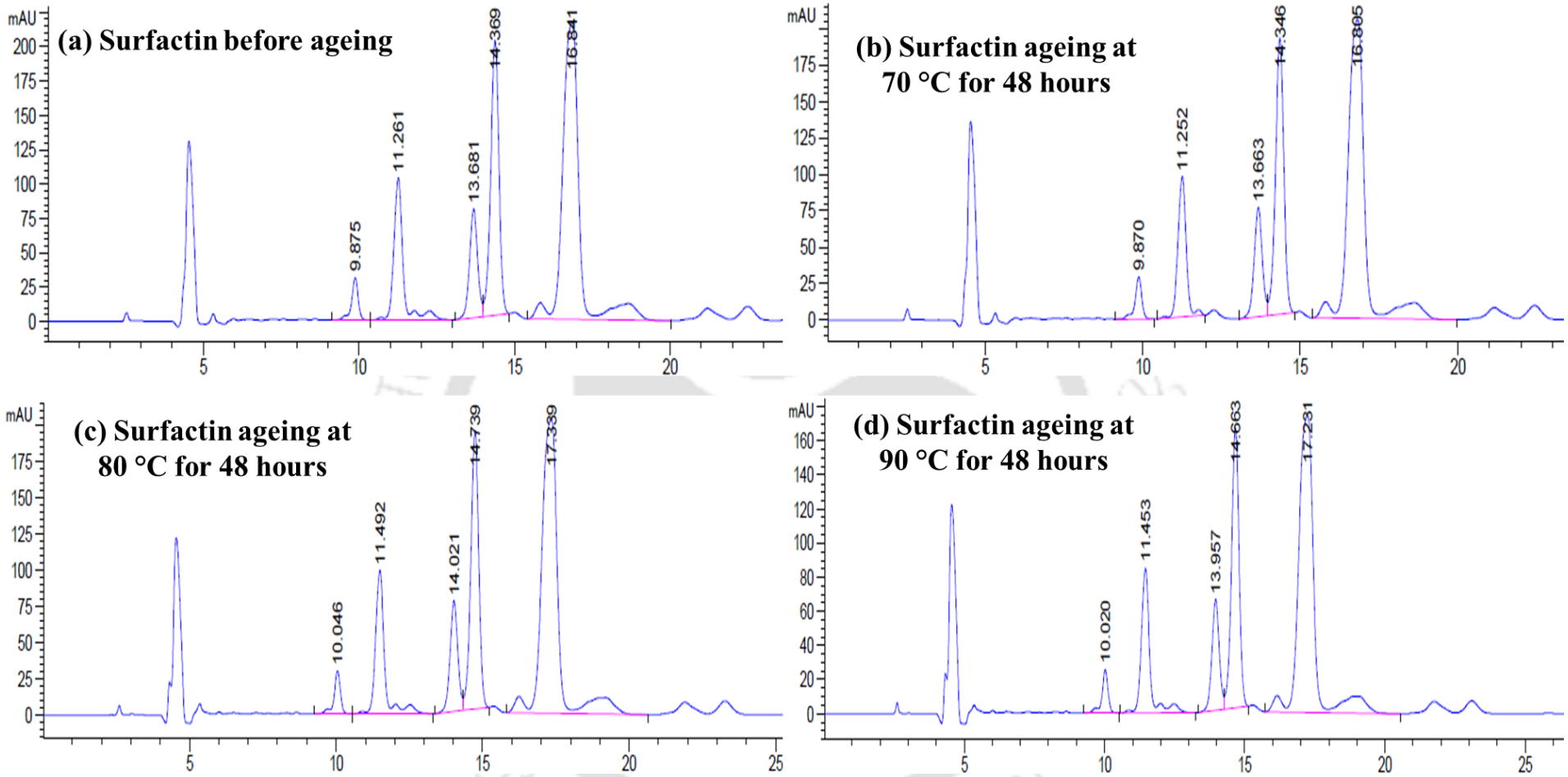


Figure 6.4. HPLC chromatograms of Surfactin produced by *Bacillus subtilis* MG 495086 (a) before ageing and after ageing at (b) 70 °C, (c) 80 °C and (d) 90 °C for 48 hours.

FTIR spectra of Surfactin samples produced from *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017, before and after thermal ageing are shown in Figure 6.5 (a) and (b). In the spectra, 3433 cm^{-1} corresponded to hydroxyl or stretching of the N-H group. 2940 and 2843 cm^{-1} indicated the presence of methyl and methylene respectively and confirmed the existence of the aliphatic group. The peptide group (CO-NH) in the produced bio-molecule was designated by 1641 cm^{-1} (Yalaoui-Guellal et al., 2020; Yilmaz et al., 2009), Symmetric nitro group or C-H bend which is common in alkyl chain containing compounds was represented by 1384 cm^{-1} and 1017 cm^{-1} denoted the characteristic vibration of the Si-O bond. Therefore, FTIR results directed the presence of aliphatic hydrocarbon along with peptide group, which is the distinctive feature of lipopeptides (Surfactin) (Liu et al., 2016b; Pereira et al., 2013). The transmittance of Surfactin was observed to be decreased with the increase in temperature and exposure duration although the peak positions remained almost the same.

Figure 6.6 and Figure 6.7 show the NMR spectra of Surfactin before and after thermal ageing synthesized from *Bacillus tequilensis* MK 729017 and *Bacillus subtilis* MG 495086, respectively. The peaks at 76.88, 77.1 and 77.31 ppm denoted the solvent peak of $CDCl_3$. In the up-field region the peak at 30.81 ppm indicated the presence of sp^3, -CH, $-CH_2$, $-CH_3$ aliphatic groups. The peaks at 50.07 and 50.15 ppm suggested the presence of amides. It was observed that the intensity of the aliphatic spectra was higher in the crude Surfactin NMR spectra however, it was reduced after thermal ageing and the amide spectra exhibited the opposite trend indicating the change of relative abundances of the Surfactin isoforms.

Overall, it can be conferred that in the FTIR and NMR spectra, and the HPLC chromatograms the peak positions do not alter even after thermal ageing at very high temperatures for a longer duration indicating exceptional thermal stability of Surfactin which endorses its suitability for promising EOR applications,

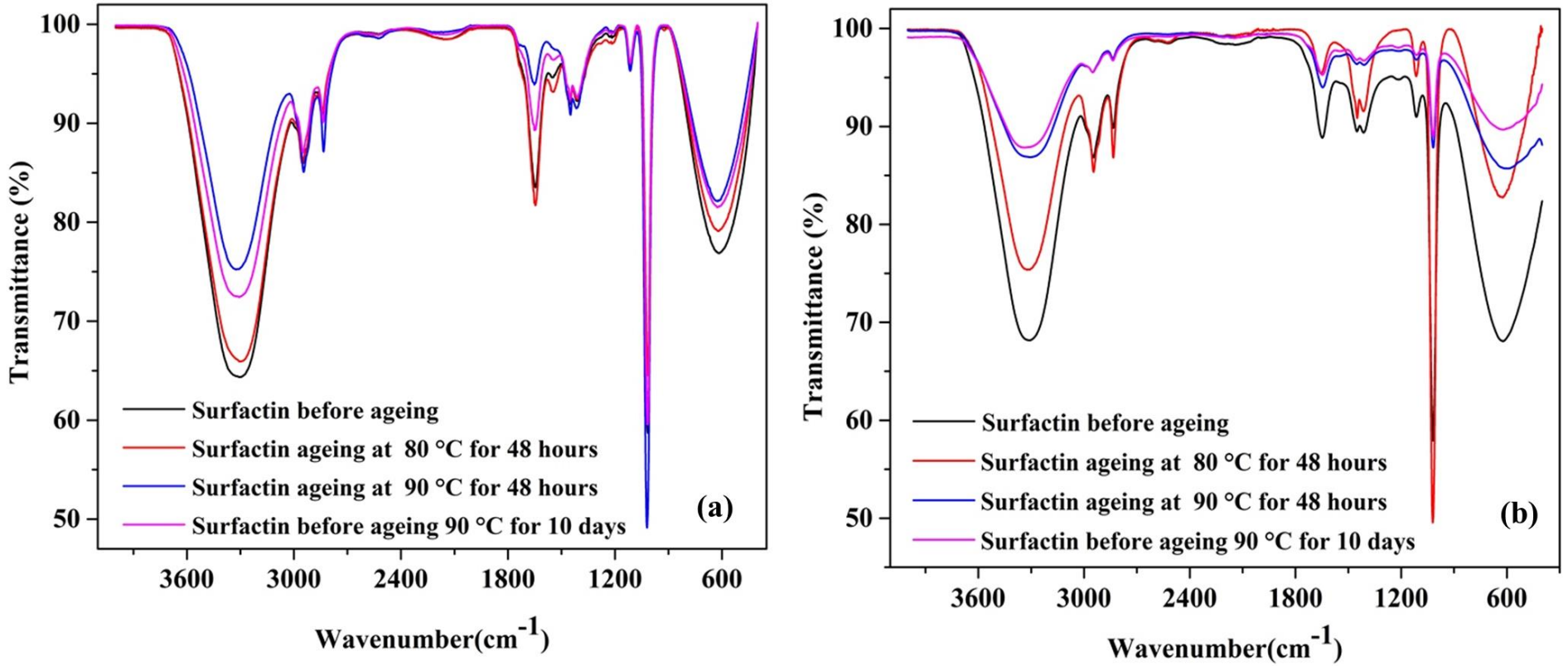


Figure 6.5. FTIR spectra of Surfactin produced by (a) *Bacillus subtilis* MG 495086 and (b) *Bacillus tequilensis* MK 729017 before and after thermal ageing

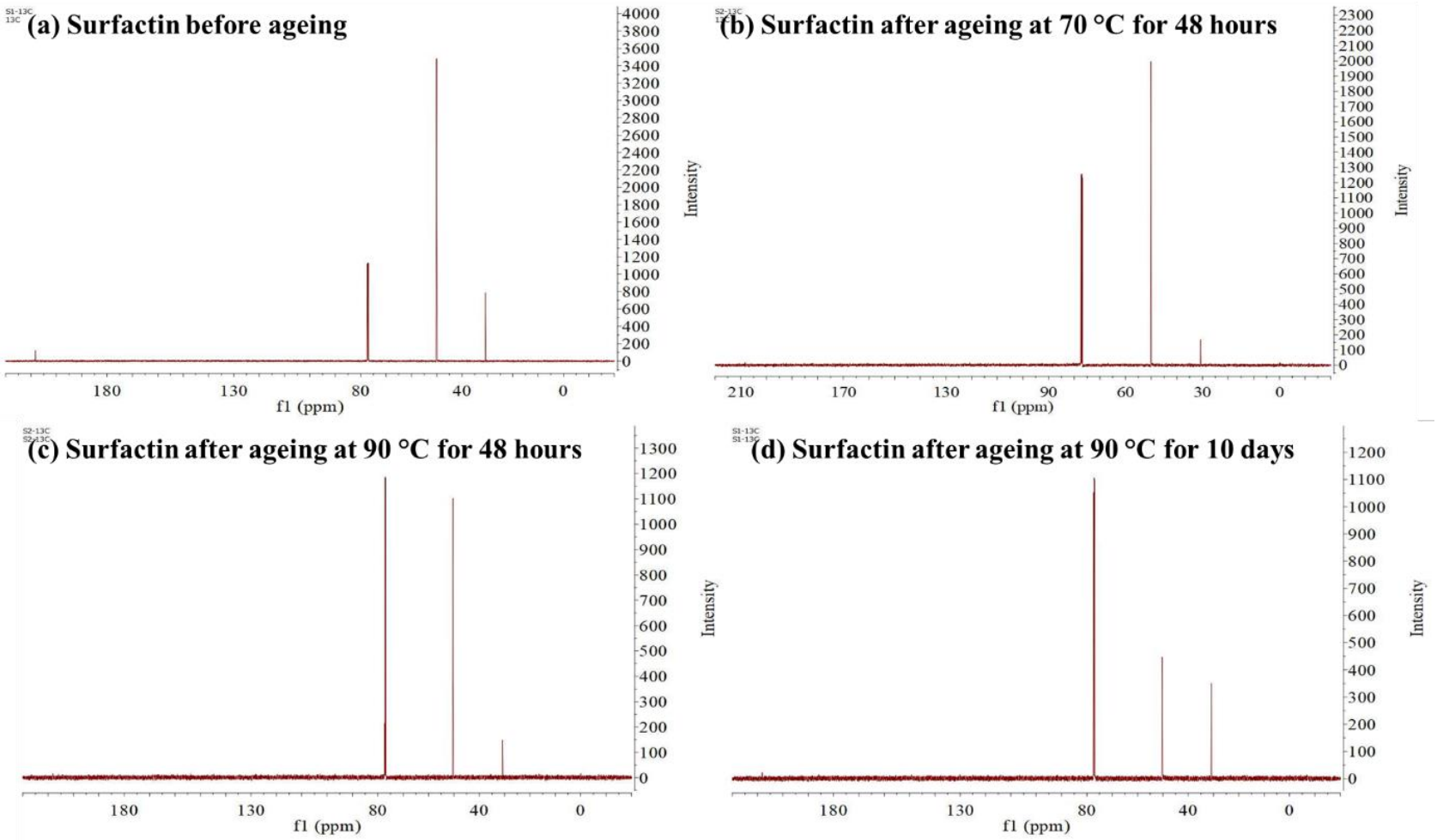


Figure 6.6. NMR spectra of Surfactin produced by *Bacillus tequilensis* MK 729017 (a) before thermal ageing and after thermal ageing at (b) 70 °C for 48 hours, (c) 90 °C for 48 hours and (d) 90 °C for 10 days

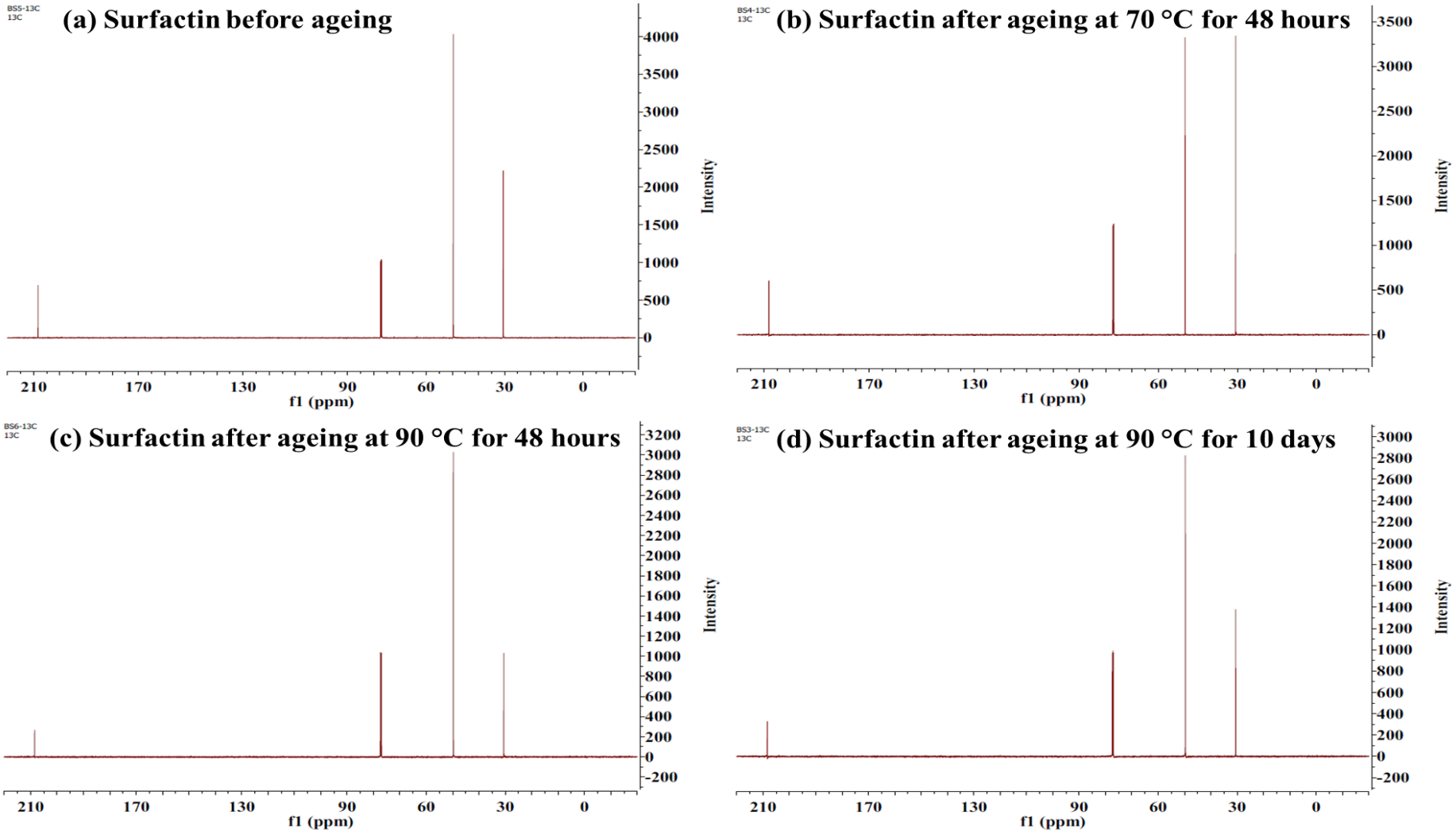


Figure 6.7. NMR spectra of Surfactin produced by *Bacillus subtilis* MG 495086 (a) before thermal ageing and after thermal ageing at (b) 70 °C for 48 hours, (c) 90 °C for 48 hours and (d) 90 °C for 10 days

6.3.1.3. Thermal stability of the produced Surfactin by TGA

Thermo-stability analysis of the biosurfactants is an important factor for its implementation in MEOR and various industrial applications (Chandankere et al., 2014). The thermal behavior of the produced biosurfactant was examined by thermal gravimetric analysis (TGA). The thermogravimetric thermograms of *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 have been exhibited in Figures 6.8 (a) and 6.8 (b), respectively. In Figure 6.8 (a), three thermal degradation phases were clearly observed during the entire heating-up process. The initial straight line indicated the negligible weight loss upto 150 °C. Next, 67.93 % weight loss was observed in the temperature range of 150 – 350 ºC. Then in the third phase of degradation, 22.02 % weight loss was observed when the temperature rose upto 450 ºC. A similar trend was followed by the biosurfactant produced by *Bacillus tequilensis* MK 729017 as presented in Figure 6.8 (b). Upto 150 °C, no mass loss was observed however in the second degradation phase from 150 to 350 °C, 87.4 % mass loss occurred. The biosurfactant was degraded at 375 °C which indicated its extremely well thermal tolerance property. According to Yernazarova, A., *et al*., (Yernazarova et al., 2016), the maximum temperature of the oil reservoir is about 100 ºC. So, the thermal analysis of the produced biosurfactant proved that the biosurfactant could be stable even at the maximum oil reservoir temperature. After the TGA analysis, the stability of the biosurfactant was examined in terms of the surface properties (oil displacement, drop collapse, emulsification index and CMC) as mentioned in Chapter 3 after exposing the sample at 90 ºC for 48 hours. The CMC was found to be almost the same (40 mg/L and 90 mg/L) as shown in Figure 6.2 (a) and 6.2 (b). Other surface properties also remained unchanged proving its thermal stability.

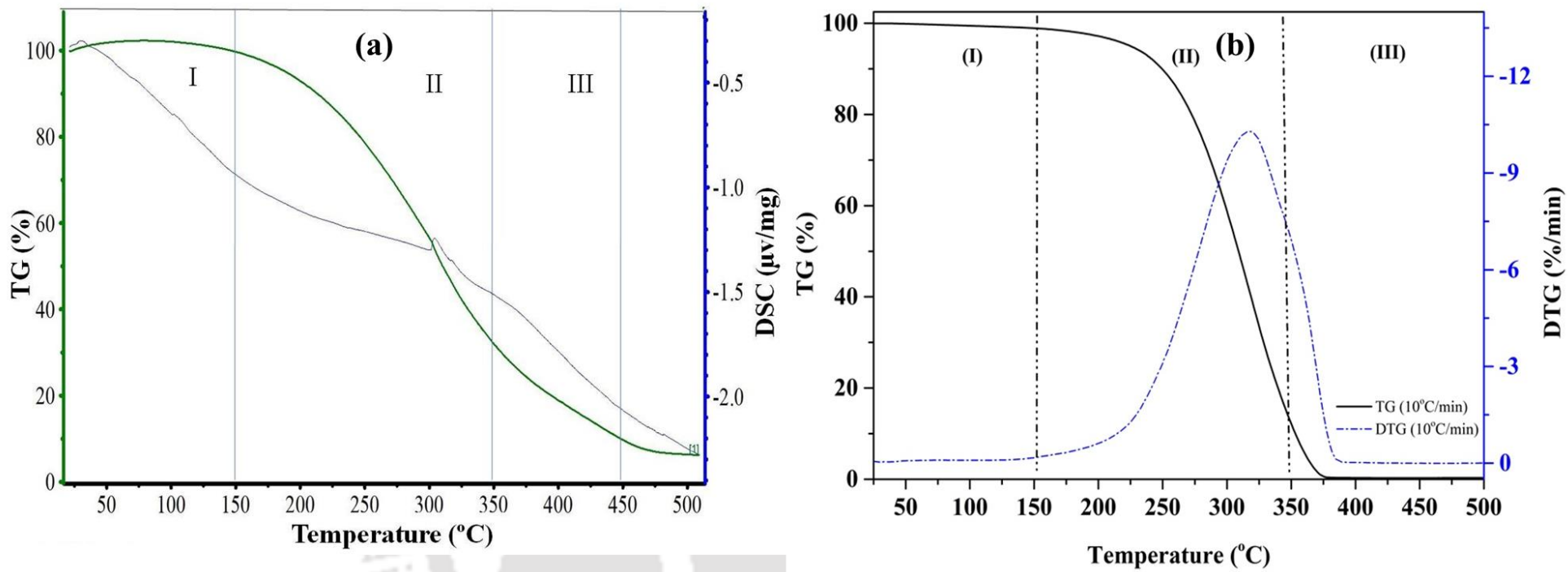


Figure 6.8. Thermogravimetric curve (thermogram) of the biosurfactants obtained from (a) *Bacillus subtilis* MG 495086 and (b) *Bacillus tequilensis* MK 729017

6.3.2. Pressure and halo stability of the produced biosurfactants

The HPLC chromatograms for Surfactin stability at 1000 Psi pressure and 10 g/L salinity have been presented in Figure 6.9 for the Surfactin from *Bacillus tequilensis* MK 729017 and Figure 6.10 for the Surfactin from *Bacillus subtilis* MG 495086, respectively. As exhibited in Figure 6.9, the peak areas were found to be 46010, 37165, 36201 and 29710 mAU*s for crude (Figure 6.9 a), thermally aged (Figure 6.9 b), halo aged (Figure 6.9 c) and pressure aged (Figure 6.9 d) Surfactin from *Bacillus tequilensis* MK 729017, respectively. Likewise, Figure 6.10 represents the peak areas of 14306, 11801, 11220 and 10639 mAU*s for crude (Figure 6.10 a), thermally aged (Figure 6.10 b), halo aged (Figure 6.10 c) and pressure aged (Figure 6.10 d) Surfactin from *Bacillus subtilis* MG 495086, respectively. The obtained results indicated that even after the exposure of the produced Surfactin at extreme temperature, pressure and salinity for a longer period, they could retain their properties in terms of the peak area and peak positions, which strongly endorsed their application for MEOR.

The FTIR spectra in Figure 6.11 also showed the stability of the Surfactins from *Bacillus subtilis* MG 495086 (Figure 6.11 a) and *Bacillus tequilensis* MK 729017 (Figure 6.11 b) in high pressure (1000 Psi) and high salinity (10 g/L) conditions. The NMR spectra in Figure 6.12 and Figure 6.13 represented the stabilities of Surfactin from *Bacillus tequilensis* MK 729017 and *Bacillus subtilis* MK 495086, respectively. These NMR spectra also supported and correlated with the HPLC and FTIR data which showed the stability of the produced Surfactin in extreme thermal, high pressure and salinity environments and highlighted their suitability for EOR related applications.

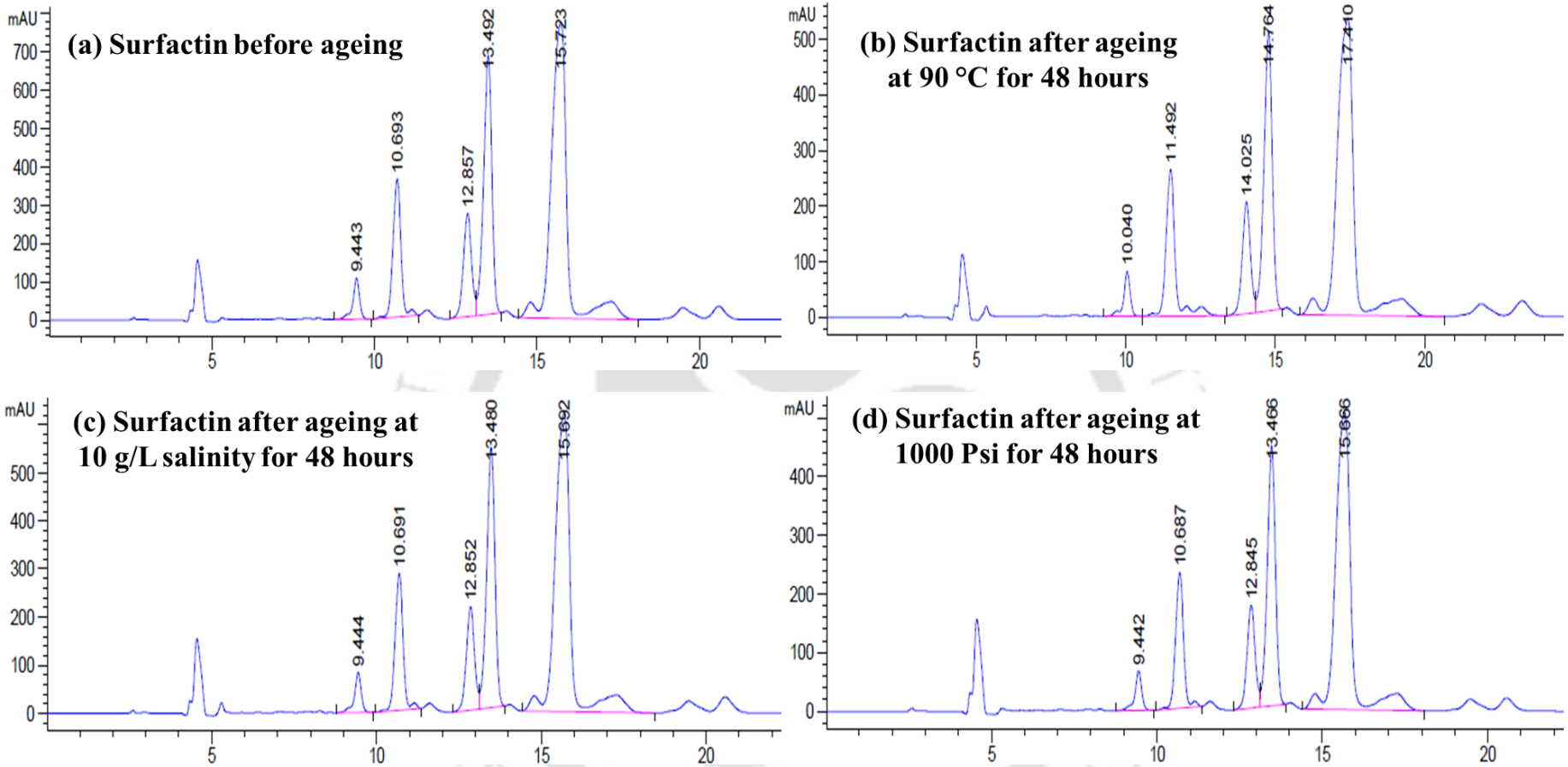


Figure 6.9. HPLC chromatograms of Surfactin produced from *Bacillus tequilensis* MK 729017 in (a) crude form, (b) thermally aged, (c) salinity aged and (d) pressure-aged form

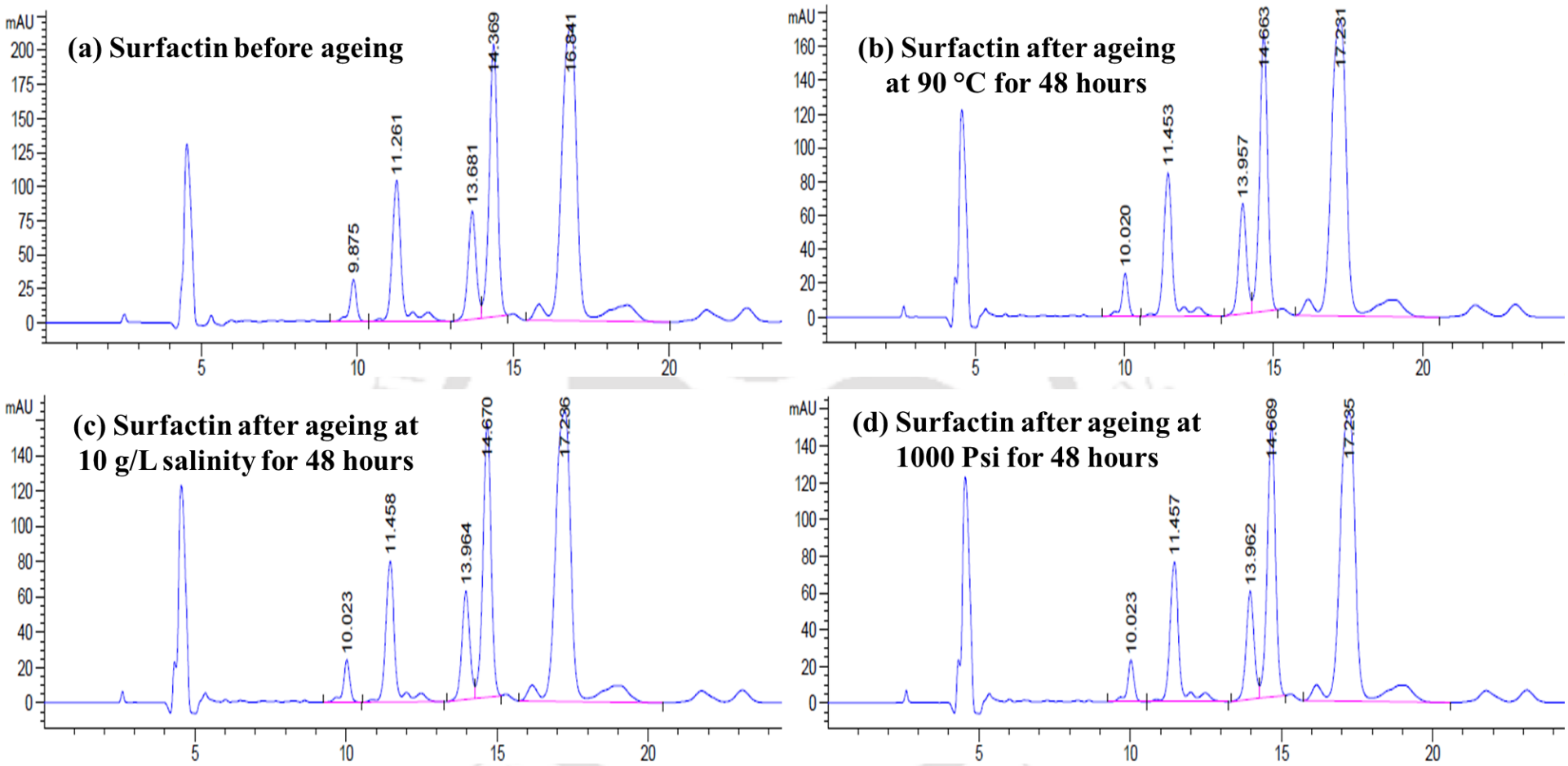


Figure 6.10. HPLC chromatograms of Surfactin produced from *Bacillus subtilis* MG 495086 in (a) crude form, (b) thermally aged, (c) salinity aged and (d) pressure-aged form

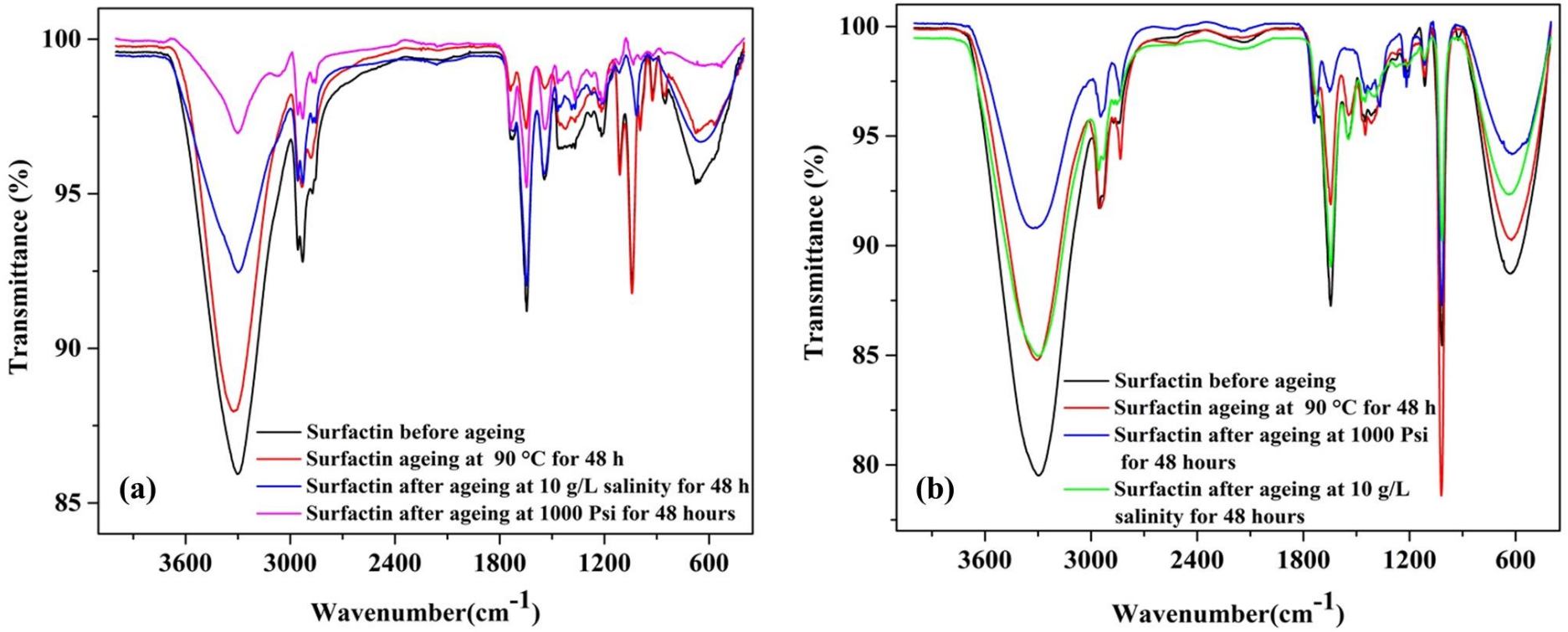


Figure 6.11. FTIR spectra of the crude, thermally aged, halo aged and pressured aged Surfactin produced by (a) *Bacillus subtilis* MG 495086 and (b) *Bacillus tequilensis* M729017

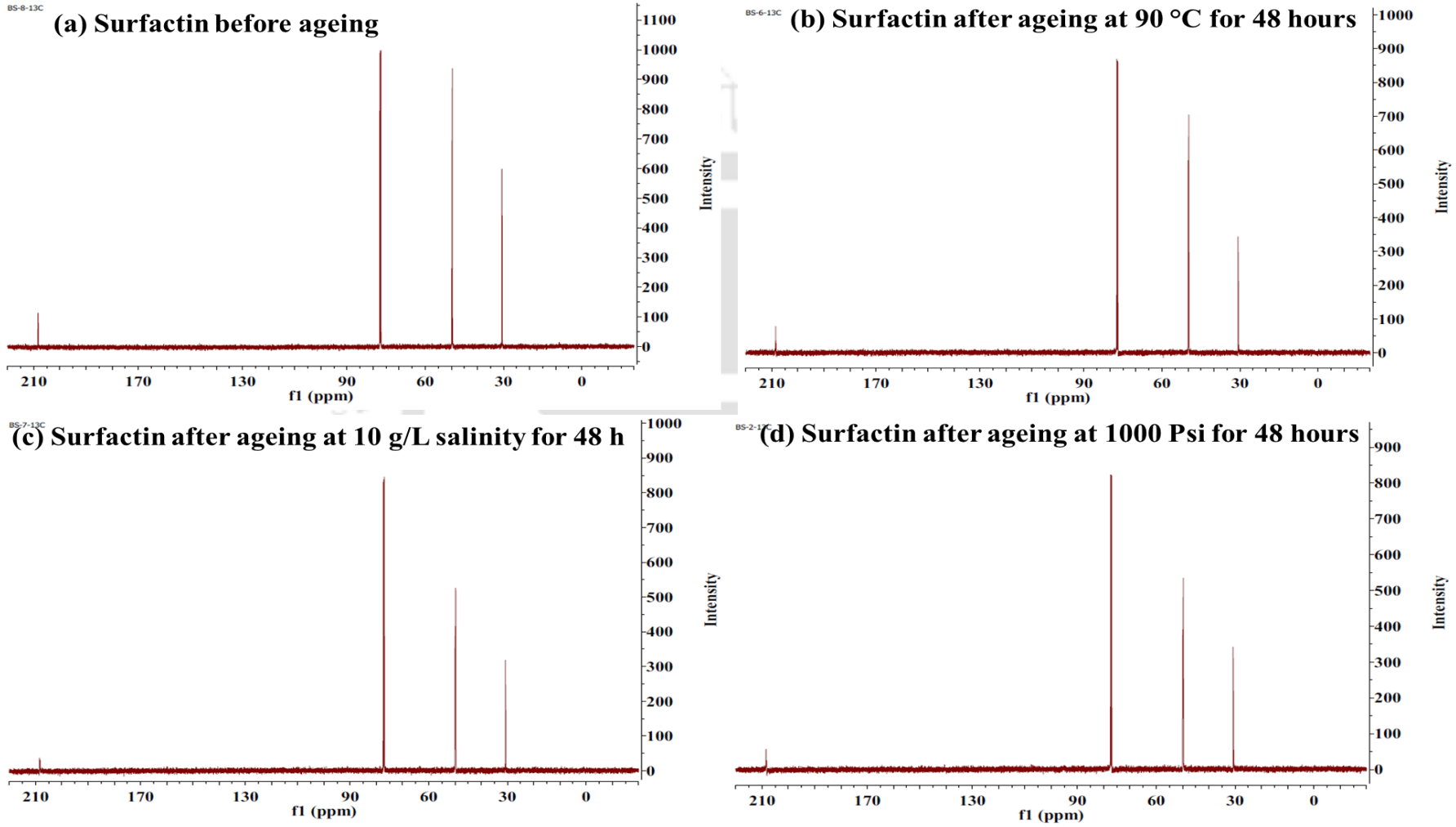


Figure 6.12. The NMR spectra of the (a) crude Surfactin, (b) thermally aged, (c) halo aged and (d) pressure aged Surfactin produced by *Bacillus tequilensis* MK 729017

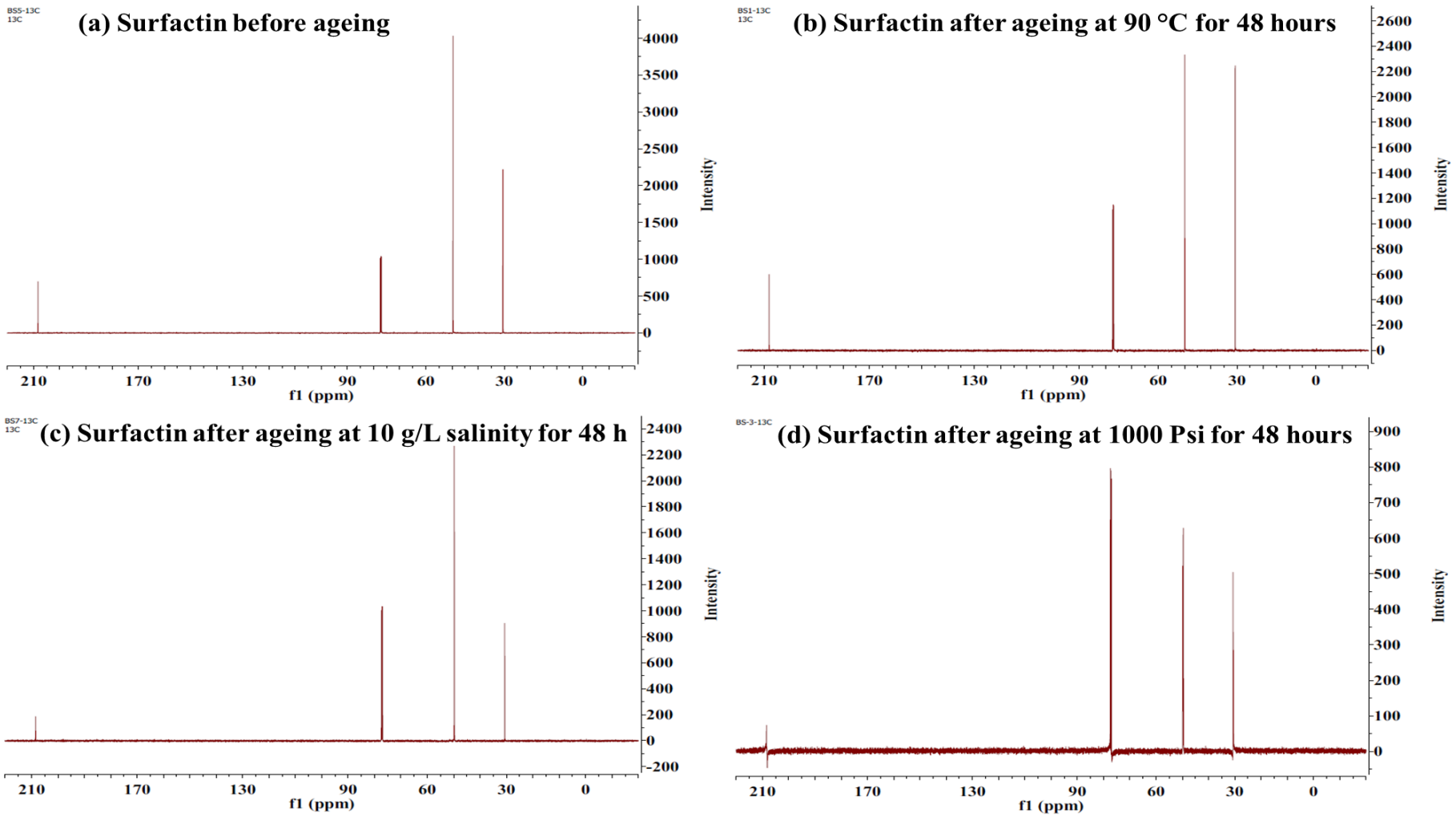


Figure 6.13. The NMR spectra of the (a) crude Surfactin, (b) thermally aged, (c) halo aged and (d) pressure aged Surfactin produced by *Bacillus subtilis* MG 495086

6.3.3. Alkaline stability of the biosurfactant produced by *Bacillus tequilensis* MK729017

Surface charge is a property of biosurfactant which helps to predict its stability over a wide range of pH. In the alkaline stability study, the zeta potential of the produced biosurfactant was studied in a wide pH range (3 to 11). The zeta potential (mV) of the produced biosurfactant (Surfactin) at pH values of 3, 5, 7, 9 and 11 were found to be -22.1 ± 1.55, -37.6 ± 1, -43.3 ± 0.6, -43.3 ± 1.3 and -44.8 ± 0.2 mV, respectively (Figure 6.14). The obtained values of zeta potential are in the agreement with the reported literature data (Fan et al., 2014) and indicated the anionic nature of the Surfactin. A surface charge on a particle of greater than ± 20 mV is considered to be stable (Saxena et al., 2018; Saxena & Pandey, 2019). This endorsed the colloidal stability of the Surfactin produced by *Bacillus tequilensis* MK729017.

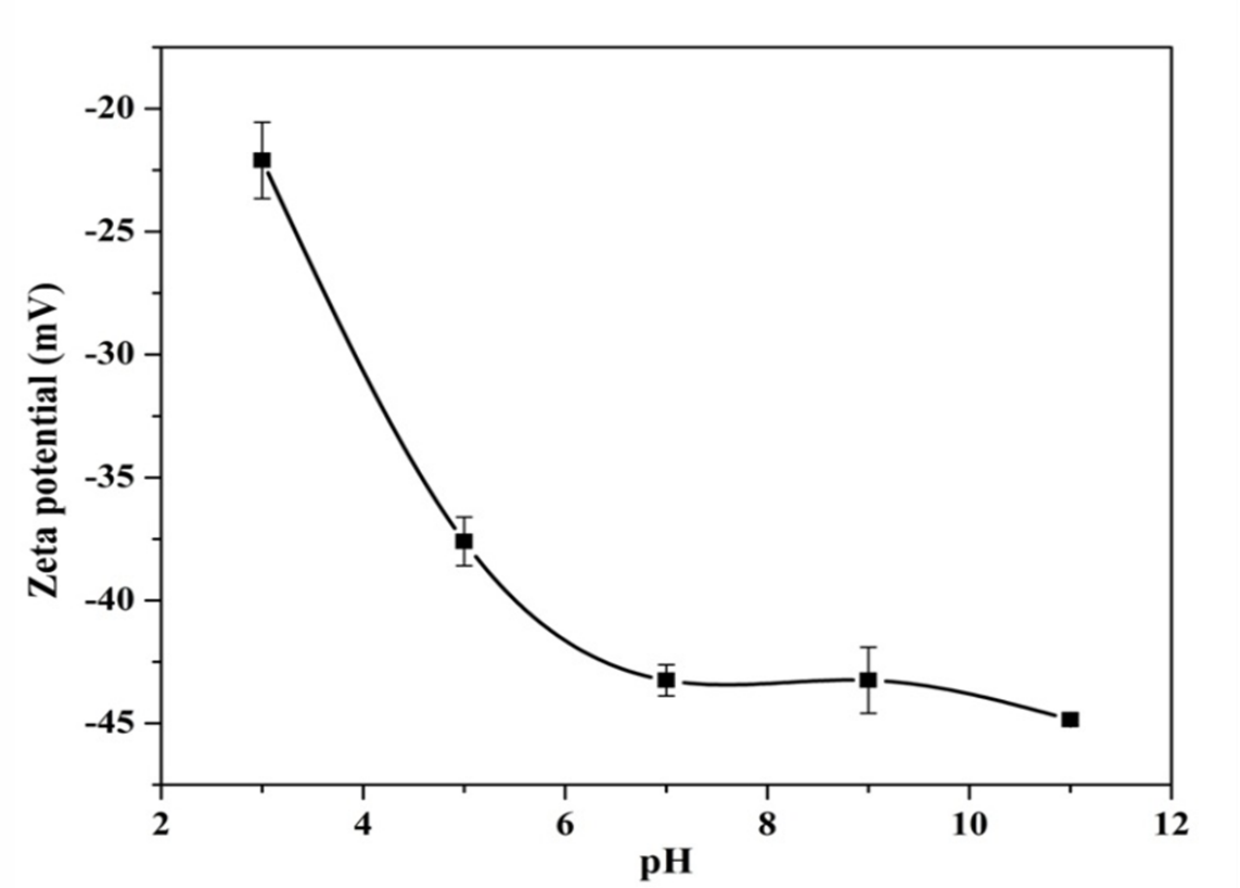


Figure 6.14. Zeta potential profile of the biosurfactant solution of *Bacillus tequilensis* MK 729017 at different ranges of pH

6.3.4. Suitability of *Bacillus subtilis* MG495086 under *in-situ* conditions

For this study, the experiments were carried out under anaerobic conditions at 80 ºC and 90 ºC for 7 days to investigate if the strain could produce biosurfactant in those conditions and whether it would be functional. MSM media with 3.8 % light paraffin oil and 1 % NaCl was employed for the production of biosurfactant at pH 7.7 using 1 % inoculum. The samples were collected every 24 hours and the biosurfactant surface properties i.e., oil displacement, surface tension, drop collapse, emulsification index was analyzed following the protocol mentioned in Chapter 3 and the obtained results are summarized in Table 6.1. On the third day, the best surface properties were observed because the strain was in the stationary phase as exhibited in the bacterial growth curve in Chapter 3. After that, the surface tension started to increase and dry biomass was found to decrease. Although the emulsification index (62.5 %) remained the same from the third day to the seventh day but in the oil-displacement test the diameter of the clear zone started to decrease gradually. The surface properties of the produced biosurfactant under *in-situ* conditions thereby indicated the suitability of *Bacillus subtilis* MG495086 for *in-situ* EOR applications. In order to access its suitability for *ex-situ* application, experiments were performed under optimized conditions as discussed in the next section.

6.3.5. Comparative analysis of the biosurfactant producing strain for EOR application

The important properties of biosurfactant produced from *Bacillus subtilis* MG495086 under optimum conditions are compared with the literature data on biosurfactants, which had been reported for EOR applications (Table 6.2). The strains mentioned in Table 6.2 are mostly isolated from the oil fields (indigenous sources) of diverse geographical locations along with the isolated strain of this study. So, the surface properties of the produced biosurfactant from the isolated strain were found to be comparable and quite similar to those from the indigenous sources. This endorses the suitability of the *Bacillus subtilis* MG495086 for *ex-situ* EOR application as well.

Table 6.1. The analyzed surface properties of Surfactin obtained from *Bacillus subtilis* MG495086 during *in-situ* experiments at 80 ºC and 90 ºC

Time (Hours)	ODT (cm)		ST (mN/m)		Dry Biomass (g)		Drop Collapse		EI Index	
	80 ºC	90 ºC	80 ºC	90 ºC	80 ºC	90 ºC	80 ºC	90 ºC	80 ºC	90 ºC
24	3.8	3.5	36.92 ± 0.04	39.79 ± 0.08	0.46	0.43	No	No	50 %	55 %
48	4.2	3.8	36.44 ± 0.07	37.29 ± 0.08	0.51	0.47	No	No	50 %	55 %
72	5.7	4.5	33.49 ± 0.08	34.27 ± 0.09	0.71	0.67	Yes	Yes	62.5 %	60 %
96	5.4	4.2	34.71 ± 0.02	35.63 ± 0.02	0.64	0.62	Yes	Yes	62.5 %	60 %
120	5.1	3.9	35.62 ± 0.04	35.49 ± 0.07	0.62	0.59	No	No	62.5 %	60 %
144	4.9	3.4	35.81 ± 0.08	36.21 ± 0.08	0.60	0.56	No	No	62.5 %	60 %

Table 6.2. Comparison of the reported/desired surface properties with the obtained results for EOR applications

Strain	Isolation source	Biosurfactant type	ODT (cm)	Drop collapse	ST (mN/m)	EI %	CMC (mg/L)	Yield (g/L)
Bacillus subtilis MG495086 (The present study)	Formation water of Assam oil reservoir	Lipopeptide	6.6	+++	29.85	72.45	40	6.1
Bacillus tequilensis MK729017 (The present study)	Soil sample of Assam oil reservoir	Lipopeptide	7.1	+++	30	66	90	6.87
Bacillus licheniformis (El-Sheshtawy et al., 2015a)	Formation water from Niage field	Lipopeptide	NA	NA	36	96	32	1
Bacillus methylotrophicus USTBa (Chandankere et al., 2014; Chandankere et al., 2013)	DaGang oil field	Glycolipid	+++	++	28	80	35	1.8
Acinetobacter baylyi ZJ2 (Zou et al., 2014)	Zhongyuan oil field	Lipopeptide	5	NA	35		90	0.09
Bacillus subtilis CN2 (Bezza & Chirwa, 2015)	Contaminated soil from a wood treatment plant	Lipopeptide	NA	+++	32	84	185 ± 10	NA
Pseudomonas aeruginosa DQ3 (Zhao et al., 2018b)	Production water of Daqing oil reservoirs	Rhamnolipid	1.83 ± 0.6	NA	33.8	58	10	0.228
Fusant strain FA-2 of *Bacillus mojavensis* JF-2, *Pseudomonas stutzeri* DQ1 (Liang et al., 2017)	Daqing Oil Field	Lipopeptide	2.6 ± 0.3	NA	31.2	58.6	60	0.382
Bacillus amyloliquefaciens TSBSO 3.8 (Alvarez et al., 2015)	Petroleum drilling mud	Lipopeptide	7.4	+++	28.5	63	NA	NA

Symbols: (++) = average result, (+++) = good result, (++++) = excellent result, NA-Not available

6.3.6. Oil washing efficiency of the produced biosurfactants (Surfactin)

In this study, the produced biosurfactants were analyzed for their suitability in oil recovery and were compared with the efficiencies of the chemical surfactants such as anionic surfactant sodium dodecyl sulfate (SDS) and cationic surfactant cetyl trimethyl ammonium bromide (CTAB). The ability of oil recovery was assessed by performing laboratory-scale oil washing from crude oil contaminated (saturated) sand. Typically, petroleum hydrocarbon compounds bind to the sand components and become very difficult to remove due to their hydrophobicity. Biosurfactants can emulsify hydrocarbons by enhancing their water solubility, reducing IFT and increasing the displacement of oil substances from the sand particles (Bezza & Chirwa, 2015).

In this experiment, the produced biosurfactants were employed for the oil washing of crude oil-saturated sand. The oil washing efficiency was estimated as the ratio of removed (washed) oil to initial oil in sand using Equation (6.1). The oil washing efficiencies at different concentrations of the produced biosurfactants (Surfactin from *Bacillus subtilis* MG495086: 40 mg/L, 100 mg/L and 200 mg/L; Surfactin from *Bacillus tequilensis* MK729017: 90 mg/L, 200 mg/L and 500 mg/L) were compared with that of two chemical surfactants (SDS: 2350 mg/L and CTAB: 335 mg/L) and a commercial biosurfactant, Rhamnolipid (200 mg/L), whereas water was used as the control for this experiment. Kinetics of oil washing using produced biosurfactant from *Bacillus subtilis* MG495086 and *Bacillus tequilensis* MK729017 with other surfactants from oil-saturated sand are shown in Figure 6.15 and Figure 6.16, respectively. The washing efficiency reached to plateau within 48 hours. The maximum oil washing efficiencies (A_{max}) were found to be 29 ± 1 %, 84 ± 1 %, 89 ± 1 %, 92 ± 1 % and 82 ± 1 % for water, produced Surfactin (200 mg/L) from *Bacillus subtilis* MG495086, SDS, CTAB and rhamnolipid (Liu et al., 2018), respectively. Similarly, 28 ± 1 %, 80 ± 2 %, 86 ± 1 %, 88 ± 1 % and 80 ± 1 % for the water, produced Surfactin (200 mg/L) from *Bacillus tequilensis*

MK729017, SDS, CTAB and rhamnolipid (Liu et al., 2018), respectively. These observations evidently highlighted the potential of the produced biosurfactants for oil recovery associated applications.

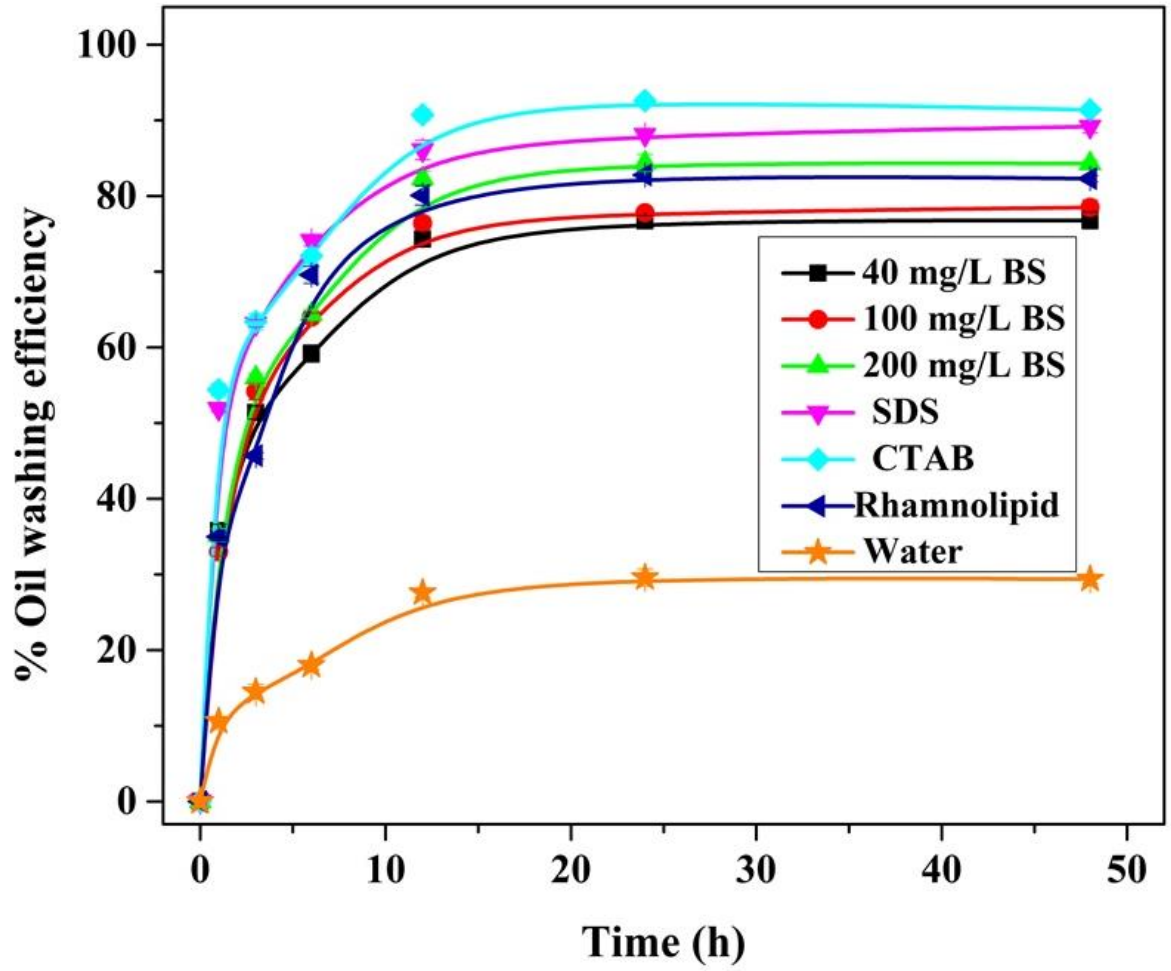


Figure 6.15. Comparison profiles of oil washing efficiency kinetics of the produced biosurfactant from *Bacillus subtilis* MG495086 at different concentrations and other surfactants

In order to understand the mechanism of oil washing, the oil washing efficiency data were collected at different duration and the efficiency versus $t^{1/2}$ curve resulted in two distinct slopes. This indicated the involvement of at least two steps in the oil washing process such as surface washing and internal (intraparticle) washing. Therefore, the kinetic data were fitted using two

exponential functions (Hasan & Pandey, 2016), which is expressed by the following Equation (6.3):

$$Oil\ washing\ (\%) = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) \quad (6.3)$$

Where k_1 and k_2 are the rate constants of two different steps. A_1 and A_2 are the constants whose sum is equal to A_{max}.

The experimental kinetics data were fitted to Equation (6.3) and solved for three unknown k_1, k_2 and A_1 or A_2. Table 6.3 summarizes the estimated kinetic parameters for the produced biosurfactants from *Bacillus tequilensis* MK729017 as well as the commercial surfactants. The rate constant data distinctively highlighted a two-step process, initial a faster (surface) washing followed by a slower (internal) washing. With the increase in the concentration of produced biosurfactant, surface washing (A_1) increased from 90 mg/L to 200 mg/L and remained constant at 500 mg/L. However, internal washing (A_2) increased with an increase in concentration, which indicated concentration-dependent internal diffusion. Similar oil washing kinetics was observed for the commercial-grade rhamnolipid (200 mg/L) biosurfactant. A_1 values of chemical surfactants were found greater than biosurfactants presumably due to higher CMC values and lower micelle sizes of the former; however, A_2 values were estimated to be lower than biosurfactants possibly due to larger emulsion sizes than biosurfactants.

Table 6.3. Oil washing efficiencies of different concentrations of the biosurfactant produced by *Bacillus tequilensis* MK 729017 and their comparison with commercial surfactants

Surfactants	Surfactant conc. (mg/L)	A_1	k_1	A_2	k_2	R^2
Produced Surfactin	90 (CMC)	42.60 ± 5.93	1.19 ± 0.09	31.07 ± 4.30	0.13 ± 0.03	0.99
	200	45.60 ± 0.94	1.41 ± 0.15	34.56 ± 1.34	0.11 ± 0.02	0.99
	500	44.64 ± 1.92	1.64 ± 0.16	36.35 ± 0.82	0.14 ± 0.01	0.99
SDS	2350	61.42 ± 1.91	1.24 ± 0.02	24.60 ± 1.51	0.14 ± 0.01	0.99
CTAB	335	68.22 ± 1.03	1.48 ± 0.13	19.46 ± 1.14	0.13 ± 0.01	0.99
Rhamnolipid	200	45.38 ± 5.81	1.64 ±0.26	33.02 ± 3.27	0.12 ± 0.03	0.99
Water	Control	12.07 ± 0.98	2.89 ± 0.09	15.93 ± 0.28	0.10 ± 0.01	0.99

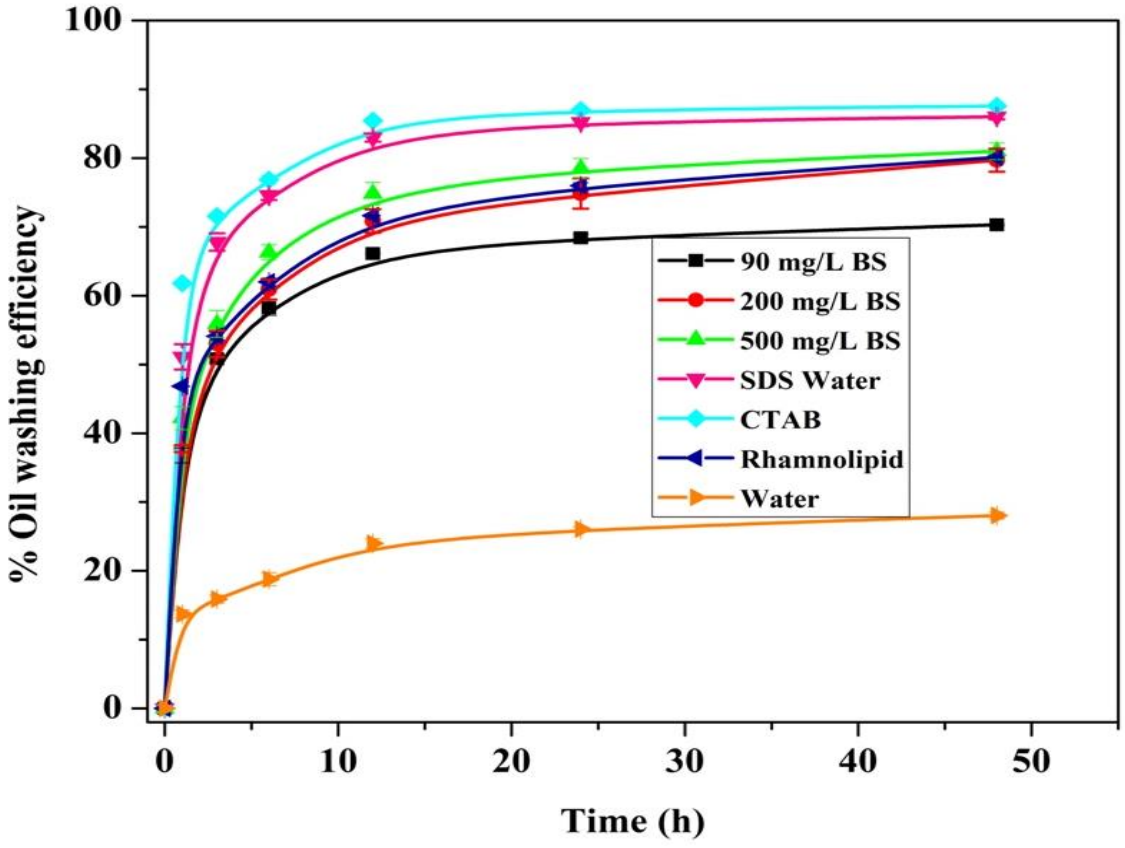


Figure 6.16. Comparison profiles of oil washing efficiency kinetics of the biosurfactant produced by *Bacillus tequilensis* MK729017 and other surfactants

Further, to investigate the insights of oil washing, micelles and water-oil emulsions of the produced biosurfactant were prepared. It is reported that the solubilization capacity of a biosurfactant for crude/motor oil can be determined by the micelle properties (Durval et al., 2019). The hydrodynamic diameter, d_H of biosurfactant solution was determined by a DLS-particle size analyzer, which provides information about the micelle size (Song et al., 2013). The d_H values for the produced biosurfactant (Surfactin) from *Bacillus tequilensis* MK729017 were estimated to be 6.8 ± 0.5 nm, which is higher than reported sizes (≤ 4 nm) of SDS and CTAB (Niraula et al., 2004). The hydrodynamic diameter is inversely related to the diffusion mass transfer coefficient by the Stokes-Einstein equation (Xie et al., 2007). This indicated that lower sizes of chemical surfactants contributed to the greater surface washing (higher values of A_1). The water-oil emulsion sizes (µm) of the produced biosurfactant from *Bacillus tequilensis* MK729017 at different concentrations of 90 mg/L, 200 mg/L and 500 mg/L were calculated to be 19.0 ± 2, 23.8 ± 1 and 33.3 ± 2, respectively whereas the emulsion sizes (µm) for SDS and CTAB were found to be comparatively larger i.e., 54.4 ± 1 and 43.3 ± 5, respectively at their CMC values (Figure 6.17). The size of the rhamnolipid emulsion was also measured and it was 40.4 ± 1 µm. This indicated that lower emulsion sizes of the produced biosurfactants contributed to the greater internal washing (higher values of A_2).

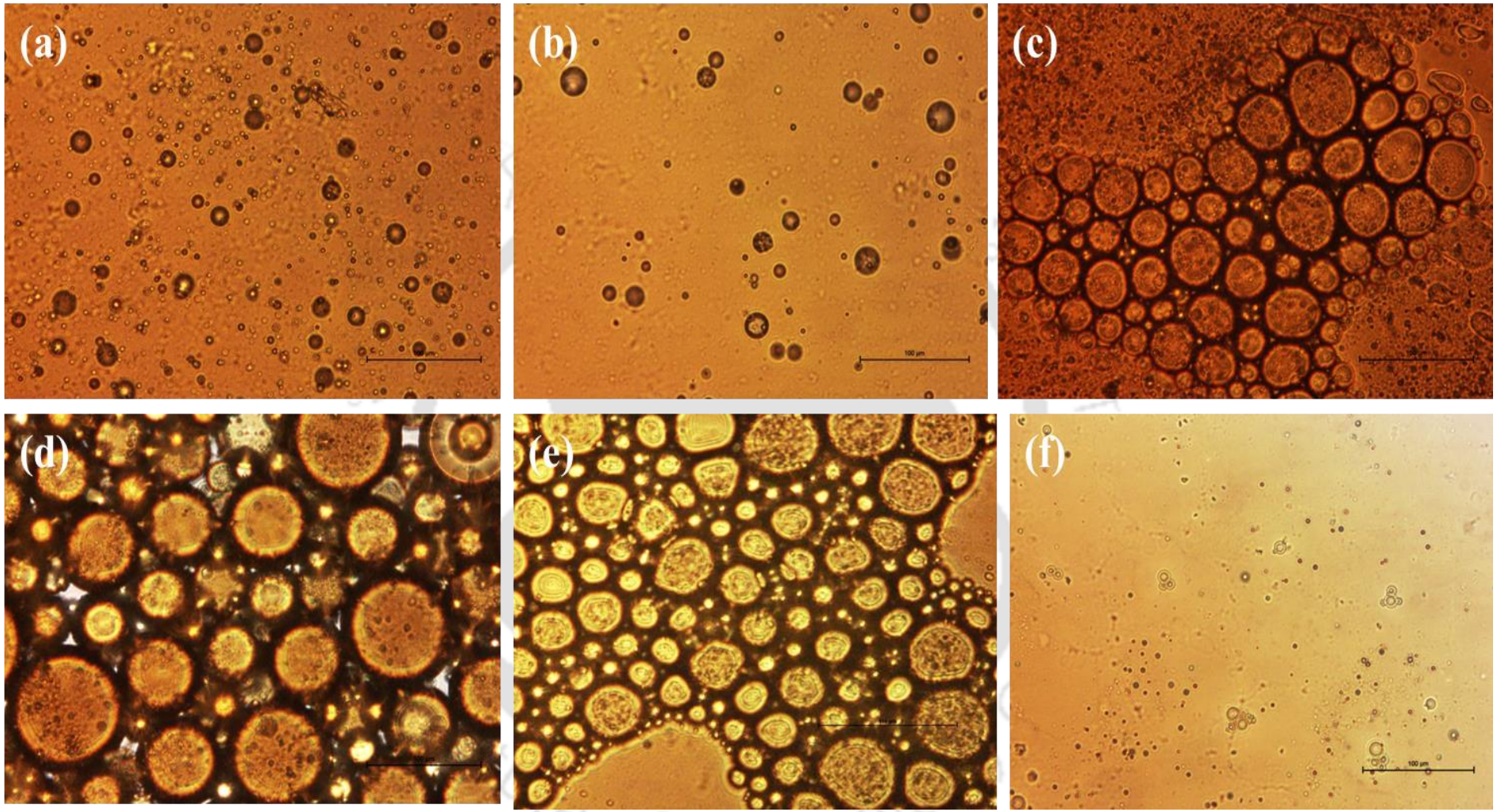


Figure 6.17. Microscopic images of emulsions of Assam crude oil and (a) 90 mg/L Surfactin, (b) 200 mg/L Surfactin, (c) Rhamnolipid, (d) SDS, (e) CTAB and (f) water where the scale bar represents 100 µm.

6.3.7. Interfacial tension measurement

Proper selection of the flooding agents is very significant for flooding experiments which would not only increase the flooding efficiency but also reduce the oil retrieval expenses. Surfactants can lower the IFT of the oil trapped in capillary pores and water surrounding the pores, hence allow the oil to be easily mobilized (Schramm, 2000). In the surfactant flooding process, the ratio of viscous force to capillary force is a critical parameter for oil recovery is termed as Capillary number (Nc). The value of Nc should be in the range of 10^{-5} for mobilizing the trapped oil. When the velocity of the displacing fluid is constant, the IFT should be lowered to maintain Nc in the above-mentioned range (Park et al., 2015). The IFT measurements of the produced biosurfactant solutions in different concentrations were carried out using Assam crude oil as the light drop phase and were compared with that of the chemical surfactant solutions to ensure their applicability for EOR applications. The minimum value of IFT has been reported to maximize the Capillary number (*Nc*). The IFT values for different combinations of flooding agents were measured and calculated using Equation (6.2) and the comparative results are shown in Figure 6.18.

Initially the IFT of three different concentrations of the Surfactins were measured and they were found to be 0.38 ± 0.01, 0.39 ± 0.01 and 0.41 ± 0.03 mN/m at 40, 100 and 200 mg/L Surfactin from *Bacillus subtilis* MG495086 and 0.31 ± 0.02, 0.31 ± 0.03 and 0.345 ± 0.02 mN/m at 90, 200 and 500 mg/L of Surfactin from *Bacillus tequilensis* MK729017, respectively. This indicated that the IFT did not get altered with the change in biosurfactant concentration beyond CMC values. The IFT for SDS and CTAB at their respective CMC values were also measured and found to be 0.52 ± 0.04 and 0.13 ± 0.01 mN/m, respectively. The IFT of SDS and CTAB with Assam crude oil at 0.3 % (w/v) surfactant concentrations were previously reported to be 0.66 and 0.075 mN/m, respectively (Saha et al., 2017a; Saha et al., 2018). The individual IFT values of NaOH and commercial grade bio-surfactant (rhamnolipid) were

reported to be 0.437 ± 0.03 and 0.43 ± 0.01 mN/m, respectively. Further, the combinations of biosurfactant - chemical surfactant, biosurfactant - alkali, biosurfactant - chemical surfactant – alkali were examined in order to achieve the minimum IFT value. The IFT values at 90, 200 and 500 mg/L Surfactin and NaOH combination were found to be 0.11 ± 0.01, 0.06 ± 0.01 and 0.061 ± 0.01 mN/m. The IFT values at 90, 200 and 500 mg/L Surfactin with NaOH and SDS were measured to be 0.021 ± 0.01, 0.028 ± 0.01 and 0.03 ± 0.01 mN/m. The IFT values at 90, 200 and 500 mg/L Surfactin along with NaOH and CTAB were calculated to be 0.018 ± 0.01, 0.013 ± 0.01 and 0.017 ± 0.01 mN/m.

The IFT value ranges using crude oil as the light drop phase were reported to be in between 0.1 to 5 mN/m for its application in EOR as shown in Table 6.4. The produced biosurfactants resulted in IFT in the mentioned range. Other surface properties (ST and CMC) of produced biosurfactants were also found to be comparable (Table 6.4). Hence the produced biosurfactants are predicted to be utilized further for EOR applications.

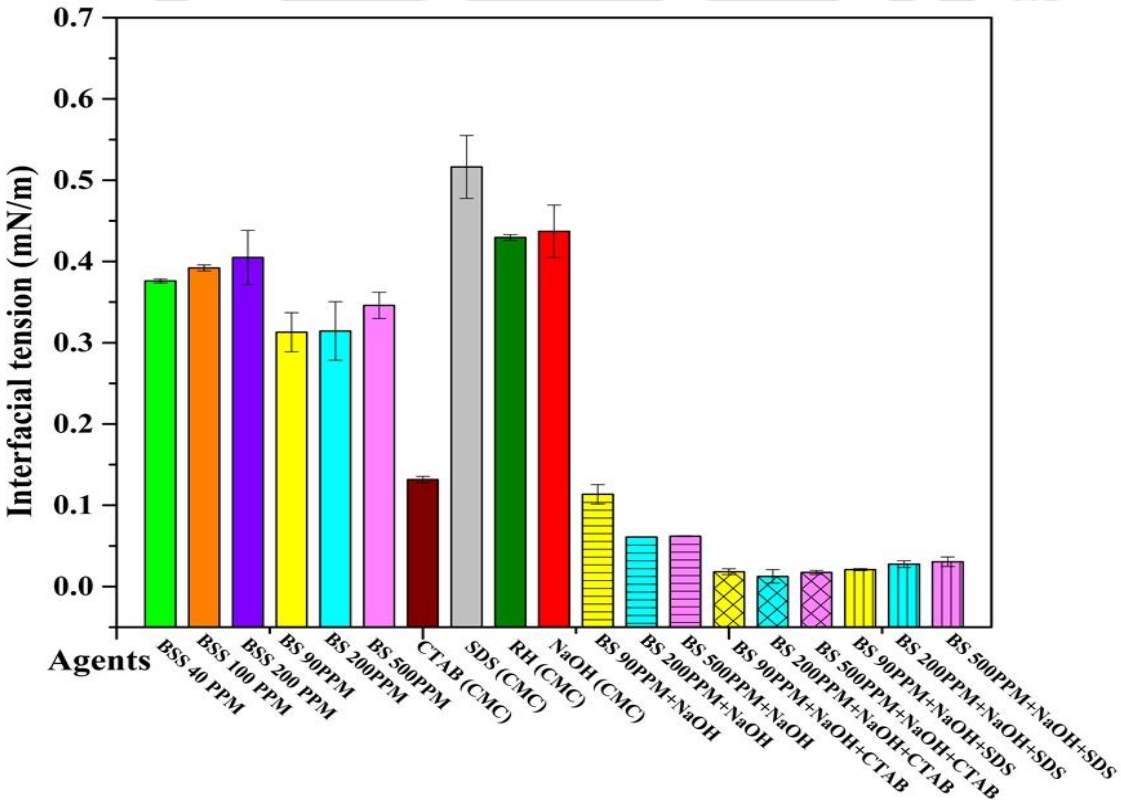


Figure 6.18. IFT variations for different combinations of agents

Table 6.4. Summary of the reported IFT data by various biosurfactant producing bacterial strains using crude oil as the light drop phase

Strain	Surfactant nature	CMC Value (mg/L)	Surface tension (mN/m)	IFT value (mN/m)	References
Bacillus mojavensis JF-2	Lipopeptide	20	28	0.16	(McInerney et al., 2005)
Pseudomonas aeruginosa L6-1	Rhamnolipid	NA	NA	0.8	(Cui et al., 2017b)
Bacillus megaterium	Lipopeptide	250	28.25	1.5	(Dhanarajan et al., 2017)
P. aeruginosa HATH	Rhamnolipid	120	25	2	(Amani, 2015)
Pseudomonas aeruginosa NCIM 5514	Rhamnolipid	NA	31	3.2	(Varjani & Upasani, 2016c)
Bacillus subtilis 20B	Surfactin	NA	29.5 – 30	4.2-4.8	(Joshi et al., 2008)
Pseudomonas aeruginosa DAB	Rhamnolipid	90	26.15	4.59	(He et al., 2017a)
P. aeruginosa, *B. subtilis and R. erythropolis*	Rhamnolipid, Trehalose lipids and Surfactin	30, 50 and 70	22 - 30	1.85, 2.87 and 4.45	(Xia et al., 2011)
Bacillus safensis	NA	96	56	11.3	(de Araujo et al., 2019)
Acinetobacter baylyi ZJ2	Lipopeptide	90	38.4	15	(Zou et al., 2014)
Bacillus tequilensis MK 729017	Surfactin	90	30.46 ± 2.25	0.32 ± 0.02	The Present Study

6.3.8. Phytotoxicity determination of the produced biosurfactant

The phytotoxicity assay was performed to investigate whether the produced biosurfactant imposes any toxic effect on the plants. For that, seed germination and shoot elongation of selected vegetable species (Chickpea and Mung bean) was used as a bioindicator parameter of phytotoxicity of the produced biosurfactant. The germination index (GI), which combines measures of relative seed germination and relative shoot elongation, has been used to evaluate the toxicity of the biosurfactant on *Cicer arietinum* (Figure 6.19 a, b, c, d) and *Vigna radiate* (Figure 6.19 e, f, g, h). A GI of 80 % has been standardized as a bioindicator to confirm the absence of phytotoxicity of the biosurfactant (Chandankere et al., 2014).

The seed germination was observed in the pot containing normal tap water, 0.4 g/L and 1 g/L produced biosurfactant. Relative seed germination (%) was determined using Equation (6A.1) which were found to be 100 %, 80 % and 60 % for *Cicer arietinum* and 90 %, 80 % and 70 % for *Vigna radiate*, respectively. Relative shoot germination was observed in the pot containing tap water, 40 mg/L and 1 g/L produced biosurfactant. Relative shoot germination was estimated using Equation (6A.2) which were 106.9 %, 90.27 % and 76.38 % for *Cicer arietinum* and 94.24 %, 86.3 % and 81.34 % for *Vigna radiate*, respectively. Germination index was determined using Equation (6A.3) involving relative seed germination along with relative shoot lengths which were calculated to be 93.54 %, 88.62 % and 78.55 % for *Cicer arietinum* and 91.24 %, 85 % and 80.21 % for *Vigna radiate*, respectively. The complete calculation for determining the relative seed germination, relative shoot elongation and germination index have been elaborated in Appendix 6A. The obtained results confirm that the biosurfactant from *Bacillus subtilis* MG 495086 does not pose any inhibitory effect on seed germination and shoot elongation and additionally the growth was observed to be quite well.

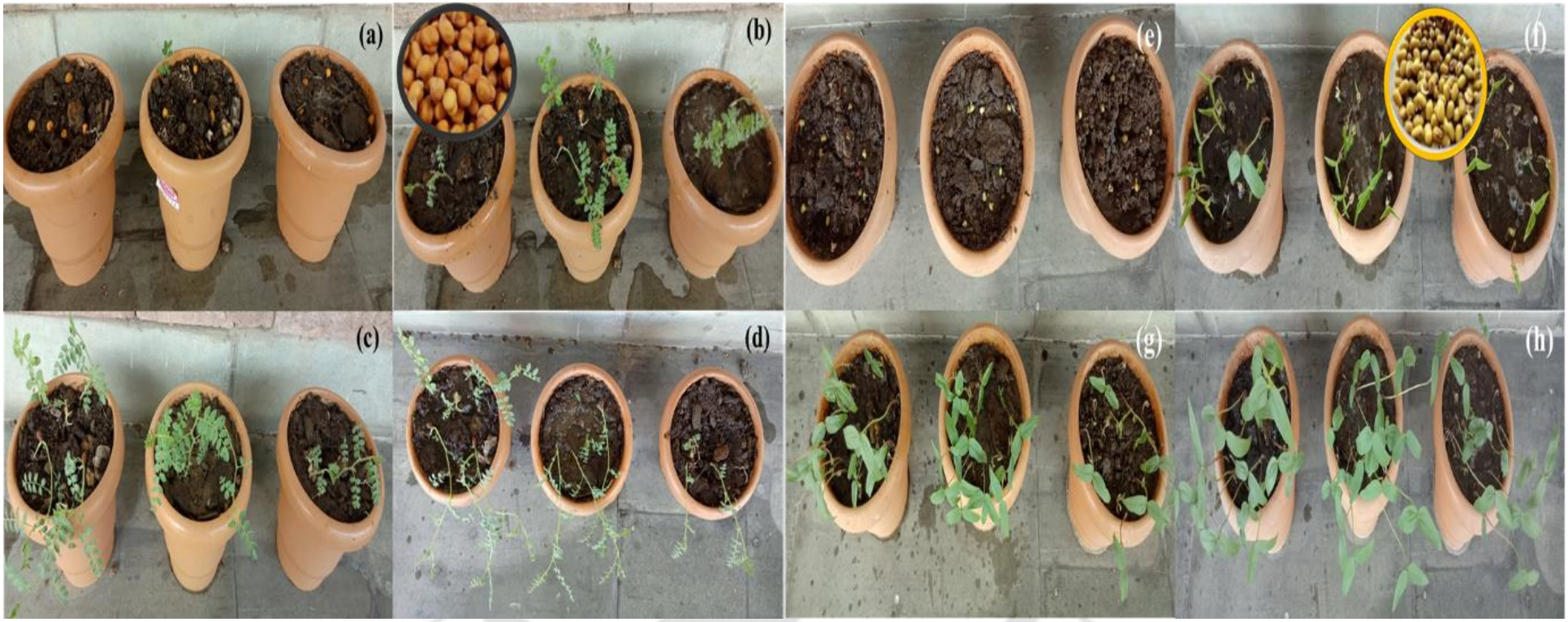


Figure 6.19. Phytotoxicity assay of biosurfactant with *Cicer arietinum* (a - d) and *Vigna radiate* (e - h): Each of the photographs of the plant growth was captured after an interval of 5 days

6.3.9. Antimicrobial activity of the biosurfactant

The biosurfactant showed antibacterial activity against the pathogenic culture of a gram-negative (*Escherichia coli*) strain. The diameters of the clear zones were calculated to be 4 ± 0.5 cm and 5 ± 0.7 cm due to the presence of biosurfactant and Kanamycin in the petri-plate comprising *Escherichia coli*. In the other hole of the petri-plates, no clear zone was observed indicating that water does not possess any antibacterial activity. These findings established that the produced biosurfactant had almost similar antimicrobial activity as kanamycin. Lipopeptide surfactants (Surfactin, Strptofactin and gramicidin) have been reported to possess excellent antimicrobial properties compared to the glycolipid producing strains (Balan et al., 2017; Hasan et al., 2018; Richter et al., 1998; Sabaté & Audisio, 2013; Sarwar et al., 2018).

6.4. Conclusions

The produced biosurfactants were characterized and assessed in advance for their applicability for EOR applications in terms of stability studies, oil washing, IFT and other wetting properties. The thermal stability of the produced biosurfactant was scrutinized by analyzing the CMC, HPLC, NMR, TGA and FTIR which revealed that the biosurfactant could retain its native property even after exposing it at a higher temperature (internal reservoir temperature) for a longer duration. Zeta potential established the alkaline stability of the produced Surfactin in a wide range of pH. *Bacillus subtilis* MG495086 was proved to be very well sustainable at extremely high temperatures (80 – 90 °C) confirming its applicability for *in-situ* MEOR applications. Whereas, *Bacillus tequilensis* MK729017 was more suitable for *ex-situ* MEOR which was investigated by its oil washing proficiency. The oil washing efficiencies (80 - 84 %) of the produced Surfactin were found to be quite comparable with chemical surfactants (SDS, CTAB) and commercial biosurfactant (rhamnolipid). The process involved two steps: initial a faster surface washing followed by a slower internal washing. The first process was dependent on micelle sizes, while the latter was regulated by water-oil emulsion size. The lower emulsion

size of Surfactin (23.8 ± 1 μm) as compared to chemical surfactants (≥ 40 μm) contributed to a greater internal washing. The IFT of the produced biosurfactants was found to be 0.32 to 0.39 mN/m. Therefore, the IFT measurements of various mixture combinations of alkali-chemical surfactants and biosurfactants were determined in order to optimize the suitable flooding agent combination based on the lowest IFT. The toxicity of the produced Surfactin was also evaluated by the phytotoxicity assay as well as antimicrobial activity. These observations endorsed the potential of the isolated strains towards biosurfactants production and their further application for MEOR related purposes.

Chapter 7

Overall Conclusions and Future Scopes of the Work

Chapter 7 summarizes the inclusive inferences drawn from the research work conducted in this dissertation. Furthermore, this chapter also emphasizes the prospects for future research in the relevant area.

7.1.Overall conclusions

Indigenous sources such as formation water and soil samples of Assam oil reservoir were explored to find out potential crude oil-utilizing and biosurfactant producing strains in order to utilize them for oil recovery purposes as well as for bioremediation. The isolation and screening of such five microbial strains were carried out depending on their surface properties such as surface tension reduction (28 – 30 mN/m), oil displacement activity, drop collapse efficiency and emulsification index (66 – 72 %). The strains were identified as *Stenotrophomonas* sp. MG520349, *Bacillus subtilis* MG520348, *Bacillus subtilis* MG495086 and *Bacillus tequilensis* MK729017, *Bacillus subtilis* MK729018. Among them, *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017 were revealed to be the most promising strains from the formation water and the soil samples of the Assam oil reservoir, respectively. Their growth profile studies were conducted in three different conditions (aerobic, anaerobic and facultative) and the aerobic condition was found to be the most suitable due to comparatively high biomass formation than the anaerobic and facultative conditions. The media optimization studies were also carried out with different hydrocarbon sources in which light paraffin oil and glycerol were reported to induce more biosurfactant production among the other hydrocarbons for *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017, respectively. Both the strains were found to be very stable in terms of their performance in a wide range of environmental parameters. The produced biosurfactants from *Bacillus* were chemically characterized with FTIR and NMR analysis which revealed the presence of both aliphatic and peptide groups

confirming them to be lipopeptide biosurfactant. Moreover, the presence of five isoforms in the lipopeptide (C_{12}, C_{13}, C_{14}, C_{15} and C_{16}) through LC-MS and HPLC analyses identified and established it to be Surfactin at very low CMC values of 40 mg/L and 90 mg/L produced by *Bacillus subtilis* MG 495086 and *Bacillus tequilensis* MK 729017, respectively. Statistical analysis with RSM-CCD and ANOVA corroborated the production optimization condition (carbon source and environmental parameters) of *Bacillus subtilis* MG495086 by determining the least surface tension (29.85 mN/m) with 3.8 % (v/v) of light-paraffin oil as the sole carbon source at 62.4 ºC and pH 7.7 with the maximum oil degradation capability of 91.3 ± 5 %. During the hydrocarbon degradation by *Bacillus tequilensis* MK729017, the residual glycerol concentration was monitored and estimated to be gradually decreasing with time and was almost exhausted in a comparatively short span of time i.e., only 96 hours along with the value-added (biosurfactant) product formation that established their suitability for microbial enhanced oil recovery. Moreover, *Bacillus tequilensis* MK 729017 provided a very good yield of 7.46 g/L in the shake flask scale utilizing extremely economical substrate (raw glycerol) indicating an enormous prospect for waste management and exploitation.

The produced biosurfactants were characterized and evaluated for their suitability for EOR applications in terms of their stability studies, oil washing, IFT and other wetting properties. The retention of native surface properties after exposing the Surfactin at a very high temperature for a longer period proved the thermal stability of *Bacillus subtilis* MG495086 by its high sustainability at extremely high temperature (80 – 90 °C) confirming its applicability for *in-situ* MEOR applications. Whereas reasonably more Surfactin production (7.46 ± 0.39 g/L) was achieved by *Bacillus tequilensis* MK729017 though it could not survive well beyond 60 °C. Hence, it was further explored for *ex-situ* MEOR application utilizing its oil washing efficiency. The oil washing proficiency of the produced Surfactin (84 %) was found to be quite comparable with the chemical surfactants (SDS, CTAB) and procured biosurfactant

(Rhamnolipid). The process consisted of two steps: initial a faster surface washing followed by a slower internal washing. The first process was dependent on micelle sizes, while the latter was regulated by water-oil emulsion size. The lower emulsion size of Surfactin (23.8 ± 1 μm) as compared to chemical surfactants (≥ 40 μm) contributed to a greater internal washing enhancing more oil retrieval. Thereafter, the IFT of the produced biosurfactants were determined to be 0.32 to 0.39 mN/m which were relatively higher than the desired IFT value for EOR. Hence, the IFT measurements of various mixture combinations of alkali-chemical surfactants and biosurfactants were estimated in order to optimize the suitable flooding agent combination based on the lowest IFT. The wettability alteration of hydrophobic rock surface from 90 ± 1º to 26 ± 1º suggested excellent interfacial interaction of *Bacillus tequilensis* MK 729017. Zeta potential demonstrated the alkaline stability of the produced Surfactin from *Bacillus tequilensis* MK 729017 in a wide range of pH indicating its performance consistency in diverse reservoir conditions.

The adsorption phenomenon of Surfactin onto model sandstone silica surface had been systematically discussed by using synthetic formation water and employing diverse reservoir thermal conditions (25, 37 and 55 °C). The mineralogical composition of the rock surface via EDX and XRD revealed the presence of silica which acts as the active site for the adsorption of biosurfactants. Freundlich model was the best fit among the other adsorption equilibrium models such as Langmuir and Temkin isotherms, which described the process to be multilayer adsorption on a heterogenous surface. The adsorption system followed Elovich kinetics when the data points were also examined with Lagergren's pseudo-first-order and pseudo second order model. Intra particle diffusion (IPD) model fitting with a high correlation coefficient substantiated the hypothesis of the oil washing process by showing that the intra-particle diffusion mechanism is not the only rate-controlling process and other processes such as boundary layer diffusion also influence the adsorption to some extent. Thermodynamic

adsorption parameters indicated the feasibility and spontaneity of the adsorption process. The adsorption characteristics of Surfactin exhibited lower adsorption capacity onto sandstone silica surfaces indicating its endorsement for further EOR applications. The toxic effects of the produced Surfactin were also assessed by the phytotoxicity assay as well as antimicrobial activity. All the analyses for both the isolated strains represented their promising suitability for MEOR purposes considering their good oil washing efficiency, better surface properties along with higher productivity and greater stability in extreme conditions. These outcomes recommended the potential of the isolated strains towards biosurfactant production and their further application for MEOR related purposes.

7.2.Future Scopes

Presently, oil-producing companies consider MEOR as a high-risk technology to achieve efficient and predictable oil recovery. While modeling approaches could predict reliable oil recovery to some extent under simulated reservoir conditions. The potential development of a "universal" formulation, constituting a mixture of nutrients, selected microorganisms and biosurfactants is an optimistic elucidation subjected to future research in the field scales. Nevertheless, the existing research gaps could be focused and fulfilled by considering the following future scopes.

a) **Bioreactor *ex-situ* scale-up -** The biosurfactant production at a larger scale (bioreactor) would be required for the field applications. Hence, suitable bioreactor (sand-packed columns) fabrication and subsequent operation could be optimized accordingly.

b) **Biological and chemical combined strategy -** Numerous core-flooding experiments have already been carried out separately utilizing chemical surfactants or biosurfactants. The combinatorial approach could be examined in the future to explore the synergistic or antagonistic effect of the chemical surfactants and biosurfactants in EOR when they would be utilized in specified proportions.

c) **Expense estimation -** Cost analysis could be predicted mainly by scrutinizing two factors. Either by monitoring the downstream processing for surfactant recovery and purification or by employing economic substrates for higher surfactant yield. The heavy petroleum fractions could also be employed for biosurfactant production. The cost analysis would help further for real field applications during scale-up.

d) **Biotechnological improvisation -** More and more indigenous unexplored microbial communities could be isolated via new culturing techniques and characterized by novel metagenomics approach both at a phylogenetic and functional level for future MEOR purposes. The development of high-yield extremophilic genetically engineered strains opens a new horizon for their sustainability inside the severe reservoir conditions as well as the development of robust industrial processes for additional biometabolite production. The discovery of petroleum-degrading extremophiles with combined tolerances, such as thermophilic-halophiles, or psychrophilic-oligotrophs would play a pioneering role to understand the microbiology of oil reservoirs so as to reveal probable microbial strains that would be new to science, as well as open opportunities to harness their potential for MEOR.

e) **Performance prediction -** Since microbial performance in laboratory experiments cannot be expected to be the same in the field, it is unrealistic to anticipate the exact outcome of the MEOR process in the field. However, employing various modeling and simulations the predictions could be done to some extent. Novel approaches for monitoring microbial performance *in-situ* are very necessary in order to determine their effectiveness in actual oil recovery. Hence, research studies that focus to understand different factors affecting MEOR success in diverse reservoir settings are recommended.

Appendices

Appendix 5A

Adsorption Behaviour of the Produced Biosurfactants

5A.1. Standard curve of Surfactin

The standard curve of the procured (purchased) Surfactin is shown in Figure 5A-1as mentioned in Chapter 5. This curve is calibrated by integrating the peak areas of Surfactin isoforms at the wavelength of 205 nm. This curve has been used to determine the concentration of Surfactin throughout the Thesis.

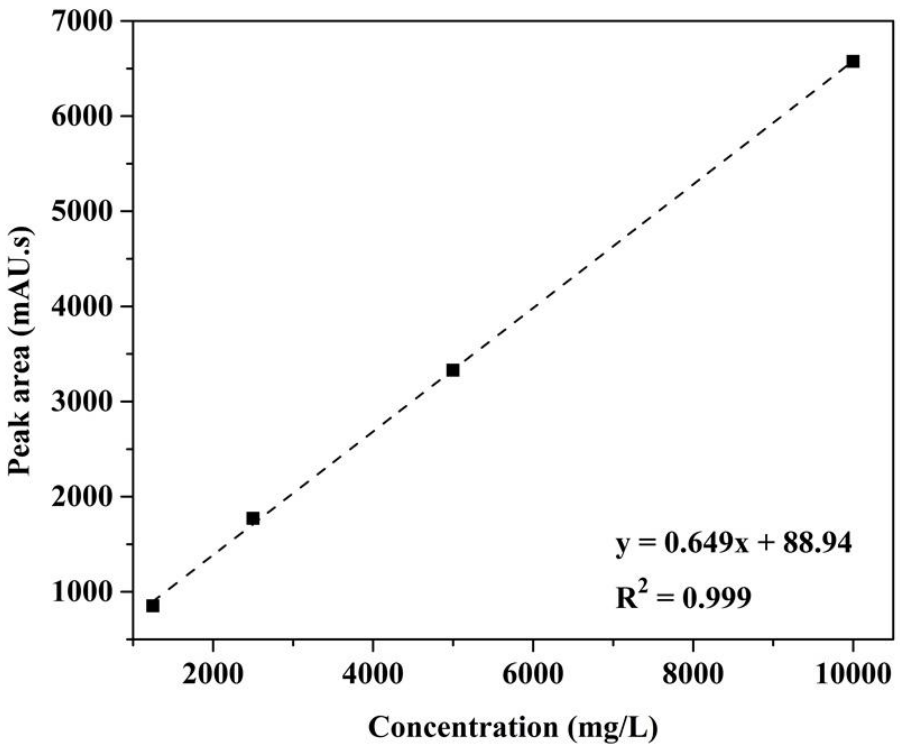


Figure 5A-1. Standard curve of procured (purchased) Surfactin

HPLC chromatograms of the Surfactin samples after adsorption have been presented in this section. Figure 5A-2 and Figure 5A-3 show the HPLC chromatograms of various concentrations of Surfactin samples after adsorption in water and formation water, respectively. Figure 5A-4 and Figure 5A-5 represent the HPLC chromatograms of the 5000 mg/L Surfactin samples after adsorption at 37 °C in water and formation water, respectively withdrawn after certain time intervals. Figure 5A-6 and Figure 5A-7 exhibit the HPLC chromatograms of the 5000 mg/L Surfactin sample after adsorption at 25 °C and 55 °C, respectively.

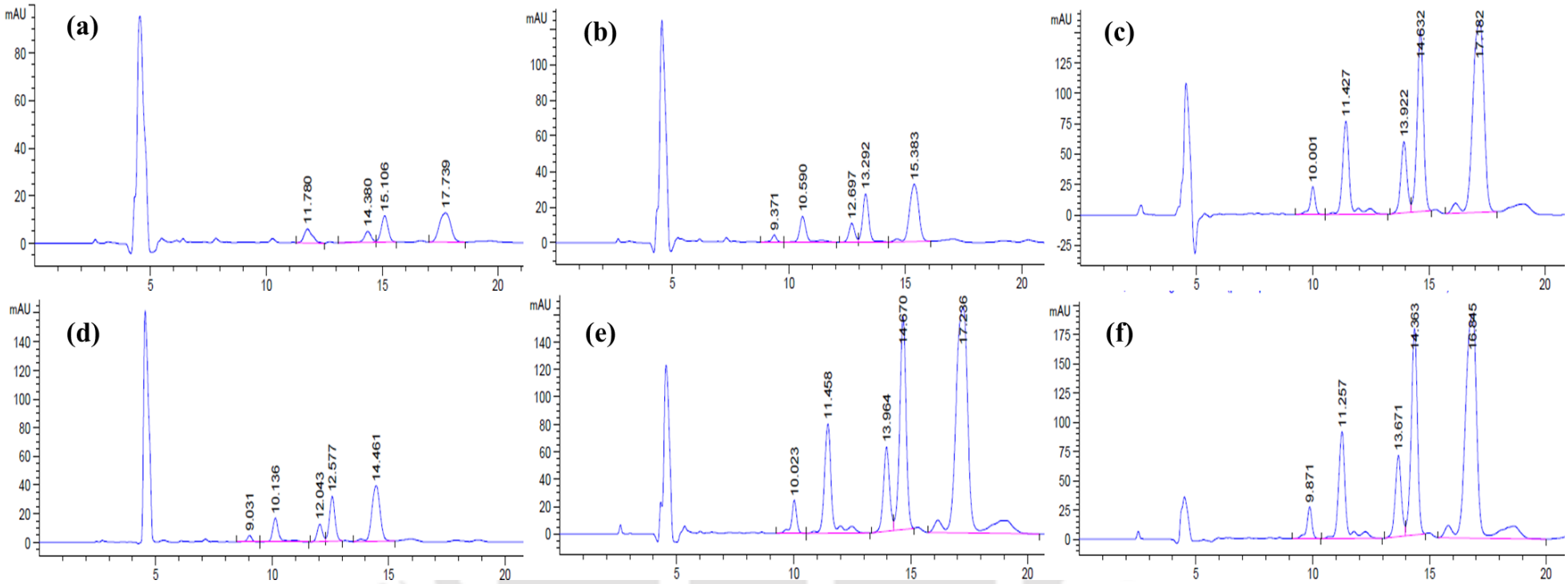


Figure 5A-2. HPLC chromatograms of the Surfactin solutions having concentrations of (a) 500 mg/L, (b) 2000 mg/L, (c) 5000 mg/L, (d) 10,000 mg/L, (e) 20,000 mg/L and (f) 25,000 mg/L after adsorption to study the adsorption equilibrium.at 37 °C

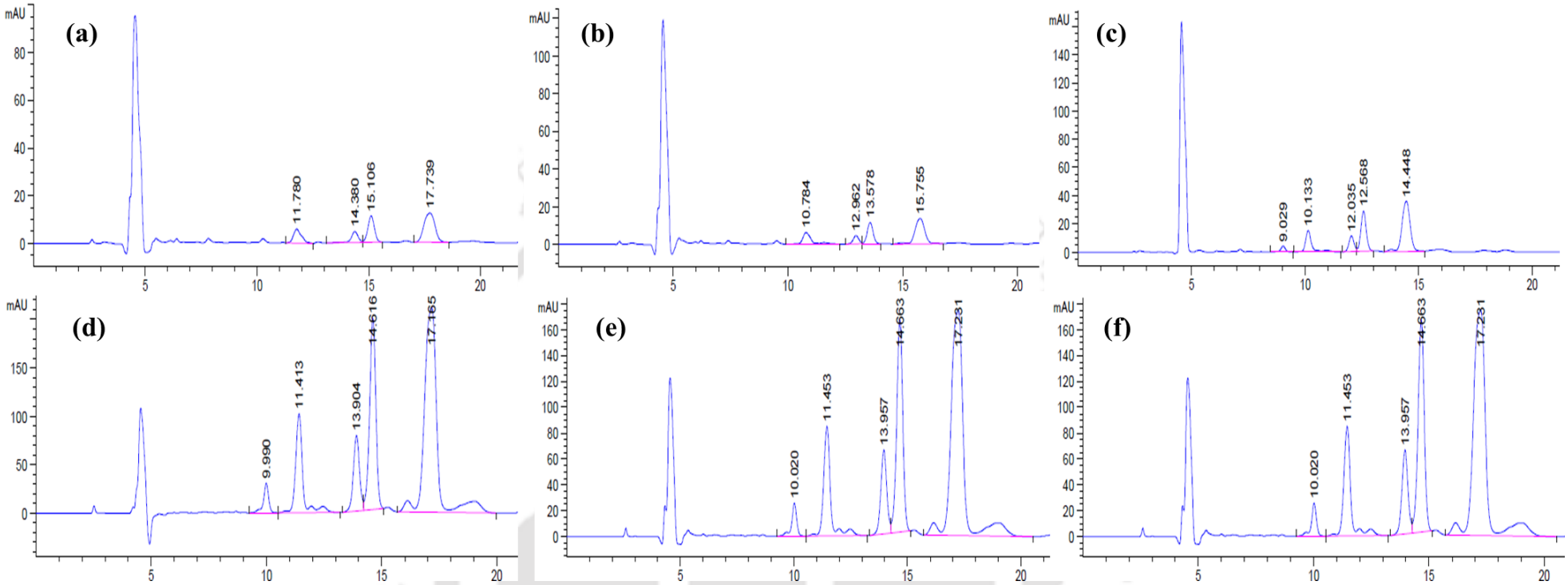


Figure 5A-3. HPLC chromatograms of the Surfactin solutions in formation water having concentrations of (a) 500 mg/L, (b) 2000 mg/L, (c) 5000 mg/L, (d) 10,000 mg/L, (e) 20,000 mg/L and (f) 25,000 mg/L after adsorption to study the adsorption equilibrium at 37 °C

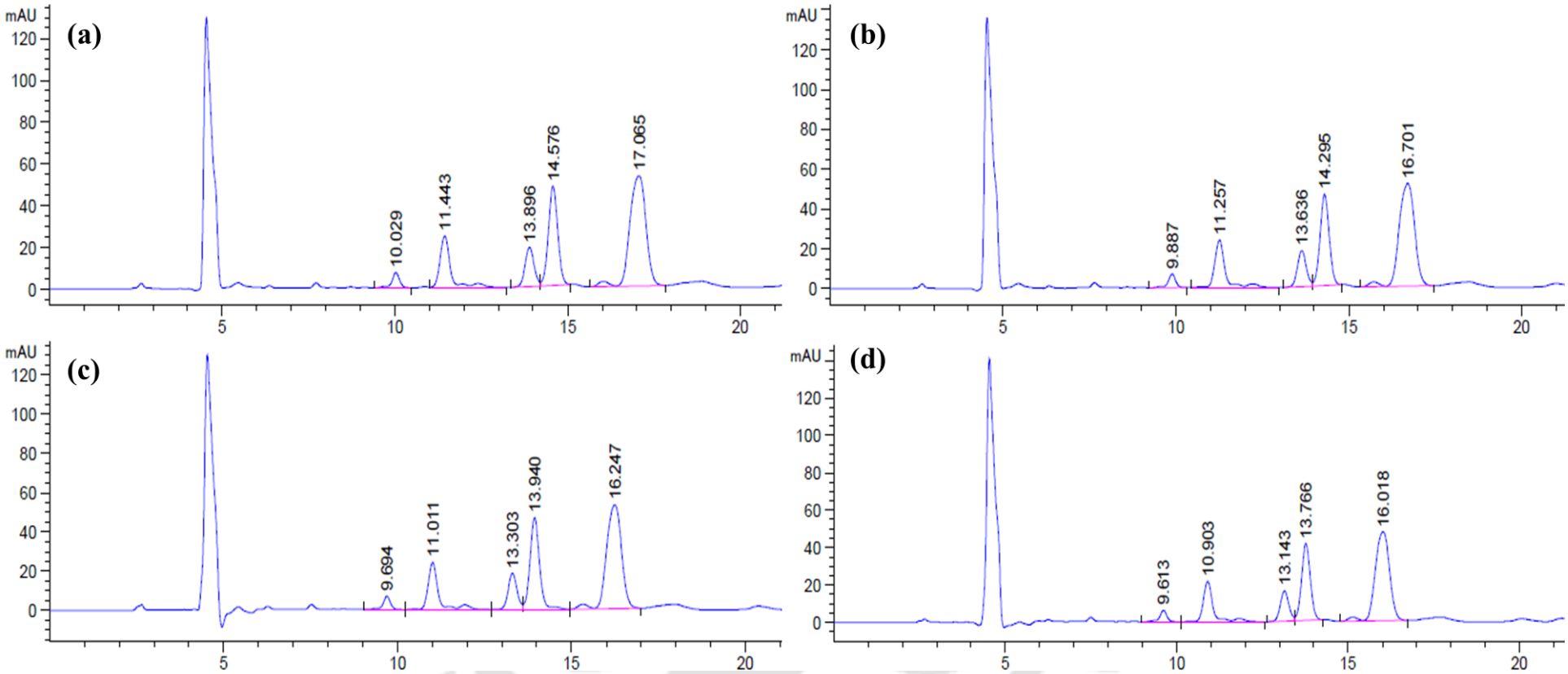


Figure 5A-4. HPLC chromatograms of 5000 mg/L Surfactin solutions, withdrawn after (a) 15 minutes, (b) 60 minutes, (c) 240 minutes and (d) 480 minutes of adsorption to study the adsorption kinetics at 37 °C

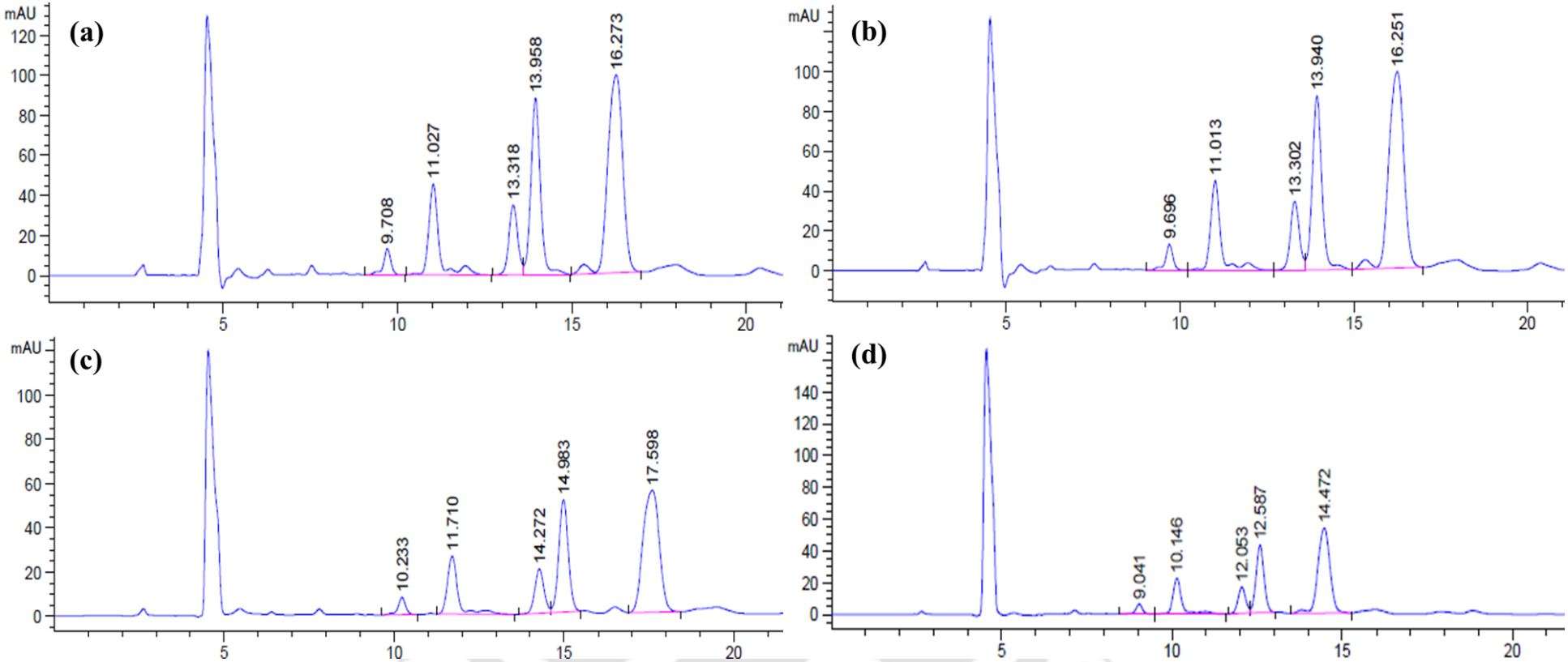


Figure 5A-5. HPLC chromatograms of 5000 mg/L Surfactin solutions in formation water, withdrawn after (a) 15 minutes, (b) 60 minutes, (c) 240 minutes and (d) 480 minutes of adsorption to study the adsorption kinetics at 37 °C

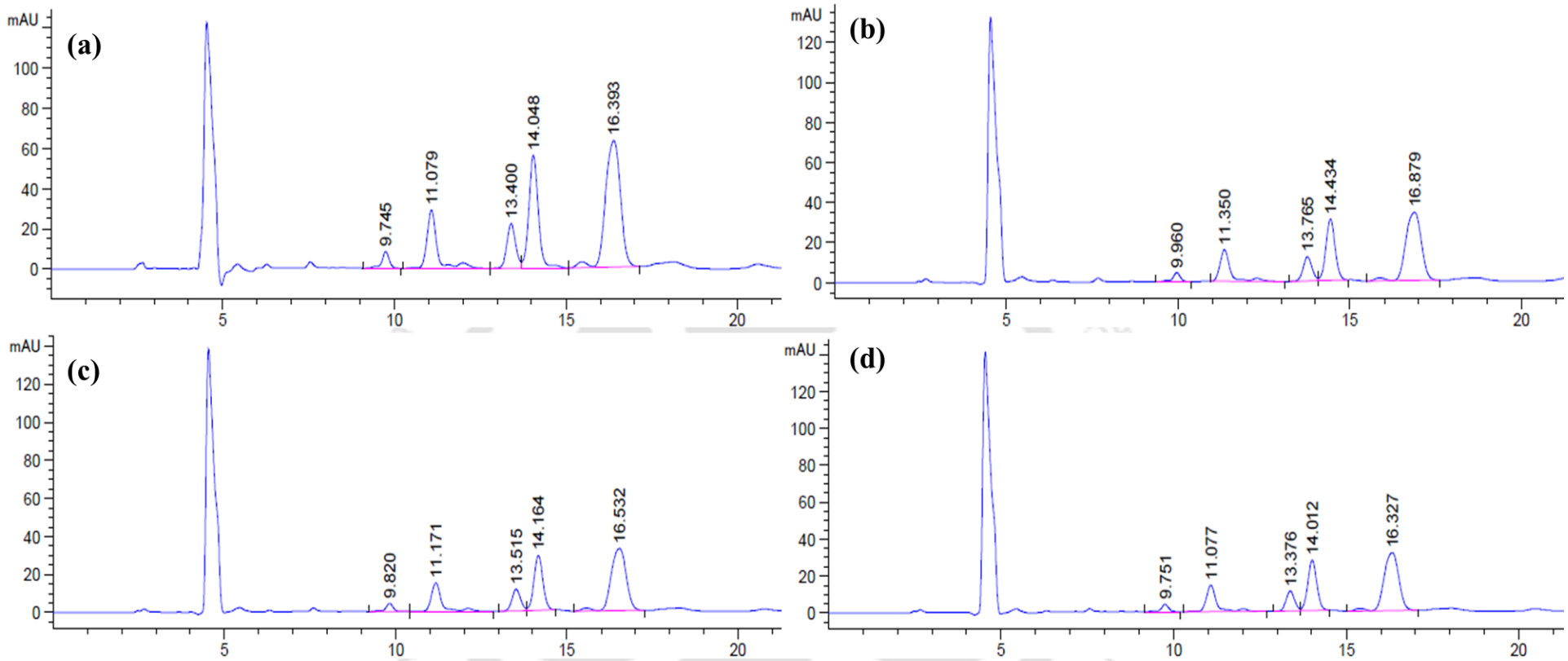


Figure 5A-6. HPLC chromatograms of 5000 mg/L Surfactin solutions in formation water, withdrawn after (a) 15 minutes, (b) 60 minutes, (c) 240 minutes and (d) 480 minutes of adsorption to study the adsorption kinetics at 25 °C

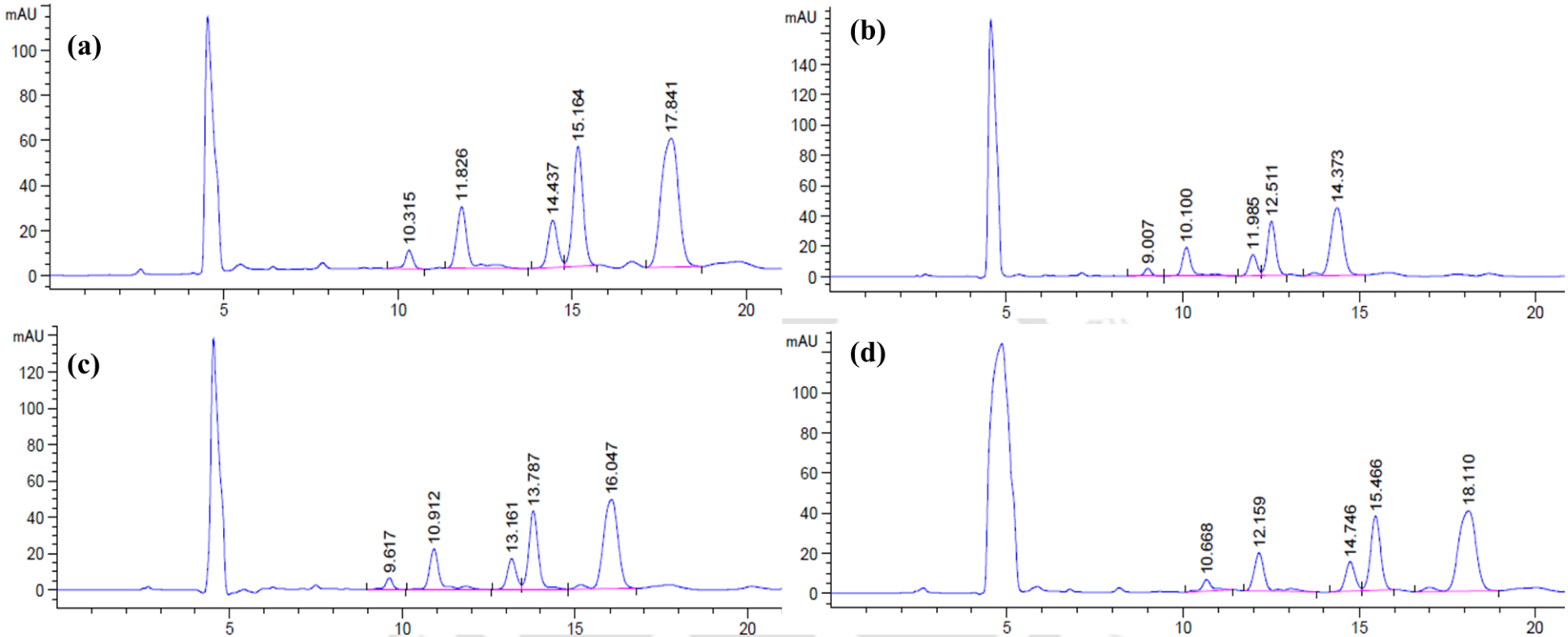


Figure 5A-7. HPLC chromatograms of 5000 mg/L Surfactin solutions in formation water, withdrawn after (a) 15 minutes, (b) 60 minutes, (c) 240 minutes and (d) 480 minutes of adsorption to study the adsorption kinetics at 55 °C

Appendix 6A

Suitability Assessment of the Potential Biosurfactants for Promising MEOR Applications

6A.1. Phytotoxicity determination of the produced biosurfactant

Three main parameters such as relative seed germination, relative shoot length and the germination index were measured using Equation (6A.1), Equation (6A.2) and Equation (6A.3), respectively as discussed in Chapter 6. The mean and standard deviation of triplicate samples were calculated.

$$\text{Relative seed germination (\%)} = \frac{\text{Number of seeds germinated in the extract}}{\text{Number of seeds germinated in the control}} = \frac{SG_e}{SG_c} \times 100 \quad (6A.1)$$

$$= \frac{4}{5} \times 100 = 80$$

$$\text{Relative shoot length (\%)} = \frac{\text{Mean shoot length in the extract}}{\text{Mean shoot length in the control}} = \frac{RL_e}{RL_c} \times 100 \quad (6A.2)$$

$$= \frac{13}{14.4} \times 100 = 90.27$$

$$\text{Mean shoot length in the extract } (RL_e) = \frac{15+15+12+10}{4} = 13$$

$$\text{Mean shoot length in control } (RL_c) = \frac{15+14+14+15+14}{5} = 14.4$$

$$\text{Germination index, GI (\%)} = [(SG_e/SG_c)/(RL_e/RL_c)] \times 100 \quad (6A.3)$$

$$= \frac{80}{90.27} \times 100 = 88.62$$

Where SG_e and SG_c are the numbers of seeds germinated in the extract and control, respectively. RL_e and RL_c are the mean shoot length in the extract and control, respectively (Chandankere et al., 2014).

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List of Publications

(A) Journal publications

1. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2018). "Isolation and characterization of biosurfactant producing and oil-degrading *Bacillus subtilis* MG495086 from formation water of Assam oil reservoir and its suitability for enhanced oil recovery". **Bioresource Technology**, 270, 439-448.
2. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2021). "Experimental Investigation on Suitability of Surfactin for Enhanced Oil Recovery: Stability, Adsorption Equilibrium and Kinetics Studies". **Journal of Environmental Chemical Engineering**, 10, 107083.
3. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2020). "Oil washing proficiency of biosurfactant produced by isolated *Bacillus tequilensis* MK 729017 from Assam reservoir soil". **Journal of Petroleum Science and Engineering**, 195, 107612.
4. Sharma, S., **Datta, P.,** Kumar, B., Tiwari, P., & Pandey, L. M. (2019). "Production of novel rhamnolipids via biodegradation of waste cooking oil using *Pseudomonas aeruginosa* MTCC7815." **Biodegradation**, 30(4), 301-312.
5. Fopase, R., Pathode, S. R., Sharma, S., **Datta, P.,** & Pandey, L. M. (2020). "Lipopeptide and essential oil-based nanoemulsion for controlled drug delivery." **Polymer-Plastics Technology and Materials**, 1-11.
6. Singh, A., **Datta, P.**, & Pandey, L. M. (2017). Deciphering the mechanistic insight into the stoichiometric ratio-dependent behavior of Cu (II) on BSA fibrillation. **International journal of biological macromolecules**, 97, 662-670.

(B) Book Chapters

1. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2021). "Microbial Biosurfactants Remediation of Contaminated Soils" In "*Biodegradation, Pollutants and Bioremediation Principles"* (pp. 160-173), CRC Press, Taylor & Francis Group.
2. Sevda, S., Garlapati, V. K., **Datta, P.,** Chandel, A. K., Pandey L. M., Rathore, D., Singh, A., & Sreekrishnan, T. R. (2020). "Bioethanol Production from Lignocellulosic/Algal Biomass: Potential Sustainable Approach" In "*Algal Biofuel: Sustainable Solution*" (pp. 107-119), TERI, New Delhi, India.
3. **Datta, P.,** Tiwari, S., & Pandey, L. M. (2018). "Bioethanol production from waste breads using *Saccharomyces cerevisiae*" In "*Utilization and Management of Bioresources*" (pp. 125-134), Springer, Singapore.
4. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2021). "Screening of Extremophiles for Microbial Enhanced Oil Recovery Based on Surface Active Properties" In "*Microbial Enhanced Oil Recovery: Principles and Potential*", ISBN 978-981-16-5464-0, Springer Nature.
5. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2021). "Effect of Reservoir Environmental Conditions and Inherent Microorganisms" In "*Microbial Enhanced Oil Recovery: Principles and Potential*", ISBN 978-981-16-5464-0, Springer Nature.
6. **Datta, P.,** Tiwari, P., & Pandey, L. M. (2021). "Recent Case Studies of *In-Situ* and *Ex-Situ* Microbial Enhanced Oil Recovery" In "*Microbial Enhanced Oil Recovery: Principles and Potential*", ISBN 978-981-16-5464-0, Springer Nature.
7. **Datta, P.,** Pannu, S., Tiwari, P., & Pandey, L. M. (2021). "Core Flooding Studies Using Microbial Systems" In "*Microbial Enhanced Oil Recovery: Principles and Potential*", ISBN 978-981-16-5464-0, Springer Nature.

(C) Conferences Proceedings

1. **P. Datta**, P. Tiwari, and L. M. Pandey, "Production and Characterization of Lipopeptide Biosurfactants from Indigenous *Bacillus* Strains of Assam Oil Reservoir and its Suitability for Microbial Enhanced Oil Recovery", presented virtually at **"Chemical Research 2020: International Conference on Chemical Engineering and Chemistry"** held during December 8 – 9, 2020.
2. **P. Datta**, P. Tiwari, and L. M. Pandey, "Understanding the Role of Biosurfactants in MEOR Application", presented at **"CHEMCON 2019"**, organized by Department of Chemical Engineering, Indian Institute of Technology, Delhi and Indian Institute of Chemical Engineers, December 16 – 19, 2019.
3. **P. Datta**, P. Tiwari, and L. M. Pandey, "Understanding the Role of Biosurfactants in MEOR Application", presented at **"Reflux 7.0" 2019** - The Annual Chemical Engineering Symposium, organized by Department of Chemical Engineering IIT Guwahati, India, September 28 - 29, 2019.
4. **P. Datta**, P. Tiwari, and L. M. Pandey, "Characterization and Optimization Study of Biosurfactant Produced by Microorganism Isolated from Formation Water of Assam Oil Reservoir for MEOR Application" presented at International Conference on Waste Management **"Recycle" 2018**, Indian Institute of Technology, Guwahati, India, February 22 - 24, 2018.
5. **P. Datta**, P. Tiwari and L. M Pandey, "Understanding the Role of Biosurfactants in MEOR Application to Assam Oil Fields" oral presentation at **"Reflux" 2017** - The Annual Chemical Engineering Symposium, organized by Department of Chemical Engineering IIT Guwahati, India, March 24 - 26, 2017.
6. **P. Datta**, S. Tiwari, & L. M. Pandey, "Bioethanol Production from Waste Breads Using *Saccharomyces cerevisiae*." 6th International Conference on Solid Waste Management

"ISWMAW" 2016, organized by Jadavpur University, Centre for Quality Management System, Kolkata, India, November 23 - 26, 2016.

7. S. Tiwari, **P. Datta** & L. M. Pandey, **"**Bioremediation of heavy metal (Lead) through bio-sorption using a novel adsorbent." National Conference on Recent Advancements in Environmental Research **"RAER" 2016**, organized by Centre for the Environment at Indian Institute of Technology, Guwahati, India-781039, June 4 - 5, 2016.
8. **P. Datta**, S. Tiwari, N. Kumar & L. M. Pandey, "Enzymatic Hydrolysis of Waste Breads Using *Saccharomyces cerevisiae* and Subsequently Bioethanol Production" presented at International Conference on Waste Management **"Recycle" 2016**, organized by Association of Civil Engineers (ACE) & Waste Management Research Group (WMRG) at Indian Institute of Technology, Guwahati, India - 781039, April 1 - 2, 2016.
9. S. Tiwari, **P. Datta** & L. M. Pandey, "Nano-bioremediation & Biosorption: New Approach towards Sustainable Waste Management" presented at **"Research Conclave," 2016**, organized by Indian Institute of Technology, Guwahati, India – 781039, March, 2016.

(E) Awards

1. **1st prize** with a certificate, cash and memento in "**NORTH EAST BIOSTART: Innovation and Talent Search Contest**" organized by Guwahati Biotech Park and Department of Science and Technology, Assam on 3 - 5 April 2018 at IASST, on the project proposal "Utilization of Waste Motor Oil & Oily Wastewater: Degradation and Product Formulation".
2. **P. Datta**, P. Tiwari, and L. M. Pandey, "Suitability Evaluation of Surfactin Produced by *Bacillus tequilensis* MK 729017 for Enhanced Oil Recovery Applications", won **Best Paper** in **"5th International Conference on Bioenergy, Environmental and Sustainable**

Technologies" (virtual mode) organized by Arunai Engineering College, Tamil Nadu, India, January 29 – 30, 2021.

3. **P. Datta**, P. Tiwari, and L. M. Pandey, "Evaluation of Oil Washing Efficiency of the Biosurfactant Produced by *Bacillus tequilensis* from Assam Reservoir Soil", won **Best Oral Presentation** in "**DST-UKIERI supported Workshop cum Symposium – 2020**; Bio-inspired Nano-materials for Environmental Applications" organized by Indian Institute of Technology Guwahati, India, February 12 – 13, 2020.
4. **P. Datta**, P. Tiwari, and L. M. Pandey, "Investigating the Potential of Biosurfactants for MEOR Application", won **Best Poster Presentation** in National Conference On "**WATER 2020":** Issues & Challenges in Water Treatment and allied research for Sustainable environment", organized by Centre for the Environment, Indian Institute of Technology Guwahati, India, January 23 - 25, 2020.
5. **P. Datta**, P. Tiwari, and L. M. Pandey, "Isolation and Characterization of Crude Oil Degrading Bacteria from Formation Water of Assam Oil Reservoir, India", won **Best Paper Award** in "Bioenergy and Biochemical Engineering" category in **CHEMCON 2017**, Haldia Institute of Technology, Haldia, West Bengal, India, December 27 - 30, 2017.