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Crude oil degradation and biosurfactant production abilities of isolated *Agrobacterium fabrum* SLAJ731

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ABSTRACT

Microbial biosurfactants have a wide range of applications as surface active agents. This study aimed to explore an indigenous bacterial strain, *Agrobacterium fabrum* SLAJ731, isolated from a core sample of Assam oil field for the biosurfactant production and biodegradation of crude oil, which were elucidated in terms of alkane hydroxylase (AH) activity. Preliminary screening was performed using drop collapse assay and oil displacement activity and culture conditions were optimized for biosurfactant production. The biodegradation of spent crude oil was explored under the optimized conditions in the presence of glucose as a co-substrate. The strain produced 5.77 ± 0.3 g/L of biosurfactant under optimal conditions of pH 6 and 30 °C using glucose and yeast extract (C:N = 2:1) with high emulsification activity of 65 ± 0.5%. The crude biosurfactant was characterized using FTIR and ^{1}H NMR and was found to be lipopeptide with a critical micelle concentration value of 650 mg/L and reduced surface tension to 34 ± 0.5 mN/m. It was revealed from FTIR and GC-MS analyses that bacterium was able to degrade oil more efficiently in the presence of glucose and degraded aliphatic (47%) and aromatic hydrocarbons (70%) as compared to abiotic. Both extra and intra-cellular AH activities were found to enhance the biosurfactant production. This study puts forward a distinctive mechanism involved in the biosurfactant production and oil degradation, in the presence of a co-substrate. The notable lipopeptide production and oil degradation ability of *A. fabrum* endorse its suitability for bioremediation and other biotechnological applications.

1. Introduction

Crude oil is composed of variety of hydrocarbons, heavy metals, non-metals along with other particulate matters and utilized as the foremost value-added substrate to meet the energy demands. The mishandling of crude oil during extraction and transportation, and accidental spills lead to hazardous exposure of its toxic components into the environment (Hou, 1982). Complex composition of spent crude oil makes it poor-biodegradable. Toxicity of the components of crude oil including particulate matters, polyaromatic hydrocarbons, volatile organic matters, oil mist poses a serious threat to the environment (Bhattacharya et al., 2019). Therefore, various environmentalists are working across the globe for the remedy of this alarming hazardous exposure.

The major issue in biodegradation of spent crude oil is its hydrophobic nature as it causes a decrease in its bioavailability for natural attenuation. Among various reported physio-chemical techniques (Arivalagan et al., 2014; Pugazhendhi et al., 2018; Sharma et al., 2018b), researchers have reported bioremediation of spent crude oil as the most efficient, economical and eco-friendly approach (Thulasinathan et al., 2019). Bioremediation mediates the employment of biological agents (i.e., bacteria, fungi or algae), which increase the bioavailability of oil by secreting biosurfactant upon utilizing them as carbon (C) source (Saravanakumar and Kathiresan, 2014; Sharma et al., 2018a, 2019). Unlike synthetic detergents, biosurfactants are less toxic, more biodegradable and stable (Fai et al., 2015). These lucrative advantages drive towards the optimization of biosurfactant production to maximize the biodegradation of crude oil. Various bacterial strains such as *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Brevibacillus* sp., *Lysinibacilllus* sp., *Stenotrophomonas* sp. have been studied for their role in biosurfactant production and crude oil degradation however, the biosurfactant

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Table 1
Culture conditions investigated for the optimization of the biosurfactant production.

Factors	Values	Experimental conditions
pH (a)	4.0, 5.0, 6.0, 7.0, 8.0, and 10.0	C = Glucose (1%); T = 37 °C
Temperature (b)	20 °C, 30 °C, 40 °C, 50 °C and 60 °C	C = Glucose (1%); pH = optimized (a)
C source (c)	Glucose, Sucrose, Glycerol, Molasses, Crude oil, and Hexadecane.	pH = optimized (a); T = optimized (b)
N source (d)	Yeast extract, Urea, Ammonium sulphate, and Sodium nitrate	pH = optimized (a); T = optimized (b); C = optimized (c)
C:N ratio (w/w)	1/1,1/2, 1/3, 1/4, 1/5, 2/1, 3/1, 4/1, and 5/1	pH = optimized (a); T = optimized (b); C = optimized (c); N = optimized (d)

production was not found to be very impressive i.e. 0.1–6 g/L. Moreover, *Agrobacterium* genus has not been exploited for the production of biosurfactant and this study is the first report on the ability of *A. fabrum* in this direction to the best of our knowledge.

Furthermore, alongside emulsifying activity of biosurfactant, the rate of biodegradation of crude oil is also attributed to the enzyme activity of certain oil degrading enzymes such as alkane hydroxylase (AH). The activation of hydrophobic crude oil involves oxygenation of its aliphatic as well as aromatic residues, which is catalysed by alkane hydroxylase (Hou, 2017). The enzyme oxidizes alkanes (medium chain length mostly C_5 to C_{16}) into alcohols, which are further metabolically oxidized to aldehydes and ketones and later fluxed to β oxidation pathway for the production of energy and other metabolic products (Hou, 2005, 2006).

In the present study, the ability of rhizosphere microbiome *Agrobacterium* spp. to produce biosurfactant has been explored. Culture conditions i.e. pH, temperature, C, Nitrogen (N) and C:N ratio for the maximal production of biosurfactant were optimized. Further, the biosurfactant was characterized using various techniques like Fourier-transform infrared spectroscopy (FTIR), Critical micelle concentration (CMC), and Nuclear Magnetic Resonance (^{1}H NMR). The synergistic effect of co-substrate glucose on the crude oil degradation and biosurfactant production ability of the present strain was also studied, which was analyzed in terms of the enzyme (alkane hydroxylase) activity.

2. Material and methods

2.1. Microbial strain and culture conditions

In this study, a Gram negative bacterium *Agrobacterium fabrum* SLAJ731 was used, which was earlier isolated by our group from a core sample of Assam oil field, India. Crude oil used in this study was obtained from Assam oil reservoir field, India. Bushnell hass (BH) media (Himedia M350) was used for all optimization study. All the other chemicals and reagents were purchased from Himedia, India and MiliQ (18 MΩ, Millipore system) water was used throughout the experiments.

Pre-culture of *A.fabrum* SLAJ731 was used as inoculum (1%, v/v) and was confirmed for biosurfactant production by allowing to grow in sterile BH media supplemented with 1% (v/v) hexadecane as a model C source, at 30 °C and 150 rpm for 24 h. After incubation, the culture was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was collected and studied for drop collapse test, oil displacement test and emulsification activity (E_{24}) (Datta et al., 2018). All the experiments were performed in triplicates.

2.2. Optimization of biosurfactant production

Upon confirming the ability of present strain for the biosurfactant production, its culture conditions were optimized using one-factor-at-a-time approach (OFAT) as described elsewhere with slight modifications (Parthipan et al., 2017; Devaraj et al., 2019; Jimoh and Lin, 2019). In each case, the 24 h grown culture of *A.fabrum* SLAJ731 was used as inoculum (5%, v/v) at 1.4×10^7 CFU/mL and incubated in BH media at listed pH, temperature and 150 rpm for 24 h (Table 1). After incubation under each listed condition, the cells were pelleted down by centrifugation at 8000 rpm for 10 min at 4 °C and weighed for the dry biomass estimation, and the obtained supernatant was tested for E_{24}.

Table 2
Experimental designs for the study of the biodegradation of crude oil.

Experimental set-ups	Culture parameters (incubated at pH 6, 30 °C and 150 rpm for 144 h)
Without glucose	BH media supplemented with crude oil (1% v/v) as a carbon source and yeast extract (1% w/v) with (5% v/v) inoculum
With glucose	BH media supplemented with (1% w/v) glucose, (1% v/v) crude oil, (1% w/v) yeast extract with (5% v/v) inoculum
Abiotic control	BH media supplemented with crude oil (1% v/v) and yeast extract (1% w/v) without inoculum

2.3. Biosurfactant extraction and characterization

After analyzing all the optimization parameters, the bacterial culture was grown in sterile BH media with optimized C and N (as per the C:N ratio obtained) and maintained at an optimized conditions for 144 h. At the end of the incubation period, the cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. The pellet was collected to estimate dry biomass and the supernatant was used for the extraction of biosurfactant, as per the protocol defined by Fernandes et al. with slight modification (Gudiña et al., 2015). The cell-free supernatant was acidified to pH 2 using 6 M HCl and was kept at 4 °C overnight for the precipitation. Further, it was extracted in an equal volume of ethyl acetate: methanol (4:1) for several times. The upper organic layer was transferred to a round bottom flask and subjected to a rotary vacuum evaporation for the recovery of honey colored crude biosurfactant.

This biosurfactant was weighed to obtain the yield and was further characterized using FTIR spectroscopy (Perkin–Elmer, Spectrum two) and ^{1}H NMR spectroscopy (Mercury Plus, Varian). Briefly, 10 μL of crude biosurfactant was mixed with 100 mg of anhydrous KBr and compressed into a pellet, which was used for FTIR analysis in the wavenumber range of 400-4000 cm^{-1}. Similarly, the crude biosurfactant was mixed with 600 μL deuterated chloroform and analyzed using 400 MHz NMR Spectrometer. The cell-free supernatant was also studied for the determination of critical micelle concentration (CMC) of the crude biosurfactant based on reduction of surface tension of water upon sequentially adding varying concentrations of crude biosurfactant till inflection point was reached using a tensiometer (Dataphysics, DCAT 11 EC) at 25 °C using a Du Nouy ring method (Pandey and Pattanayek, 2013b).

2.4. Biodegradation analysis of crude oil

The ability of *A.fabrum* SLAJ731 to utilize crude oil as carbon source was performed by inoculating at 1.4×10^7 CFU/mL inoculum (5%, v/v) in sterilized BH media (100 mL) supplemented with 1% crude oil. The experimental set ups were prepared as described in Table 2. In order to study the effect of optimized C source, as co-substrate on the oil biodegradation ability, a comparative experimental setup was kept with glucose (1%). Upon incubation at pH 6 and 30 °C, 150 rpm for 144 h, the grown cultures were centrifuged at 8000 rpm, 4 °C for 10 min to pellet down the cells. The cell-free supernatant was collected and studied for the biosurfactant production by acid precipitation and solvent extraction methods, as discussed in section 2.3. The ability of *A.fabrum* SLAJ731 to utilize crude oil as C source was investigated in terms of weighing the residual crude oil based on a gravimetric method wherein

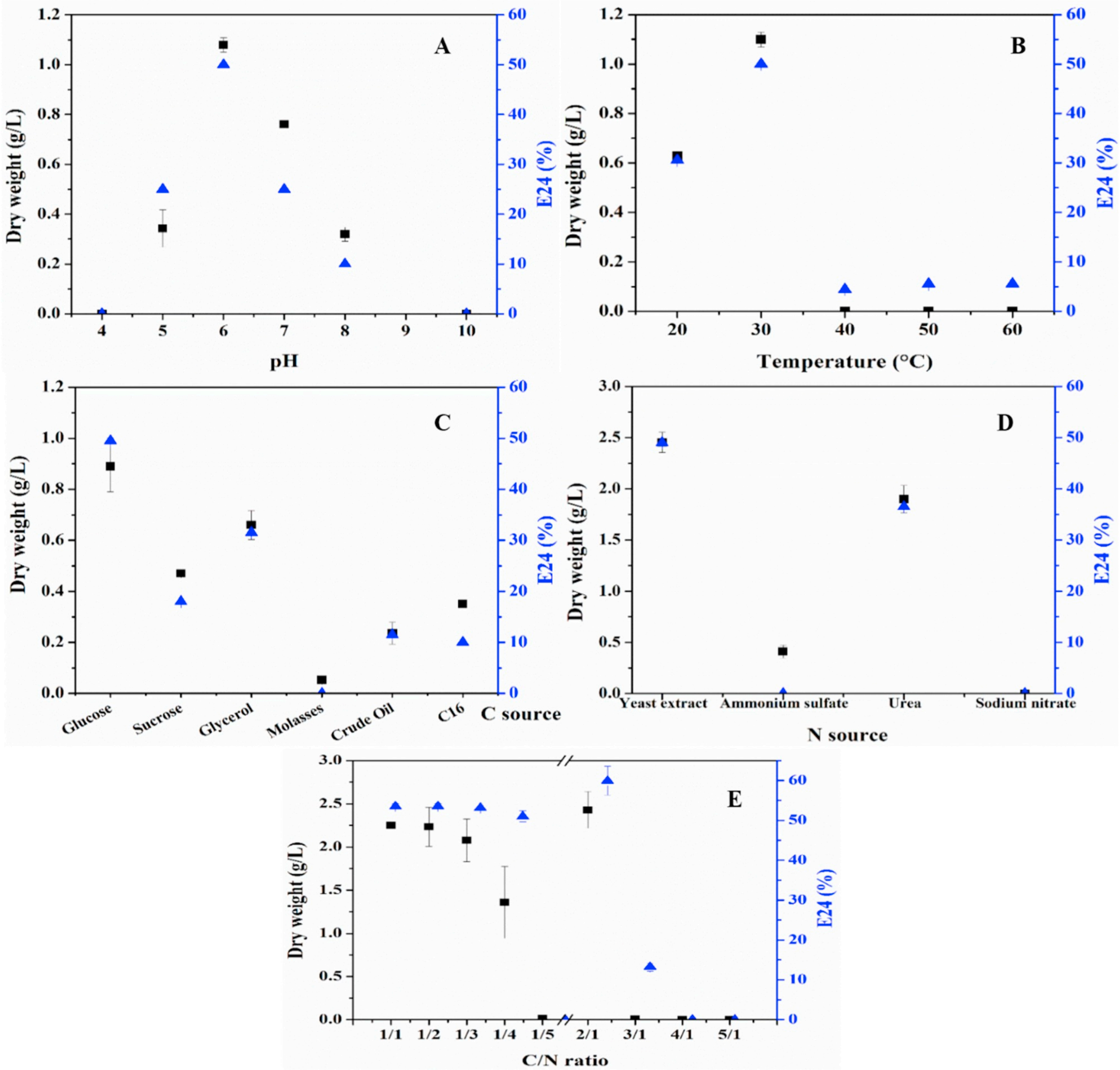

Fig. 1. Effect of different parameters on the biosurfactant production (A) pH; (B) Temperature; (C) Carbon sources; (D) Nitrogen sources; (E) C:N ratio.

the residual crude oil in the cell-free supernatant was extracted in an equal volume of n-hexane and separated using a rotary vacuum evaporator. Furthermore, the extracted residual crude oil was further analyzed using FTIR spectroscopy and Gas chromatography–mass spectrometry (GC-MS) analyses, to elaborate the change in its chemical composition. For GC-MS study (model 7890B, Agilent Technologies), DB5 column (30 m × 0.25 mm × 0.25 μm) used. The injector temperature was maintained at 280 °C with a split ratio of 1:10. Initial oven temperature was maintained at 80 °C for 2 min and was increased with the ramping of 5 °Cmin^{-1} to 280 °C and was held for 30 min. The detector was maintained at 300 °C, and the Helium (carrier gas) flow rate was maintained at 1 ml min^{-1}.

2.5. Determination of alkane hydroxylase activity

To investigate the ability of the strain to degrade crude oil, one of the major enzyme involved in its degradation *i.e.* alkane hydroxylase, was assessed. Its enzyme activity during crude oil degradation was studied in the absence and presence of glucose, as described elsewhere with slight modification (Jauhari et al., 2014; Singh and Tiwary, 2017). After incubation and centrifugation, cell pellets were washed with sterile distilled water twice and later re-suspended in 20 mM Tris-HCl (pH 7.4) and sonicated at 4 °C for 15 min to disrupt the cell wall and collect cellular proteins. It was further centrifuged at 10000 rpm at 4 °C for 30 min to separate cell debris from the protein extract, which was studied for intracellular enzyme assay as discussed below. The pelleted debris was dried in a hot air oven to quantify the dry biomass. The total protein in the crude protein extract was estimated using QuantiPro BCA assay kit (Sigma, India, Cat no. 100134331) using BSA as standard applying a protocol as described elsewhere. (Pandey and Pattanayek, 2011, 2013a; Pandey, 2012; Pandey et al., 2012, 2013; de Andrade et al., 2016; Hasan et al., 2018). Briefly, the reaction mixture was prepared by adding 20 mM Tris HCl (pH 7.4), 0.15% CHAPS buffer (pH 7.4), 0.1 mM Nicotinamide adenine dinucleotide (NADH) along with 10 μL of hexadecane substrate (1% hexadecane diluted with 80% DMSO) to 50 μL of crude enzyme extract. In similar way, the extracellular alkane

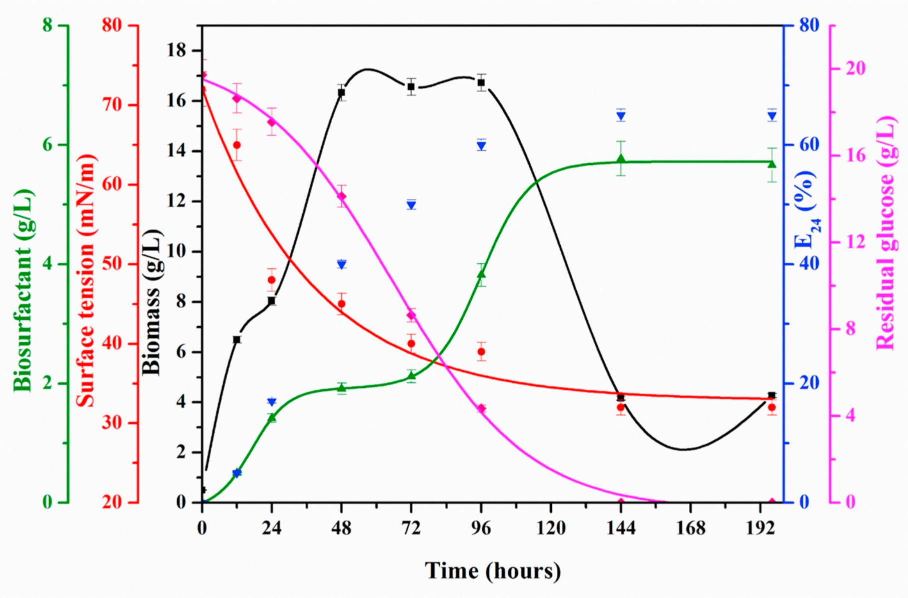

Fig. 2. Biomass growth, biosurfactant, surface tension, residual glucose and E_{24} profile under optimized conditions of pH 6 and 30 °C using 2:1 ratio of glucose: yeast extract in BH medium.

hydroxylase activity was also estimated using cell free supernatant as the enzyme extract. The enzyme activity of alkane hydroxylase was analyzed in terms of decrease in the absorbance at 340 nm of NADH consumed per minute as a result of substrate oxidation, as stated below (Meng et al., 2017).

$$RH\ (Alkane) + O_2 + H^+ + NADH \xrightarrow{Alkane\ hydroxylase} ROH(Alcohol) + H_2O + NAD^+$$

2.6. Statistical analysis

The statistical analyses were carried out using OriginPro 8.5 software. All the experiments were performed in triplicates and their values are reported as average ± standard deviation. Statistical significant difference was determined using analysis of variance (ANOVA) and considered to be significant when significance level, p value, was <0.05.

3. Results and discussion

The biosurfactant production ability of the present bacterial strain, *A. fabrum* SLAJ731 was evaluated using standard screening methods such as oil drop collapse activity, oil displacement method, and emulsification index. The bacterial strain showed positive results for all tests mentioned above with an oil displacement diameter of 1.5 ± 0.3 cm, causing flat morphology of oil drop with few seconds of adherence and a remarkable E_{24} of 50 ± 2.5% within 24 h of incubation. Hence, upon validation of the present strain for biosurfactant production, its culture conditions were optimized for the biosurfactant production.

3.1. Optimization of culture conditions for biosurfactant production

In order to enhance the biosurfactant production ability of the present strain, the culture conditions were optimized based on pH, temperature, C, N and C:N ratio (Table 1). Fig. 1 shows the optimization results for the aforementioned parameters and selections of the best

Table 3
A summarized literature of biosurfactant production utilizing oil based carbon sources.

Strain	Substrate used	Biosurfactant type	Biosurfactant yield (g/L)	E_{24}%	Surface tension (mN/m)	Ref.
Agrobacterium fabrum SLAJ731	Glucose	Lipopeptide	5.77	65	32	Present work
	Crude oil	Lipopeptide	4.15			
Lysinibacillus sphaericus IITR51	Glycerol	Rhamnolipid	1.6	48	52	Gaur et al. (2018)
Bacillus subtilis MG495086	Light-paraffin oil	Surfactin	6.3	72.45	29.85	Datta et al. (2018)
Serratia marcescens	Glycerol	NA	1.42	NA	28.4	Almansoory et al. (2017)
Brevibacillus sp. AVN 13	Used engine oil	Lipopeptide	1.26	72	36	Vigneshwaran et al. (2018)
Bacillus pumilus 2IR	Glucose and crude oil	Lipopeptide	1.06	NA	34	Fooladi et al. (2018)
Staphylococcus capitis	Diesel oil	Lipopeptide	0.1	NA	39.9	Chebbi et al. (2018)
Bacillus cereus SNAU01	Peanut oil cak	Lipopeptide	NA	65	NA	Nalini et al. (2016)
Pseudomonas aeruginosa KVD-HR42	Karanja oil	Rhamnolipid	5.9	NA	30.14	Deepika et al. (2016)
Pseudomonas cepacia	Soybean waste frying oil	Rhamnolipid	5.2	NA	29.77	(e Silva et al., 2014)
Acinetobacter junii B6	Iranian light crude oil	Glycolipid	NA	51	38	Ohadi et al. (2017)
Pseudomonas cepacia CCT6659	Waste frying oil	Rhamnolipid	8	90	25	Rita de Cássia et al. (2017)
Brevibacterium luteolum	Mineral oil	Lipopeptide	NA	NA	27	Unás et al. (2018)
Streptomyces sp. DPUA1566	Soybean waste frying oil	Lipopeptide	1.9	NA	28	Santos et al. (2019)

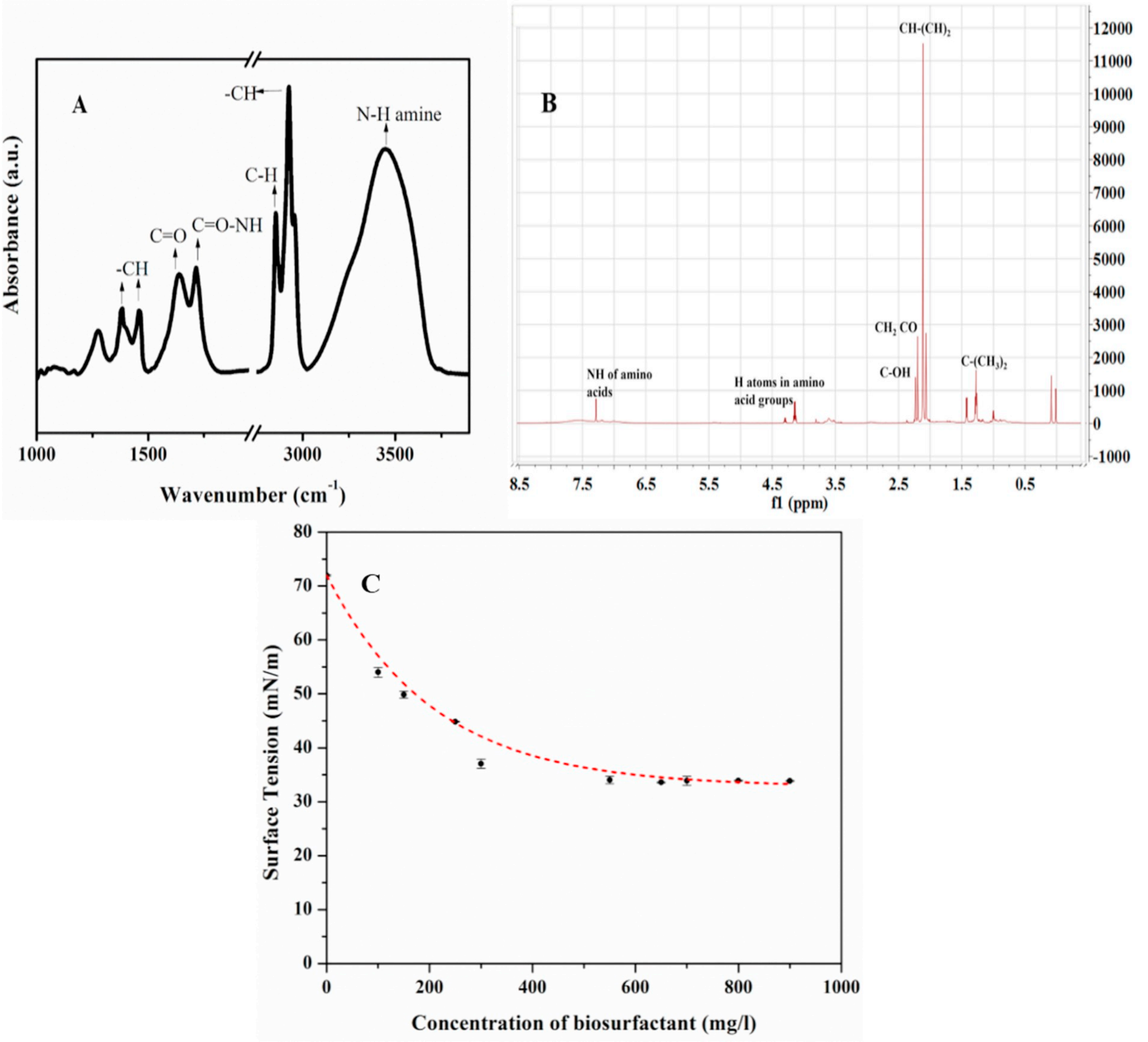

Fig. 3. Characterization analyses for crude biosurfactant confirming the presence of lipopeptide (A) FTIR, and (B) ^{1}H NMR spectroscopy (C) CMC.

conditions were done based on the E_{24} value and dry biomass yield, which responds to biosurfactant synthesis ability and the cell growth, respectively. The optimized conditions for the present strain were obtained as: pH 6, temperature 30 °C (mesophilic), glucose as C source, yeast extract as N source and C:N ratio of 2:1.

Under these optimized conditions, *A. fabrum* SLAJ731 was studied for its growth behavior (based on dry biomass) and subsequent production of biosurfactant, which was analyzed by a change in surface tension, E_{24} and biosurfactant yield analyses as shown in Fig. 2. It was observed that cells showed a short lag phase (~6 h) and exhibited a specific growth rate of 0.01 h^{-1} reaching the maximum cell mass of 16.7 ± 0.2 g/L within 96 h of incubation. Parallely, residual substrate (glucose) analysis was performed to understand the effect of substrate on bacterial growth. It was observed that glucose was consumed at the rate of 0.14 ± 0.1 g/L/h and completely depleted after 144 h. On the contrary, the biosurfactant yield increased from 48 h to 120 h at the rate of 0.07 ± 0.02 g/L/h, suggesting the biosurfactant as a secondary metabolite produced during the stationary phase. This rapid onset of extracellular biosurfactant production during stationary phase was due to the release of cell-bound biosurfactant as reported in few other studies (Thavasi et al., 2011). Hence, the E_{24} activity was found to be increasing with increase in biosurfactant production and the maximum E_{24} was found to be 65 ± 0.2% at 144 h. Similarly, the surface tension was found to follow the reverse trend concerning to E_{24} and reached to 32 ± 0.5 mN/m from 72 ± 0.4 mN/m within 144 h of incubation.

Under these optimized conditions, the overall crude biosurfactant yield was obtained as 5.77 ± 0.3 g/L, which is found to be higher than that produced by other strains utilizing oily substrate as the only 'C' source (Table 3). In comparison to the reported values, the present strain was found to be producing a notable amount of biosurfactant upon utilizing crude oil as the C source. Utilizing spent crude oil as C source for biosurfactant production is not only economical but also ecofriendly, as its degradation decreases the detrimental effects of its exposure to the environment.

3.2. *Characterization of the produced biosurfactant*

Based on the different types of functional groups present in the crude biosurfactant, its chemical nature was investigated. In Fig. 3(A), the distinctive bands near 3443 cm^{-1} designated the presence of aliphatic primary amine moiety of the peptide structure. Peaks near 1717 cm^{-1} and 1645 cm^{-1} represent the amine group and fatty acid linkage (-C═O–NH), respectively. Also, peaks at 2933-2853 cm^{-1} and 1465-1387 cm^{-1} range represent the aliphatic –CH group of the fatty acid long

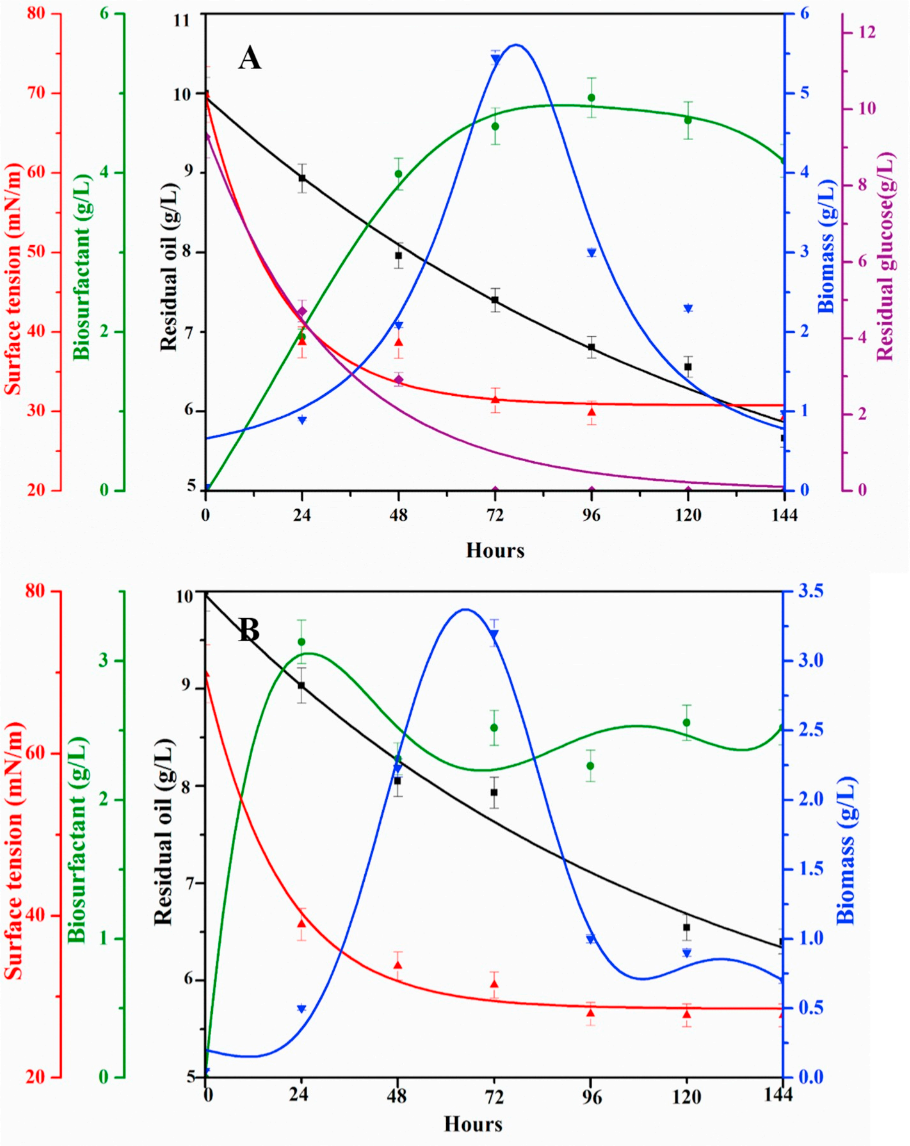

Fig. 4. Biomass growth, biosurfactant, surface tension, residual glucose and crude oil degradation profile under optimized conditions with (A) 1% glucose supplemented with 1% crude oil in BH medium (B) Control: only 1% crude oil.

chains. Similar peaks were observed in reported literature for lipopeptide biosurfactant. These characteristic peaks indicated the produced biosurfactant to be lipopeptide in nature (Nalini et al., 2016). Similarly, the NMR analysis showed the presence of peaks at position $\delta = 7.1–7.5$ signifying the C═O–NH signals of the peptide moiety bonding with fatty acid chain. Likewise, the peaks at chemical shift $\delta = 2.1–2.5$ range represented the $CH_2C{=}O$ bond as shown in Fig. 3(B). These peaks are similar to peaks reported for lipopeptide nature of biosurfactant (Datta et al., 2018). Hence the biosurfactant produced by *A. fabrum* SLAJ731 was classified as lipopeptide in nature. Similarly, CMC of crude biosurfactant was obtained as the lowest concentration at which the surface tension of water reached to a minimum value. The produced lipopeptide showed a CMC of 650 ± 10 mg/L reducing the surface tension of water from 72 mN/m to 34 ± 0.5 mN/m (Fig. 3(C)). Similar results have been reported in literature, indicating the produced lipopeptide has a decent CMC value (Santos et al., 2019).

3.3. Biodegradation analysis of crude oil

The ability of *A. fabrum* to produce a biosurfactant by utilizing crude oil as C source was explored. The experimental set ups were performed as (A) crude oil supplemented with a co-substrate, glucose, (B) control-1: only crude oil and (C) control-2: with no inoculum (abiotic). As shown in Fig. 4(A) and (B), it was observed that sets (A) and (B) followed similar growth pattern, however the specific growth rate was ~1.7 folds higher for set (A) ($\mu = 0.1 \pm 0.04\ h^{-1}$) as compared to set (B) ($\mu = 0.06 \pm 0.01\ h^{-1}$). Upon completion of 144 h, the residual oil and produced biosurfactant in the supernatant were examined using gravimetric as well as chromatographic methods. These studies were carried out with abiotic sample taken as control.

In order to study the kinetics of substrate (crude oil) utilization and product (biosurfactant) formation, the samples were taken at a regular interval of 24 h. The residual crude oil and the produced biosurfactant in the culture, were estimated by n-hexane extraction and ethyl acetate extraction methods, respectively. The crude oil degradation rate was higher in the presence of glucose (0.004 ± 0.001 g/L/h) as compared to its absence (0.002 ± 0.001 g/L/h) as shown in Fig. 4. The overall degradation of crude oil was obtained as 58 ± 5% in the presence of glucose and 40 ± 4% in its absence. Similarly, the biosurfactant yield after 6 days of incubation was obtained as 2.5 ± 0.5 g/L in the absence of

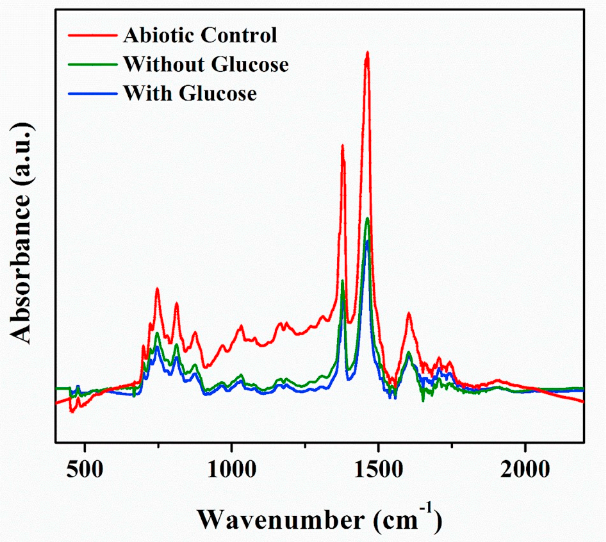

Fig. 5. FTIR analysis of residual crude oil in case of abiotic, and in the presence and absence of glucose.

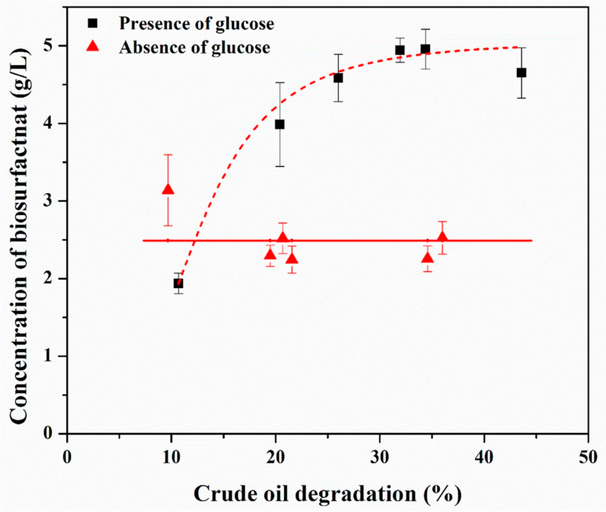

Fig. 6. Biosurfactant production with respect of crude oil (C-source) degradation in the presence and absence of glucose.

glucose, which enhanced to 4.15 ± 0.2 g/L in the presence of glucose.

To further analyze the degradation, a FTIR analysis of the residual crude oil was performed. Fig. 5 shows the change in peak area in the range of 2200–400 cm^{-1}. Peaks at 1465 cm^{-1}, 1450 cm^{-1}, and 1375 cm^{-1} suggest the presence of –CH_2 group and a –CH_3 group of alkanes, respectively. Peak at 1601 cm^{-1} shows the presence of C═C bonds of aromatic groups and peaks at 880 cm^{-1}, 830 cm^{-1}, and 742 cm^{-1} indicate the presence of aromatics out of plane, *i.e.,* "oop" bonds of the residual crude oil. Upon comparing the peak area of the mentioned peaks, the overall decrease was the highest in the presence of glucose i.e., about 65%, which agreed with gravimetric analysis.

The GC-MS analysis of residual crude oil was also studied based on a reduction in peak area (intensity) as tabulated in the Supplementary Table S1. The changes in peak intensity are attributed to the breakdown of compounds into their subsequent subunits. The GC-MS analysis showed 47% degradation of aliphatic hydrocarbons ($m/z = 57, 71, 141$) (Nicolescu, 2017) and 70% decrease in aromatic hydrocarbon ($m/z = 155, 156, 169, 170, 193, 207$) contents (Pavlova and Papazova, 2003) in the presence of co-substrate glucose, however, in the absence of any co-substrate, the decrease in aliphatic and aromatic compositions were obtained as 27% and 34%, respectively with respect to abiotic. Hence, the chromatogram revealed that microbial consumption of crude oil components occurred more effectively in the presence of glucose as compared to its absence. The reduction in total peak area of the crude oil incubated in the presence of glucose was found to be about 58%, which also complemented the FTIR and gravimetric analyses. Conclusively, based on the gravimetric and spectroscopic analyses, it was established that natural degradation of oil components was lesser than microbial biodegradation. However, the present *Agrobacterium* strain showed comparatively better degradation in the presence of glucose (optimized C source). Hence, the presence of glucose intensified the biodegradation ability of oil by the present strain. Similar results have been reported in literature stating the presence of glucose increases the crude oil degradation (Khan et al., 2006; Jeroh et al., 2011; Farag et al., 2018). However, considerable attention has not been given to the mechanism involved in the above phenomenon and hence this remains a bottleneck. In this direction, the present study provides an insight into the relationship involved in biosurfactant production and oil degradation in the presence of glucose.

3.3.1. Relation between biosurfactant production and crude oil degradation

In order to understand the effect of glucose in the degradation of crude oil, a graph of biosurfactant production with respect to oil degradation was plotted. From Fig. 6, it was observed that biosurfactant production enhanced significantly ($p < 0.05$) in the presence of glucose. Even though there was lesser production of biosurfactant in the initial 48 h, which could be due to the utilization of available glucose, as C source. However, with the depletion of glucose in the media, the cells started utilizing crude oil with subsequent enhanced biosurfactant production. On the other hand, in the control containing only crude oil, no significant change ($p > 0.07$) in biosurfactant production was observed with change in oil degradation. This revealed a breakthrough *i. e.* the presence of glucose significantly enhances the biosurfactant production and crude oil degradation. However, biosurfactant is not the sole factor involved in oil degradation. Hence, we further explored the role of alkane hydroxylase.

3.4. Determination of alkane hydroxylase activity

The principle cause for microbial degradation of crude oil components is due to the presence of degradative enzymes. For analyzing the biodegradation ability of the present strain, the activity of one enzyme, alkane hydroxylase, was focused. The present strain was found to synthesize AH both extracellularly (eAH) as well as intracellularly (iAH). The activity of alkane hydroxylase (AH) is regulated by the presence of 'C' substrate in the media. Hence, we performed a comparative study on the effect of glucose supplemented crude oil on the activity of AH.

It was observed that in the presence of glucose, the AH enzyme activity remained active both extracellulary as well as intracellularly. However, the maximum enzyme activity was observed at 72–96 h of incubation, with intracellular enzyme activity of 9 ± 0.1 U/mL and extracellular enzyme activity of 4.5 ± 0.02 U/mL. In contrast, in the absence of glucose, the maximum extracellular enzyme activity was found at day 1 *i.e.* 4.0 ± 0.1 U/mL, (which decreased gradually afterwards) whereas the maximum intracellular activity was observed at day 2 (3.4 ± 0.2 U/mL). Interestingly, the presence of glucose enhanced the overall extracellular AH activity. It was anticipated that it was due to the availability of cofactor (NADH) regeneration system during the process of catabolism of glucose to gluconic acid, as also supported in literature (Iyer and Rajkumar, 2019). The substrate continuously provided NADH for the effective activity of extracellular expressed AH. Furthermore, we observed that constitutive expression of both intracellular and extracellular AH activity was required for better degradation of crude oil, as experienced in the presence of glucose than its absence.

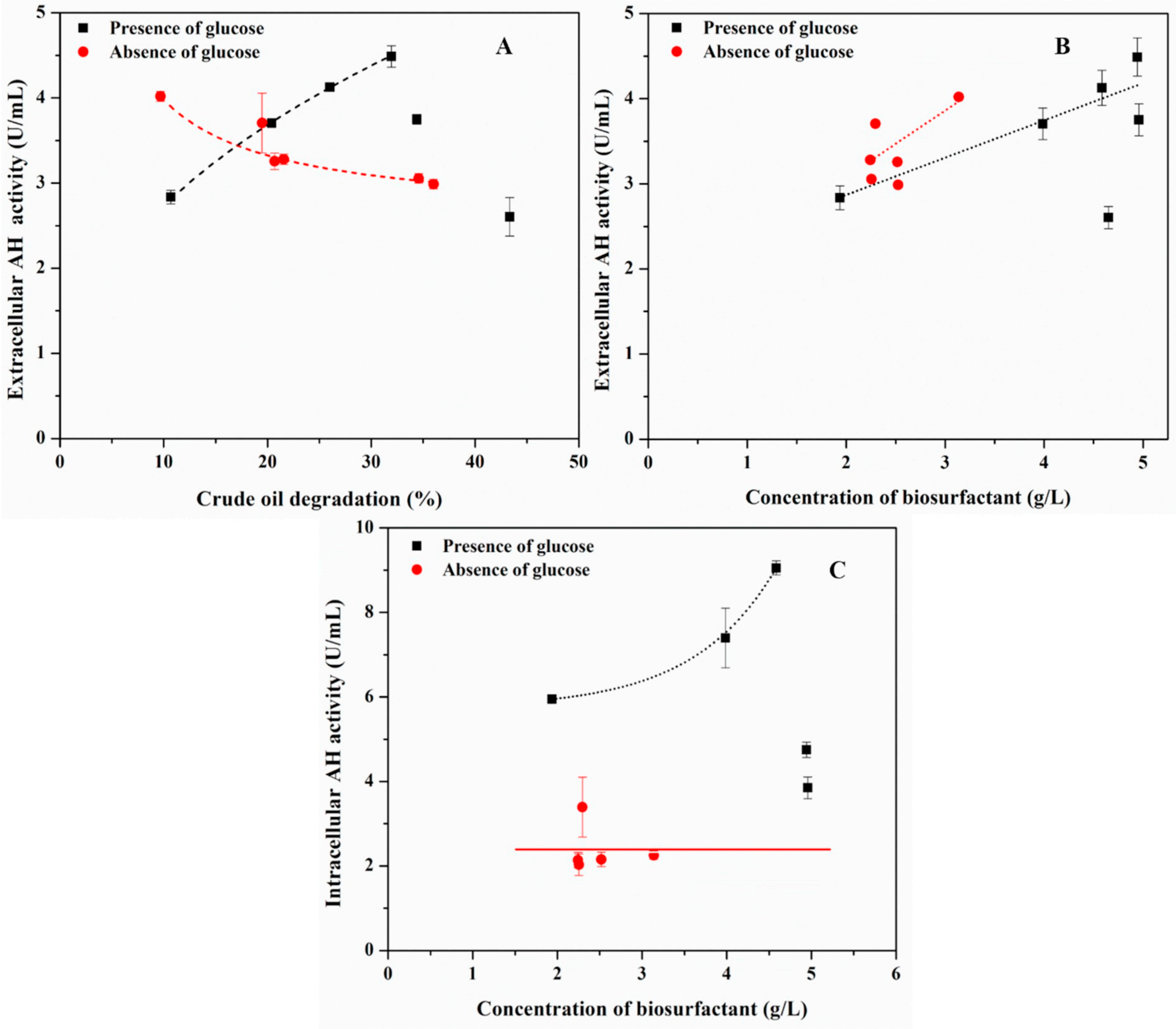

Fig. 7. Study of effect of (A) extracellular Alkane hydroxylase (eAH) activity on crude oil degradation; (B) intracellular Alkane hydroxylase (eAH) activity on biosurfactant production; (C) intracellular Alkane hydroxylase (eAH) activity on biosurfactant production.

3.4.1. *Relationship between alkane hydroxylase (AH) and oil degradation and biosurfactant production*

As a mean to understand the cause of oil degradation in the presence of glucose, we studied the enzyme activity of eAH hydroxylase, one of the key enzymes in the hydrocarbon degradation pathway. From Fig. 7 (A) it was inferred that eAH activity initially increased significantly ($p < 0.01$) with increase in the oil degradation ability, signifying that the oil degradation ability of *A. fabrum* is regulated by the activity of eAH. However, no significant change ($p > 0.12$) in the activity of eAH in the control (containing only crude oil), suggested that presence of glucose enhanced the activity of eAH. This could be explained in the terms of depletion of cofactor, NADH. Furthermore, eAH activity was also found to be responsible for the production of biosurfactant. It was observed that the AH activity increased with increase in biosurfactant as shown in Fig. 7(B). Summarily, presence of glucose showed overall enhancement in activity of eAH, which in turn uplifted the biosurfactant production and crude oil degradation in comparison to control.

Similarly, it was observed that the iAH activity got up-regulated by ~2 folds in the presence of glucose with crude oil (9.0 U/mL), than the control (3.4 U/mL). Interestingly, the expression of iAH enhanced the biosurfactant production, as shown in Fig. 7(C). However, the decrement in intracellular activity occurred as the cells reached their death phase. In the absence of glucose, biosurfactant production was found to be independent of iAH activity preassembly due to a lower activity of iAH. The iAH activity showed a similar trend for the degradation of crude oil in the presence and absence of glucose (data not shown). This revealed that both iAH and eAH regulated the biosurfactant production ability of the present strain.

4. Conclusions

In this study, *Agrobacterium fabrum* is investigated for its ability to produce biosurfactant and biodegrade spent crude oil. Screening experiments such as oil displacement assay, drop collapse test and E_{24} confirmed the biosurfactant production ability of the present bacterium. Further, optimization studies revealed that the mesophilic present strain showed the maximum biosurfactant production of 5.77 ± 0.3 g/L at pH 6, and 30 °C using glucose and yeast extract at a C:N ratio of 2:1. The present strain was also found to degrade crude oil by synthesizing AH. The microbe showed an enhanced biosurfactant production in the presence of co-substrate, glucose. Similarly, the bacterium showed an augmented crude oil biodegradation of 58 ± 5% within 6 days of incubation in the presence of glucose. The crude oil degradation was further confirmed by investigating the regulation of the AH enzyme activity. Glucose upon supplementing with crude oil uplifted the biomass density, biodegradative metabolism as well as the biosurfactant production, through enhancing the AH activity. Moreover, it was revealed that cofactor availability was the major driving force responsible for the overall metabolism. These outcomes suggest glucose rich feedstock can be used as C source to augment the overall biodegradation of spent crude oil. The obtained results postulate the suitability of the present strain in the field of enhanced oil recovery, bioremediation, biodegradation and other industrial applications.

Conflicts of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101322.

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