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ORIGINAL ARTICLE

Glycolipopeptide biosurfactant from *Bacillus pumilus* SG: physicochemical characterization, optimization, antibiofilm and antimicrobial activity evaluation

Sanaz Gharaie[1] · Mandana Ohadi[2] · Mehdi Hassanshahian[1] · Mojtaba Shakibaie[3,4] · Poorandokht Shahriary[1] · Hamid Forootanfar[3,4]

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Abstract

The *Bacillus pumilus* SG isolated from soil samples at the Persian Gulf was analyzed for its ability to produce biosurfactant. Various screening techniques were used for evaluating biosurfactant production and confirming biosurfactant presence in the culture supernatant. Most n-alkanes in the bacterial culture media were effectively degraded in the presence of biosurfactant acquired from the bacteria. The highest interfacial tension (IT) reduction (42 mN/m) was obtained at 24-h fermentation time (exponential phase) and did not change significantly afterwards. The glycolipid structure of the biosurfactant was revealed through NMR and FTIR spectroscopy analysis. Two-level factorial design was then applied for optimization of biosurfactant production, where a maximal reduction of culture broth IT (30 mN/m) acquired in the presence of crude oil (0.5%, v/v), $NaNO_3$ (1 g/L), yeast extract (1 g/L), peptone (2 g/L) and temperature of 25 °C. The produced biosurfactant that exhibited a critical micelle concentration of 0.1 mg/ml was thermally stable. The glycolipid biosurfactant also displayed significant antibacterial activities against both Gram-positive and Gram-negative bacteria. The maximum inhibition of glycolipids biosurfactant was found against *Acinetobacter* strains (zone of inhibition, 45 mm). In addition, antibiofilm activities with a 50–90% biofilm reduction percent were indicated by the glycolipid biosurfactant. In conclusion, the glycolipid biosurfactant produced by *B. pumilus* SG revealed a wide range of functional properties and was verified as a good candidate for biomedical application. In conclusion, the glycolipid biosurfactant produced by *B. pumilus* SG showed a wide range of functional properties in this study, and in the case of further in vivo studies, it can be investigated a good candidate for biomedical applications such as use against biofilm or in pharmaceutical formulations.

Keywords Glycolipopeptide biosurfactant · Optimization · *Bacillus pumilus* SG · Antimicrobial · Antibiofilm

Introduction

Global environmental issues have obliged the industry to replace chemical compounds with biodegradable counterparts in recent years (Jayasekara et al. 2022).

Sanaz Gharaie and Mandana Ohadi contributed equally as first author.

✉ Mehdi Hassanshahian
mshahi@uk.ac.ir

✉ Hamid Forootanfar
h_forootanfar@kmu.ac.ir

[1] Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

[2] Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran

[3] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran

[4] Pharmaceutical Sciences and Cosmetic Products Research Center, Kerman University of Medical Sciences, Kerman, Iran

Microorganisms produce amphiphilic compounds called biosurfactants, either released extracellularly or located on their cell surface (Srivastava et al. 2022). These molecules play a critical role in the survival of their producing microorganisms by interfering in microbe–host interactions and facilitating nutrient transport (Teng et al. 2022). Due to their low toxicity, biodegradability and natural origin, biosurfactants are viewed as "green" compounds (Kashif et al. 2022). Lipopeptides and glycolipids are the two main biosurfactant classes (Handore et al. 2022). Glycolipid biosurfactants have attracted great attention for environmental and industrial applications. They consist of a carbohydrate moiety linked to a fatty acid by a glycosidic bond (Ashby and Solaiman 2020). Besides the common surfactant properties, glycolipids also affect the cell membranes of several organisms and interact with their environment. Hence, these bioactive molecules can be considered promising antimicrobial and antibiofilm agents (Gharaei et al. 2022).

Despite the various advantages that biosurfactants provide for many sectors prior to the possibility of widespread exploitation for many biosurfactants, numerous difficulties still need to be looked at. These include manufacturing costs, achievable yields and safety concerns for some of the strains used for production (Sajadi Bami et al. 2022). Their high production costs are the biggest barrier to their use in many manufacturing processes (Ahmadi Borhanabadi et al. 2023). In most biotechnological processes, the cost of the raw materials is thought to be between 30 and 40 percent of the whole manufacturing cost. Therefore, it is preferable to use the inexpensive raw materials and optimizing production in order to decrease this cost (Goyal and Singh 2022; Jain et al. 2013). Low productivity (low yield) of biosurfactants is always one of the main bottlenecks for future commercial applications. So, scientists have attempted to use various techniques including culture condition optimization and microorganism gene manipulation to enhance the yield of biosurfactants (Gupta et al. 2022; Sajadi Bami et al. 2022). Estimating variables and their effects necessitates a higher number of experimental runs in a classic optimization design. In the optimization of culture conditions, among the best techniques used is the statistical design of experiments (Ohadi et al. 2017). The system response is assessed using statistical techniques after concurrent changes in independent parameters are studied (Pardhi et al. 2022).

Our hypothesis in this study was to optimize biosurfactant production conditions assisted by the marine strain *B. pumilus* SG bacterium isolated from the Persian Gulf. Also, determination of the optimal cultural conditions to maximize the production of the biosurfactant was performed. This study used FTIR and NMR spectroscopic techniques for analysis to examine the glycolipid biosurfactant's structure and explain some of its physicochemical characteristics. The study also assessed the antibiofilm and antimicrobial activities of the resulting biosurfactant.

Materials and methods

Microorganism and growth medium

Samples of oil-contaminated soil were collected from the Persian Gulf (26°150N; 54°150E) to isolate biosurfactant-producing bacteria. The Bushnell Haas (BH) medium was used with 1% (v/v) crude oil (National Iranian Oil Company) as the sole carbon source to isolate biosurfactant-producing bacteria. The BH medium consisted of $FeCl_3$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2$ (0.02 g), NH_4NO_3 (1 g) and KH_2PO_4 (1 g) per liter of distilled water. Pure cultures were attained by spreading serial culture dilutions on BH agar plates and incubating them at 37 °C for 24 h (Datta et al. 2018).

Confirming biosurfactant production

The preliminary screening methods of oil spreading (clear-zone forming ability) and hemolytic activity (blood cell hydrolysis in blood agar) were conducted to determine the production of biosurfactants (Arifiyanto et al. 2020). Complementary methods were used on bacterial isolates with higher activity in preliminary tests. The biosurfactant acquired from the bacteria was tested for interfacial tension (IT) activity, which is the known method commonly used in studies (Liu et al. 2022). Wilhelmy's plate technique was used to measure IT (at room temperature) in comparison to the negative control (culture media devoid of bacteria) using a tensiometer (krüss® tensiometer k100). Prior to each measurement, the device's accuracy in reading surface tension was tested using pure water and 100% ethanol (Ahmadi Borhanabadi et al. 2023; Gharaei et al. 2022).

Molecular identification of the selected isolate

Identification of the most potent biosurfactant-producing strain was carried out by 16S rDNA sequence analysis. The 16S rDNA sequencing was performed using universal primers of 27F (5′ AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGYTACCTTGTTACGACTT-3′). The obtained specific sequences were compared with known 16S rDNA using a basic local alignment search tool (BLAST) (Gharaei et al. 2022).

Strain growth profile

BH media supplemented by crude oil (1% v/v) was used to study the growth profile by analyzing changes in the cell

population of the selected strain in a fixed period (37 °C). The absorbance data were taken at 600 nm by UV–vis spectrophotometer (UV-1800, Shimadzu Co., Tokyo, Japan) for 60 h. Seed culture (1% v/v) was used for the growth experiments (Gharaei et al. 2022). Ensuring continuous production of biosurfactants was done by performing 24-h readings of IT alteration (Ohadi et al. 2017).

Statistical optimization of biosurfactant production

Production of biosurfactants was optimized with a two-level factorial design. In these experiments, physical factors such as temperature and efficient nutrients, (such as crude oil, yeast extract, $NaNO_3$ and peptone), were used for optimization. Table 1 presents the actual levels and the coded values of each parameter. Nineteen experiments were devised using factorial design in Design Expert 7.0.0 software (Stat-Ease, Inc., Minneapolis, MN, USA). The accuracy of attained results was ensured by comparing the median ratios of three repetitions. The obtained results then underwent analysis of variance (ANOVA) (Zargar et al. 2022). Validation and confirmation of the attained results were done by conducting three experiments with unspecified values.

Crude oil treatment and GC–MS (gas chromatography-mass spectrometry) analysis

After cultivation of the selected isolate in the optimized culture medium for 10 days, it was centrifuged at 10,000 rpm for 10 min to acquire the culture broth. Dichloromethane 10% v/v was used to extract the oil layer. After the addition of anhydrous Na_2SO_4 (3 g) to the solutions, they were incubated at room temperature overnight to remove residual water in the resulting solution. Whatman paper (No. 1) was used to filter the contents of the Erlenmeyer flask, and the filtered contents were kept at room temperature for the dichloromethane to evaporate. At the end, GC–MS apparatus was used to test for residual crude oil in the sample. The GC program was as follows: CP SIL 5 CB cp8740 (30 m × 0.32 mm × 0.1 m) Varian capillary column, FID detector, helium as the carrier gas, 100 °C initial temperature for 1 min, 300 °C transfer temperature, 280 °C injection temperature, 70 °C column storage temperature for 2 min followed by 7 min storage at 290 °C, and 290 °C final temperature and 3 ml/min flow rate. The GC peaks were compared to the control, and the decomposition percentage was calculated (Gharaei et al. 2022).

Table 1 Parameters used and level applied in the full factorial design

Variables	Symbol	Unit	Test levels of each parameter		
			− 1	0	+ 1
Yeast extract	(A)	g/l	1	1.75	2.5
Peptone	(B)	g/l	0.5	1.25	2
$NaNO_3$	(C)	g/l	0.2	0.6	1
Temperature	(D)	(°C)	25	31	37
Crude oil	(E)	(V/V %)	0.5	2.75	5

Obtained biosurfactant extraction

The extraction of biosurfactant produced in the optimized conditions was done by acid precipitation (6 M HCl) and an ethyl acetate and methanol (3:1 ratio) mixture as a solvent in solvent extraction methods (Ohadi et al. 2018). To separate the biomass, the culture was firstly centrifuged for 20 min at 8000 rpm at 4 °C. Using 6 M HCl, the cell-free supernatant was then adjusted to pH 2 and stored at 4 °C for precipitation overnight. After that, an equal volume of solvent was added to the supernatant and kept for 4 h in a shaker incubator at 180 rpm. The biosurfactant was recovered by removing the solvent using a rotary evaporator. The biosurfactant obtained using this method was stored in a dry cool place for later tests after being weighed (Ohadi et al. 2018).

Analytical methods

FTIR spectroscopy (Bruker Inc., Massachusetts, USA) was used to determine the new biosurfactant's structural features. The biosurfactant's chemical composition was examined by ^{1}H NMR and ^{13}C NMR spectroscopy (Singh and Tiwary 2016). About 10 mg of the biosurfactant was heated at 10 °C/min in an aluminum pan from 10 to 500 °C in air, and a simultaneous thermal analyzer device (BAHR STA 503 Hullhorst, Germany) was used to test its thermal features. The simultaneous thermal analysis serves as the measurement of changes in mass and temperature of a sample in the function of the temperature (Resolution: mass = 0.5 µg, temperature = 0.05 °C) (Khademolhosseini et al. 2019).

Critical micelle concentration (CMC) measurement

The Du Nouy ring method was used to measure the glycolipid biosurfactant's CMC using a tensiometer (Tensiometer K100, KRUSS, Hamburg, Germany). Concentrations between 0.05 and 0.4 mg/ml of the biosurfactant were made in distilled water to measure the IT in each sample at room temperature (Balan et al. 2019).

Antibacterial activity

Agar well diffusion assay

A panel of human pathogenic bacterial strains was acquired from Kerman Medical University to evaluate the antimicrobial

activity of the biosurfactant. *Streptococcus pneumoniae* and *Bacillus cereus* as Gram-positive strains and *Acinetobacter* sp., *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* as Gram-negative bacteria were the strains used in the investigation. Manivasagan et al.'s (2014) well-diffusion technique was used to assess the glycolipid biosurfactant's antibacterial activity. A suspension of 1.0×10^8 CFU/mL was reached by the overnight culture of the bacterial strains at 37 °C. Uniform dispersion of the bacterial inoculum on Mueller–Hinton agar plates was done using a sterile cotton swab. The agar surface was then punched with a sterile tip, to produce an aseptic hole of 6 mm in diameter. Afterward, the desired wells were filled with 10 µL glycolipid biosurfactant (12.5 µg/ml) and incubated at 37 °C for 12–24 h for the inhibition zone to be measured. The mean of inhibition areas in triplicate tests was reported (Zampolli et al. 2022).

Minimum inhibitory concentrations (MIC)

The glycolipid biosurfactant MIC against different organisms was determined by conducting a broth microdilution assay. In a 96-well microplate, 200 µL glycolipid biosurfactant in nutrient broth medium stock solution (50 mg/ml) aliquot was added to well No. 1 in each row. Then, the subsequent wells received 100 µL of nutrient broth medium to produce a serially diluted broth. Then, except for the negative control column, all wells received 1×10^6 CFU/ml bacterial suspension (10 µL) to reach a final 5×10^5 CFU/well inoculum size. After incubation for 24 h at 37 °C the growth in biosurfactant-free control wells was compared with the visible growth in the microdilution trays. The lowest glycolipid biosurfactant concentration at which there is no visible growth of the test strain is considered as the endpoint MIC (Athira et al. 2021).

Glycolipid biosurfactant antibiofilm activity

Five biofilm-forming bacteria (*Acinetobacter* sp., *E. coli*, *P. aeruginosa*, *S. pneumoniae* and *B. cereus*) were tested to measure the antibiofilm activity of the glycolipid biosurfactant using the 96-well microtiter plate method. After measurement of the bacterial MIC, the 10^6 CFU/ml bacterial suspension (100 µL) was separately added to each of the 96-well microplates wells. Then, 50 µl of biosurfactant solution (final concentration of 20 µg/ml) was added to the wells. The negative control well contained a sterile medium instead of the bacterial suspension. The formed biofilm was fixed after incubation at 37 °C for 48 h and then stained with crystal violet and methanol. Using the following formula, biofilm inhibition was calculated after reading the absorbance at 570 nm (Ohadi et al. 2020b):

$$\text{Biofilm inhibition (\%)} = \left[\left(OD_{570}\text{control} - OD_{570}\text{treated}\right)/OD_{570}\text{control}\right] \times 100$$

Results and discussion

Biosurfactant-producing strain screening and identification

Fifty strains of hydrocarbon-utilizing bacteria were screened after isolation from the collected soil samples for their biosurfactant-producing ability. The highest relative growth on the BH culture medium supplemented with crude oil, oil spreading tests (23 mm) and hemolytic activity (19 mm) among the screened strains was observed in the isolated bacterium designated as SG. Enrichment of cultures using hydrophobic substrates as the sole carbon source is very promising for the screening of biosurfactant-producing bacteria (Walter et al. 2010). The literature review showed that almost all biosurfactant-producing bacteria are isolated from hydrocarbon-contaminated soils (Ohadi et al. 2017; Sajadi Bami et al. 2022). As one indicator of biosurfactant production is growth on hydrophobic compounds, enrichment of cultures with hydrophobic substrates serves as an indirect screening method; however, this method does not always indicate biosurfactant production (Kumar et al. 2016). Thus, more specific screening tests such as oil displacement and hemolytic activity assays were used to investigate biosurfactant production (Ben Ayed et al. 2014). Almost all biosurfactant producers have been demonstrated by previous studies to have positive hemolytic activity, while there are hemolytic species that do not produce biosurfactants (Derguine-Mecheri et al. 2018). Thus, one preliminary test should be a hemolytic activity test (Kachrimanidou et al. 2022). The oil displacement method is based on the replacement of oil with a biosurfactant-containing solution, which enables the latter to spread in water. The hemolytic activity test has shown a comparatively clear association with qualitative oil displacement tests in this study (Płaza et al. 2006). Similar results were reported by Chittepu (2019) and (Sharma and Pandey 2020) who isolated *Bacillus pseudomycoides* OR 1 and *Bacillus subtilis* RSL-2, respectively as bacterial producing biosurfactants from soil samples. In order to determine the concentration of biosurfactant necessary to produce lysis zones, the results of the lipase and hemolysis activity experiments would be used. All 10 isolates in Chittepu (2019) investigation yielded positive findings from all screening techniques.

Comparison of strain SG's 16S rDNA gene sequence with the sequences in the GenBank database revealed a

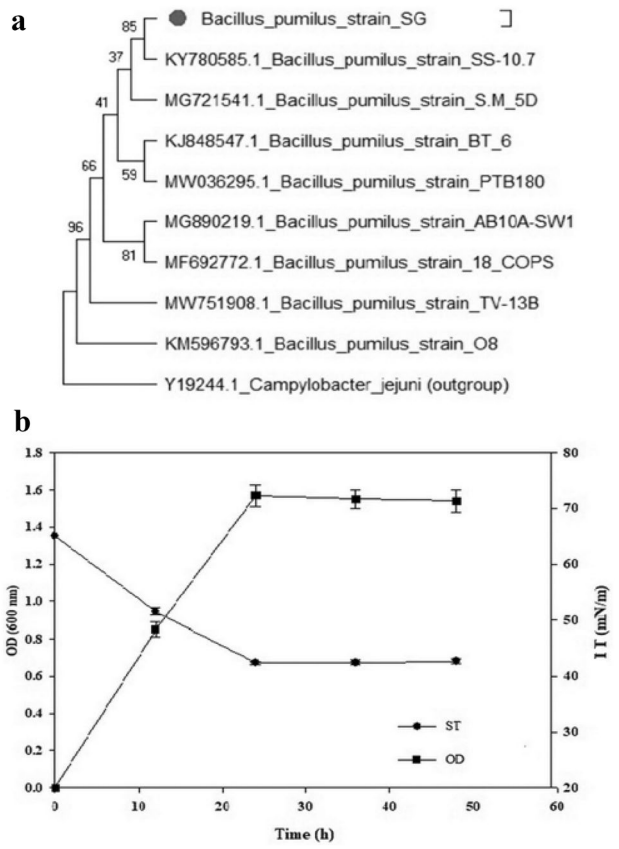

Fig. 1 **a** Phylogenic tree of the *B. pumilus* SG based on the 16S rDNA gene sequences. **b** Growth profile and IT alteration of *B. pumilus* SG

98% similarity with the *B. pumilus* sequence (Fig. 1a). This sequence was submitted to GeneBank of NCBI (accession number EMBL LK391615).

The biosurfactant production profile of *B. pumilus* SG

Through the assessment of IT changes and bacterial growth (OD_{600}), the biosurfactant production profile of *B. pumilus* SG was studied (Fig. 1b). The highest IT reduction (42 mN/m) was obtained at 24 h fermentation time (exponential phase) and not change significantly afterward (Fig. 1b). In this study, a direct relation was found between cell growth and biosurfactant production. Thus, cell growth and biosurfactant production happened simultaneously, especially in the exponential growth phase, decisively decreasing the IT. Similar growth-associated biosurfactant production was observed for the production of glycolipid biosurfactant by *Bacillus aryabhattai* strain ZDY2 (Yaraguppi et al. 2020). *Lactobacillus delbrueckii* was isolated and screened for glycopeptide biosurfactant production. Biosurfactants were stable in harsh condition and reduced surface tension (Goyal and Singh 2022). Biosurfactants may facilitate microorganism survival through the support of nutrient transport. The biosurfactant produced assists in cell growth and also improves the use of hydrocarbon, such as crude oil in this case (Kudakwashe et al. 2022).

Culture condition optimization

Regular two-level factorial design with 3 center points gave 19 different experimental runs. The results of the experimental runs were used to screen and select the most crucial factors affecting the production of the biosurfactant or their interactions with each other. The experiment design and responses are presented in Table 2. Based on the ANOVA test (Table 3), the statistically significant factors were combined to form a model constructed to describe the observed responses. The constructed model demonstrated that of the five studied factors, biosurfactant production was significantly affected by three factors—oil, temperature and peptone—and two interactions—yeast extract with $NaNo_3$ (AC) and yeast extract with peptone (AB). Temperature (P value = 0.0007) and crude oil (P value = 0.002) as the medium carbon sources showed the highest significant levels, 37% and 14% contribution, respectively. Fit statistics showed a reasonable agreement of the adjusted R^2 of 0.9697 with the predicted R^2 of 0.9839 with less than 0.2 difference (Fig. 2). The optimized medium contained crude oil (0.5%, v/v), $NaNO_3$ (1 g/L), yeast extract (1 g/L) and peptone (2 g/L) incubated at a temperature of 25 °C and was predicted to result of ST 33 mN/m with desirability of 0.95. The 3D surface plots in Fig. 3 show how biosurfactant production is affected by the combination of different factors. The studied nitrogen sources were classified into organic (yeast extract and peptone) and inorganic (sodium nitrate) sources (Shatila et al. 2020). The role of nitrogen sources in influencing biosurfactant production is quite evident. In the present study, it was found that the mixture of both organic and inorganic nitrogen sources showed a significant improvement in the production of biosurfactants (Ru et al. 2022). Similarly, Zhuet et al. (Zhao et al. 2021) showed that the use of a nitrogen source combination increased the biosurfactant production of *B. subtilis*. The carbon selection experiment used crude oil as the carbon source. Most hydrocarbonoclastic microbes use the hydrocarbon mixtures in crude oil as excellent energy and carbon sources (Ilori et al. 2005). The results of the present study were in line with those of Gharaei et al. (2022) who demonstrated that used as a carbon source, crude oil produced the lowest level of ST. Temperature is among the most influential factors in bioprocesses (Chand et al. 2020). The results of the present study demonstrated that growing the strain at 25 °C leads to

Table 2 Experimental design and results of the full factorial design

Run	Coded value of independent factor					IT (mN/m)
	A	B	C	D	E	Experimental
1	− 1	− 1	− 1	− 1	− 1	35.1
2	1	1	− 1	− 1	− 1	37.3
3	− 1	1	1	1	− 1	33.3
4	− 1	1	− 1	− 1	1	35.1
5	1	1	1	− 1	− 1	34.6
6	1	1	1	− 1	− 1	39.2
7	0	0	0	0	0	36.5
8	1	1	− 1	− 1	1	31.0
9	− 1	− 1	− 1	1	1	39.4
10	− 1	− 1	1	− 1	1	34.4
11	− 1	− 1	1	1	− 1	38.5
12	1	− 1	− 1	− 1	1	38.8
13	− 1	1	1	− 1	1	32.8
14	− 1	1	− 1	− 1	1	39.4
15	1	− 1	− 1	1	− 1	33.6
16	− 1	− 1	1	1	− 1	35.2
17	0	0	0	0	0	38.2
18	1	1	1	1	1	39.2
19	1	1	1	1	1	36.2

Table 3 Analysis of variance (ANOVA) for full factorial design

Source	Sum of squares	df	Mean square	*F* value	*p* value Prob > *F*	
Model	152.3	14	10.88	39.85	< 0.0056	Significant
B-peptone	7.90	1	7.90	28.91	0.0126	
C-$NaNO_3$	0.65	1	0.65	2.37	0.2211	
D-temp	57.30	1	57.30	209.83	0.0007	
E-oil	22.52	1	22.52	82.44	0.0028	
AB	5.88	1	5.88	21.53	0.0189	
AC	5.48	1	5.48	20.05	0.0208	
AD	0.60	1	0.60	2.20	0.234	
AE	0.83	1	0.83	3.03	0.180	
BC	1.19	1	1.19	4.35	0.128	
BD	3.78	1	3.78	13.08	0.036	
Residual	19.22	16	1.20			
Lack of fit	0.44	1	0.44	1.62	0.292	Not significant
Pure error	0.82	2	0.41			
Cor total	153.62	18				

a peak in biosurfactant activity (IT = 33 mN/m). Ram et al. (2019) reported similar findings, with a decrease in IT of the *Shewanella* sp. culture medium to 28.6 mN/m at temperatures ranging from 25 to 30 °C.

Biodegradation of crude oil by *B. pumilus* SG

Comparing the abiotic control culture's gas chromatograms made it possible to calculate the crude oil n-alkanes degradation percentage (Fig. 4). As can be seen in Fig. 4b, almost all n-alkanes in the crude oil was degraded by *B. pumilus* SG. The results of the present study are in contrast with Kiamarsi et al.'s (2019) findings. They found that n-alkanes with middle-chain hydrocarbons in soils contaminated with crude oil were degraded faster than those with long-chain hydrocarbons. They concluded that the hydrophobic nature of long carbon chain hydrocarbons delays their degradation, making rapid degradation difficult for

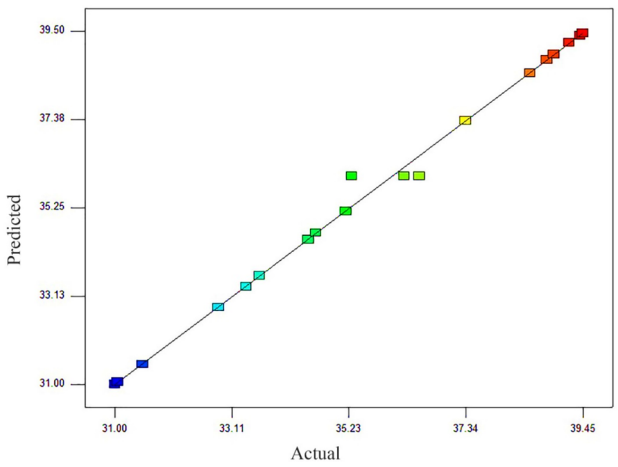

Fig. 2 The plot of actual vs. predicted surface tension by the experimental design

microbes. Tong Wang et al. (2022) reported that almost all n-alkanes in crude-oil-contaminated soil were effectively biodegraded in the presence of biosurfactant produced by *Bacillus* sp. XT-2. Literature review showed that incubation time and hydrophobic nature have a decisive role in the degradation of crude oil by bacteria that produce biosurfactants (Bayat et al. 2015). One of the apparent biosurfactant functions in microorganism that use hydrocarbon substrates for growth or to exist in oily substrates is to make these substrates available for them to metabolize (Sah et al. 2022). Other roles include motility in viscous environments or for the purpose of controlling the quorum sensing mechanisms of cells, which adjust gene expressions depending on cell density or on their surrounding environment (Balan et al. 2021; Yesankar et al. 2023).

Glycolipid biosurfactant's structural characterization

FTIR spectroscopy determined the main functional groups of the biosurfactant produced by *B. pumilus* SG (Fig. 5a).

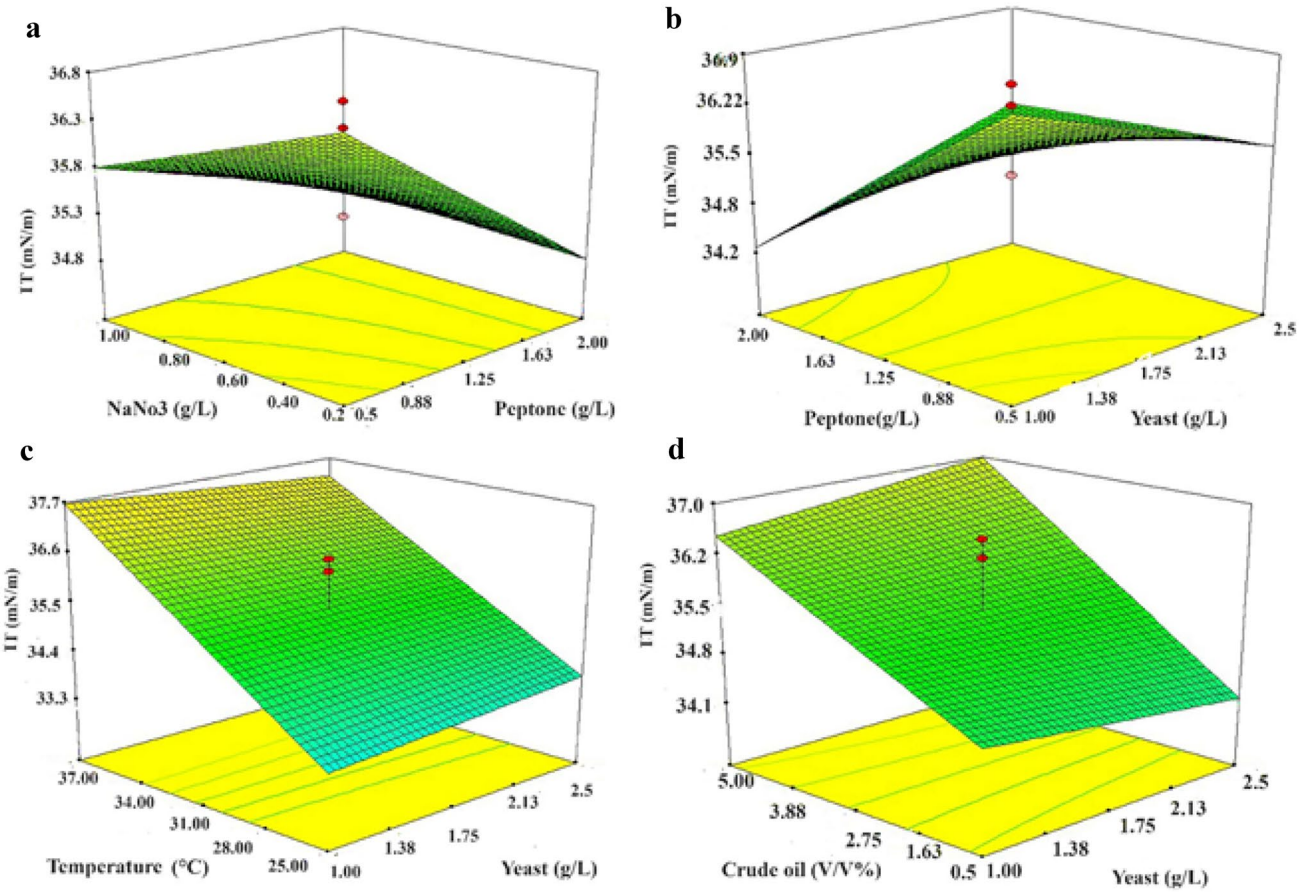

Fig. 3 The related 3D plots of **a** $NaNO_3$ concentration and peptone, **b** peptone and yeast extract concentration, **c** yeast extract concentration and temperature and **d** yeast extract and crude oil concentration versus IT as response

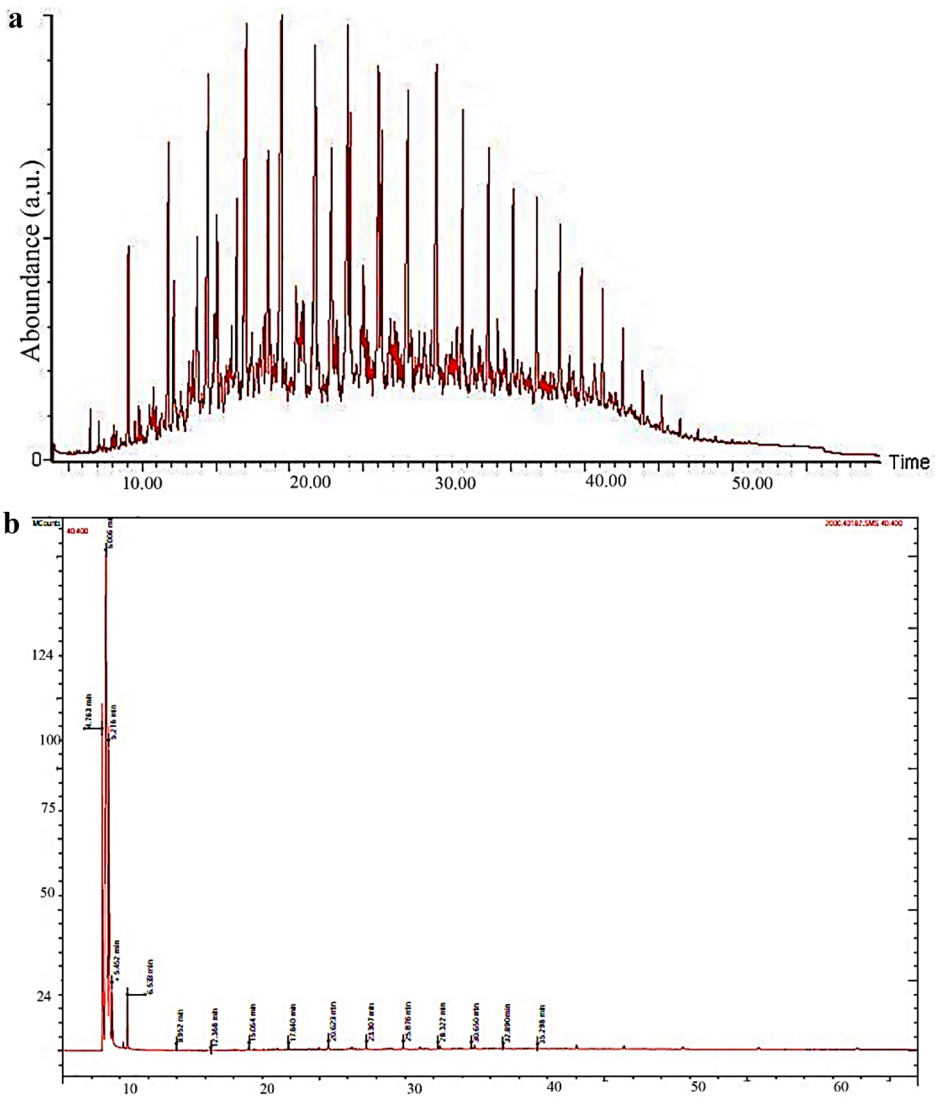

Fig. 4 **a** Gas chromatogram of negative control medium in the absence of *B. pumilus* SG. **b** the residual crude oil after cultivation of *B. pumilus* SG

The FTIR spectrum exhibited absorption from stretching vibrations of the hydroxyl group (–OH) at 3373 cm^{-1} (Balan et al. 2019). Hydrocarbon chain C-H stretching vibrations are the probable source of the strong adsorption peaks at 2928 cm^{-1}. A peak at 1730 cm^{-1} represented the carbonyl functional group (C=O). The molecule glycosidic bond (C–O–C) probably produced the characteristic 1038 cm^{-1} absorption band. This biosurfactant has a structure comprised of a sugar moiety with long hydrocarbon chains similar to the previously reported glycolipids (Khademolhosseini et al. 2019).

^{1}H NMR analysis was used to probe the structure of the glycolipid in the analyzed biosurfactant mixture (Fig. 5b). A chemical shift was identified in the ^{1}H NMR spectra at 7.2 ppm, confirming the existence of a carboxylic group. The chemical shift obtained in the 0.8–1.4 ppm range corresponded to a hydrocarbon chain and methyl group (–CH3). The 3.5 ppm signal indicated a sugar moiety in the biosurfactant composition (Fig. 5b) (Balan et al. 2019). The chemical shifts in the ^{13}CNMR analysis (Fig. 5c.) were at 38.91 ppm (CH_2), 77.08 ppm (C3′) and 61.55 ppm (C1) (Khademolhosseini et al. 2019). Similarity with the standard glycolipid biosurfactant types was apparent in all ^{13}C NMR and ^{1}H NMR spectra (Morita et al. 2008).

Stability and thermal properties analysis

An initial weight loss of 2.47% at 160 °C due to moisture and solvent removal was observed in the extracted biosurfactant's thermogravimetric analysis [Fig. 5d(1)]. At temperatures higher than 200 °C major weight loss is observed and as demonstrated by the TGA curve, the maximum change of weight, associated with rhamnose structure decomposition, takes place at 280 °C [Fig. 5d(1)]. The decomposition of the hydrocarbon chain accounts for the last major weight loss after 600 °C. Other weight losses are associated with the

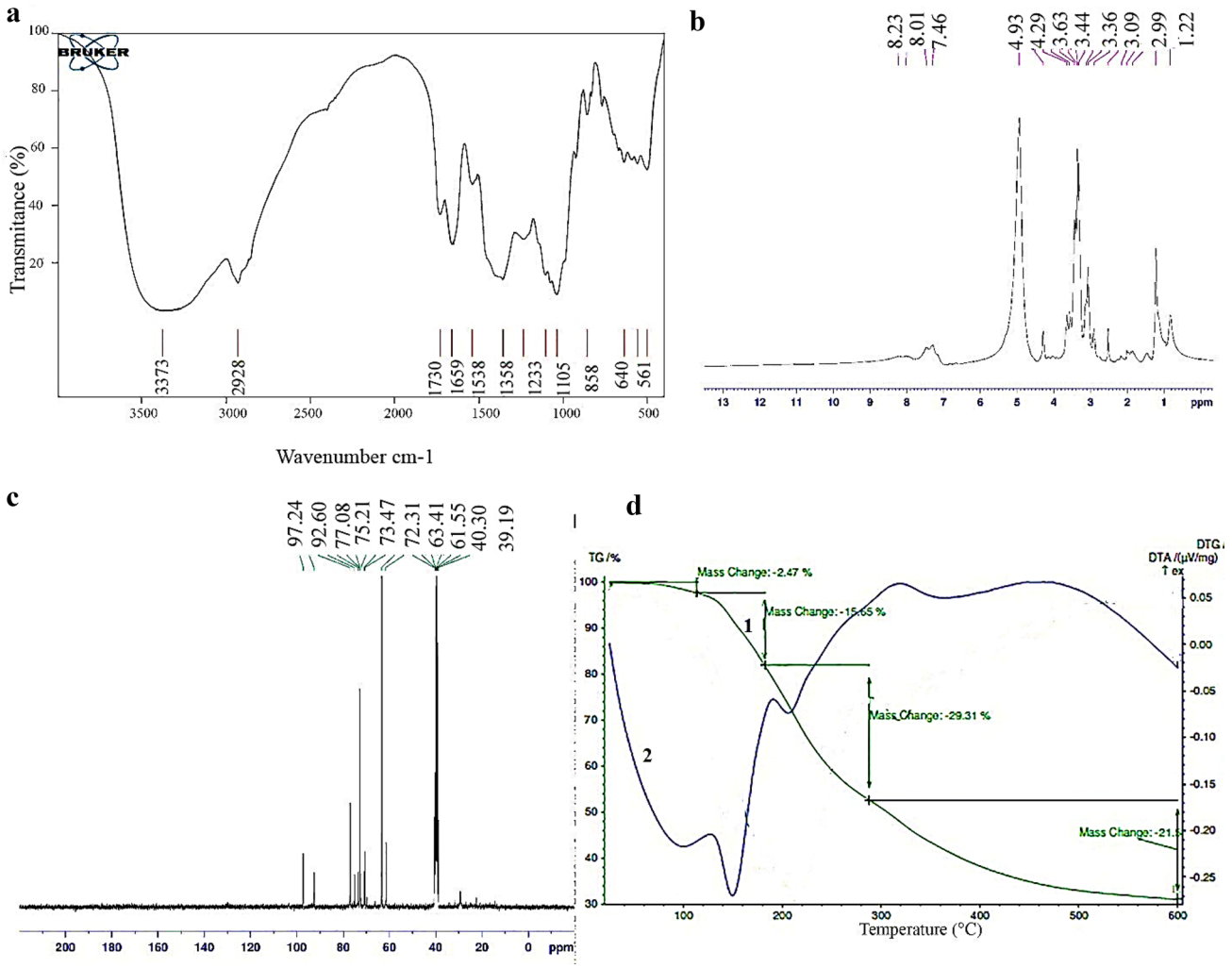

Fig. 5 **a** FTIR spectrum, **b** ^{1}HNMR profile, **c** ^{13}CNMR spectrum of glycolipid biosurfactant produced by *B. pumilus* SG. **d** Thermal gravimetric analysis (TGA, 1) and differential thermal analysis (DTA, 2) of glycolipid biosurfactant

decomposition of the biosurfactant's unstable components. Two heat reactions were observed at 120 °C and 360 °C, in the biosurfactant and an endothermic reaction was indicated at 145 °C, as presented in the DTA diagram [Fig. 5d(2)]. Thermostability has been demonstrated to play a crucial role in the industrial and environmental applications of biosurfactants in previous studies (Haloi and Medhi 2019).

Determining CMC

CMC measurement results showed a minimum IT of 26 mN/m. There was a rapid decrease in IT as the glycolipid biosurfactant concentration increased (Fig. 6a). For concentrations of biosurfactant above 0.1 mg/ml, no significant changes were observed in IT (Fig. 6a). This signifies the occurrence of CMC, a crucial feature of biosurfactants. The molecules' chemical nature influences the CMC concentration, where maximum reduction of IT is achieved. Smaller quantities of biosurfactants were necessary for reaching CMC to reflect biosurfactant efficiency and effectiveness (Fooladi et al. 2016). The CMC of 0.1 mg/ml for the *B. pumilus* SG glycolipid biosurfactant was in line with the CMC reports between 0.005 and 0.2 mg/ml in the literature (Kashif et al. 2022). For instance, a glycolipid biosurfactant produced by *Staphylococcus saprophyticus* SBPS-15 showed ST of 30.9 mN/m at a CMC of 0.02 mg/ml (Gharaei et al. 2022).

Glycolipid biosurfactant's antimicrobial activity

The inhibition area and the MIC values for the glycolipid biosurfactant produced by *B. pumilus* SG against selected bacterial strains were summarized in Table 4. It was found that the glycolipid biosurfactant inhibited the growth of both

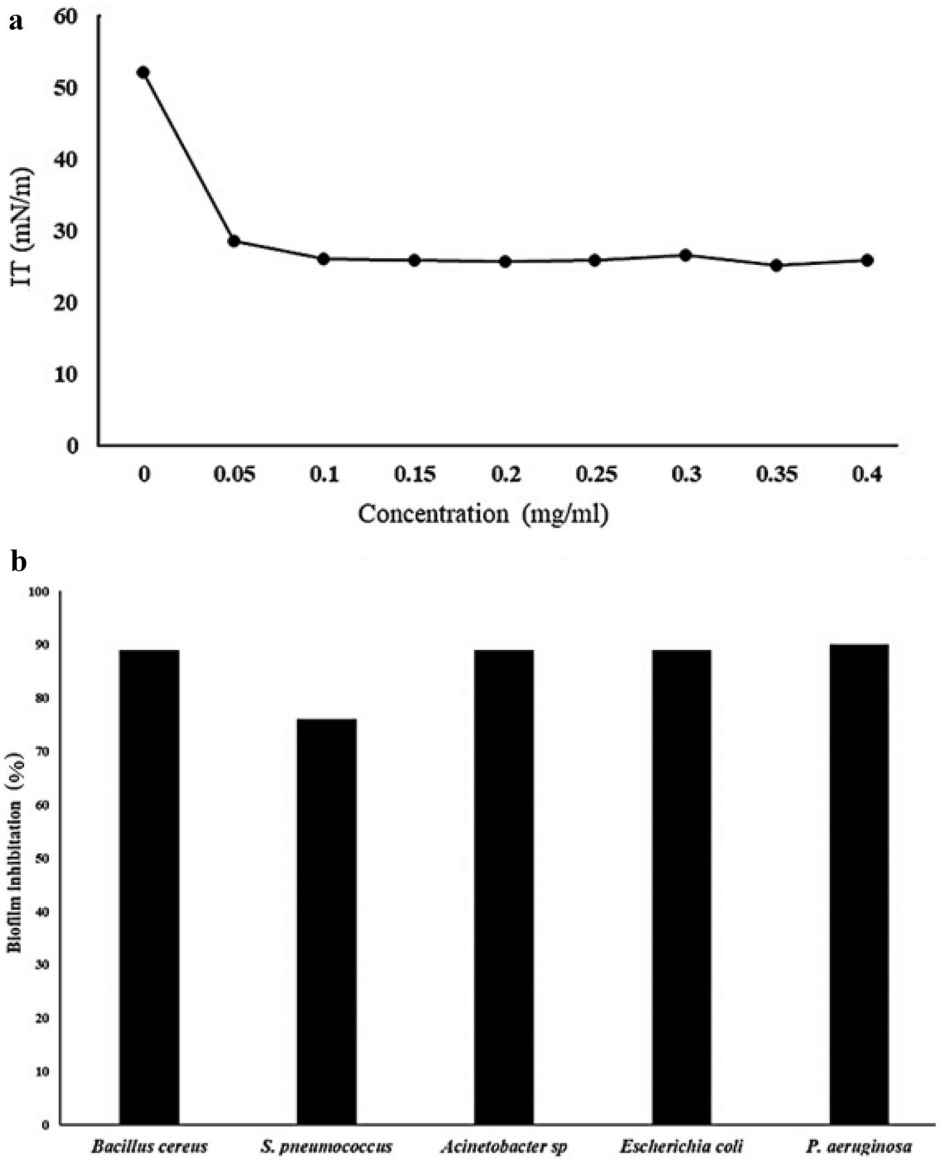

Fig. 6 **a** CMC evaluation of the glycolipid biosurfactant produced by *B. pumilus* SG. **b** Antibiofilm activity of the glycolipid biosurfactant produced by *B. pumilus* SG

Table 4 Antimicrobial activity of the glycolipid biosurfactant produced by *B. pumilus* SG. By agar well diffusion assay

Microorganisms (1.0×10^8 CFU/mL)	MIC (µg/ml)	Zone of inhibition (mm)
Acinetobacter sp.	12.5	45
S. pneumoniae	12.5	35
B. cereus	12.5	30
E. coli	12.5	32
P. aeruginosa	12.5	25
K. pneumoniae	12.5	24

Gram-positive and Gram-negative bacteria to varying inhibition, most probably due to the ability of the biosurfactant to permeabilize the cell membrane (Ohadi et al. 2020a). According to the literature survey, the amphiphilic properties of biosurfactants are the source of their antimicrobial effects because they modify the permeability of the cytoplasmic membrane by interacting with phospholipids (Naughton et al. 2019). The maximum inhibition (zone of inhibition, 45 mm) of glycolipids biosurfactant was found against *Acinetobacter* strains (Table 4). The Gram-negative pathogen *Acinetobacter* causes a range of infections and septicemia, leading to illnesses in humans (Mea et al. 2021).

Glycolipid biosurfactant antibiofilm activity

The antibiofilm activity of the glycolipid biosurfactant of *B. pumilus* SG was evaluated utilizing a biofilm inhibition assay (Fig. 6b). The glycolipid biosurfactant effectively reduced the biofilms produced by all the strains tested, generally showing higher than 50% inhibition percentage, with a maximum inhibition of 90% on *P. aeruginosa* strain. Glycolipid biosurfactants have shown evident antibiofilm activity against several drug-resistant pathogens. Paraszkiewicz et al. (2021) reported the antibiofilm characteristics of glycolipid biosurfactants and biological applications. The mechanism of biosurfactant to destroy the target organism maybe related to their amphiphilic structure. This study showed that due to their amphipathic nature, biosurfactants can enhance bacterial surface hydrophobicity and destabilize lipid packing. Eventually, the permeability of the cell membranes is modified, leading to a decline in microbial adhesion to solids. The glycolipid biosurfactant produced by *Burkholderia* sp. WYAT7 has been reported by Ashitha et al. (2020) to inhibit the biofilm production of *Staphylococcus aureus* (MTCC 1430). In another study, Petal et al. (2021) reported the antibiofilm properties of the glycolipid biosurfactant produced by *Lactobacillus rhamnosus*.

Conclusion

This study, identified, characterized the biosurfactant of the *B. pumilus* SG as a glycolipid and optimized its production. The GC–MS data demonstrated the ability of aliphatic hydrocarbons to eliminate in the presence of glycolipid biosurfactants. Due to its antibiofilm and antibacterial potential, the produced glycolipid biosurfactant can significantly reduce clinical bacterial pathogens. As pathogens are exhibiting resistance against regular antibiotics, the biomedical field can make use of these new findings, and the biosurfactant produced by *B. pumilus* SG can act as an potential alternative to antimicrobial and therapeutic agents.

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Declarations

Conflict of interest The authors declare no competing interests.

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Consent to participate I confirm that the final manuscript has been seen and approved by all the authors.

Consent to publish We hope that you will find our manuscript acceptable for publication in the above journal.

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