

# Production and partial characterization of biosurfactant produced by *Streptomyces* sp. R1

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**Abstract** The present study focused on developing a wild-type actinomycete isolate as a model for a non-pathogenic filamentous producer of biosurfactants. A total of 33 actinomycetes isolates were screened and their extracellular biosurfactants production was evaluated using olive oil as the main substrate. Out of 33 isolates, 32 showed positive results in the oil spreading technique (OST). All isolates showed good emulsification activity ( $E_{24}$ ) ranging from 84.1 to 95.8%. Based on OST and  $E_{24}$  values, isolate R1 was selected for further investigation in biosurfactant production in an agitated submerged fermentation. Phenotypic and genotypic analyses tentatively identified isolate R1 as a member of the *Streptomyces* genus. A submerged cultivation of *Streptomyces* sp. R1 was carried out in a 3-L stirred-tank bioreactor. The influence of impeller tip speed on volumetric oxygen transfer coefficient ( $k_La$ ), growth, cell morphology and biosurfactant production was observed. It was found that the maximum biosurfactant production, indicated by the lowest surface tension measurement ( $40.5 \pm 0.05$  dynes/cm) was obtained at highest  $k_La$  value ( $50.94 \text{ h}^{-1}$ ) regardless of agitation speed. The partially purified biosurfactant was obtained at a concentration of  $7.19 \text{ g L}^{-1}$ , characterized as a lipopeptide biosurfactant and was found to be stable over a wide range of temperature (20–121 °C), pH (2–12) and salinity [5–20% (w/v) of NaCl].

**Keywords** Actinomycetes · *Streptomyces* · Lipopeptide biosurfactant · Bioreactor · Fermentation · Morphology

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## Introduction

Biosurfactants are a diverse group of secondary metabolites with surface active properties of microbial origin. They have amphiphilic structures, having both hydrophobic and hydrophilic domains. The presence of these domains causes the molecules to line themselves at the water–oil or water–air interfaces, consequently reducing the interfacial or surface tensions in aqueous and oil mixtures [1]. Such characteristics make them good candidates in diverse industrial applications spanning petroleum, pharmaceutical, biomedical and food industrial processes [2]. In recent decades, the increasing awareness of eco-friendly processes has resulted in an increased interest in the production of biologically synthesized surfactants. Microbes have the ability to utilize various organic compounds. These microbes self-replicate under certain conditions, collectively producing copious amounts of biosurfactants in their vicinity. Microbial biosurfactants offer several advantages over synthetic surfactants, namely lower toxicity, inherent good biodegradability and ecological acceptability [3]. Hence, in many instances, biosurfactants are more preferred to synthetic surfactants.

Although biosurfactant production is more environmental friendly compared to that for its chemical alternatives, industrial production via fermentation is challenging due to the high production cost. As is the case with many fermentation products, downstream processing accounts for a major portion of the total biosurfactant production costs [4, 5]. Hence, reducing product recovery costs remains paramount in the effort to reduce overall production cost. The filamentous growth nature of actinomycetes can offer significant savings in product recovery, especially for extracellular microbial products since the separation of biomass from product typically become much easier.

Rhamnolipids are one of the best known biosurfactants which are produced generally by *Pseudomonas* sp., an opportunistic human pathogen [6]. This presents a concern, especially in large-scale production of rhamnolipids. Safety concerns limit the crude product use to controlled environmental applications rather than mass commercial production for distribution. In this respect, the actinomycetes can be an appropriate candidate for safer microbial producers of biosurfactants due to their accustomed non-pathogenicity to human.

*Streptomyces*, a Gram-positive filamentous bacterium, is the most dominant group among actinomycetes. This genus is often screened for secondary metabolite production and is an industrially important antibiotic producer [7]. Among the many secondary metabolites, *Streptomyces* has also been reported to produce biosurfactants. Table 1 shows a list of biosurfactant-producing *Streptomyces* spp. The first report was by Richter et al. [8], who found the extracellular hydrophobic peptide compound, streptofactin, from *Streptomyces tendae* Tu 901. Glycolipid biosurfactants have been produced by *Streptomyces* sp. ISP2-49E, *Streptomyces* sp. MAB36, *Streptomyces* sp. B3, *Streptomyces matensis* and *Streptomyces coelicoflavus* [6, 9–12]. These examples highlight the ability of filamentous bacteria as biosurfactant producers. However, there is no study to date focusing on improving biosurfactant production through bioprocessing strategies. Being a filamentous organism, the impeller tip speed is anticipated to be an important factor in biomass growth and consequently, metabolite production. The result of this study is the first to report the effect of Rushton turbine tip speed on extracellular biosurfactant production in a filamentous microorganism grown on a water-immiscible substrate.

The main focus of the present study is to use a wild-type actinomycete isolate as a model filamentous

non-pathogenic organism for biosurfactant production. The locally isolated actinomycetes were screened for extracellular biosurfactant production. The isolate showing the highest potential as a biosurfactant producer was then identified using morphological characteristics and 16S rRNA gene sequence analysis. The batch cultivation of actinomycete was done in a well-controlled stirred-tank bioreactor using palm oil as the sole carbon source. In batch cultivation, the influence of impeller tip speed on  $k_L a$ , growth, cell morphology and biosurfactant production was observed. The biosurfactant produced by this filamentous bacterium was then characterized.

## Materials and methods

### Microorganisms

A total of 33 isolates of actinomycetes isolated from oil-contaminated soil were used in this study. All isolates were obtained from oil-contaminated soil and water samples from Penang and Kedah, Malaysia. The isolates were maintained on starch casein agar (SCA) slant until further use [20].

### Screening of biosurfactant producers

Seven-day-old actinomycetes were cultivated into starch casein broth with 3% (v/v) olive oil as a biosurfactant inducer to screen the production of biosurfactant [13]. The culture was incubated for 7 days on a rotary shaker (B. Braun Certomat® R, Germany) agitated at 200 rpm at a temperature of 28 °C. After 7 days of incubation, cells were separated by centrifugation (Eppendorf Centrifuge 5424, Germany) at 8000×g for 15 min at 30 °C. Cell-free

**Table 1** List of biosurfactant-producing *Streptomyces* spp

No	<i>Streptomyces</i> sp.s	Biosurfactant	References
1	<i>Streptomyces tendae</i> Tue 901/8c	Streptofactin	[8]
2	<i>Streptomyces fradiae</i> S72	Biosurfactant	[13]
3	<i>Streptomyces</i> sp. VITDDK3	Biosurfactant	[7]
4	<i>Streptomyces gedanensis</i>	Biosurfactant	[14]
5	<i>Streptomyces rochei</i>	Biosurfactant	[15]
6	<i>Streptomyces</i> sp. B3	Biosurfactant (Glycolipid)	[10]
7	<i>Streptomyces</i> sp. MAB36	Biosurfactant (Glycolipid)	[9]
8	<i>Streptomyces</i> VITSSB2	Biosurfactant (Decanal)	[16]
9	<i>Streptomyces rochei</i> PN-18	Biosurfactant	[17]
10	<i>Streptomyces</i> sp. ISP2-49E	Biosurfactant (Rhamnolipid)	[6]
11	<i>Streptomyces coelicoflavus</i>	Biosurfactant (Rhamnolipid)	[12]
12	<i>Streptomyces matensis</i>	Biosurfactant (Rhamnolipid)	[11]
13	<i>Streptomyces</i> sp. S5	Biosurfactant	[18]
14	<i>Streptomyces</i> sp. V2	Biosurfactant	[19]

supernatant was analyzed for the presence of biosurfactant using oil spreading technique (OST) and emulsifying index ( $E_{24}$ ) tests.

### Oil spreading technique

Forty millilitre distilled water was added to a Petri dish. Then, 10  $\mu\text{L}$  of crude oil was added to the surface of the water. Ten-microlitre culture broth samples were added onto the centre of the oil film. The diameter of the clear zone on the oil surface was measured with a calliper rule and compared to the clear zone using the uninoculated medium as the control [21].

### Emulsification index ( $E_{24}$ )

Two millilitre of mineral engine oil was added to 2-mL cell-free culture broth. The solution was mixed thoroughly for 2 min on a vortex mixer and left to stand for 24 h. The  $E_{24}$  index is given as a ratio of the emulsified layer height (cm) to the total height of the liquid column (cm) expressed as a percentage [22].

### Identification and characterization of potential biosurfactant-producing actinomycete

Phenotypic characterization of the model biosurfactant producer was based on cell morphology. The selected biosurfactant-producing actinomycete was grown on SCA and incubated for 7 days at 28 °C. After 7 days of incubation, the isolate was identified based on macroscopic and microscopic morphological characteristics. Macroscopic characters such as size, shape, colour, the absence and presence of aerial mycelium and the extent of spore formation were observed on the SCA plates [23]. Microscopic features including fragmentation or non-fragmentation of substrate and aerial mycelium, the presence of sclerotia, and spore chain morphology were observed under light microscope (Olympus model BX41, Germany) using coverslip and slide culture technique. The spore surface was observed under scanning electron micrograph (SEM) (Leo, Supra 50VP, UK).

The genomic DNA of isolate was extracted using cetyltrimethylammonium bromide (CTAB) method [24]. PCR amplification of 16S rRNA gene sequence was done in DNA Engine™ Peltier Thermal Cycler Model PTC-100 (USA) using the primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGGTCWTCARCC-3'). The 16S rRNA gene sequence was amplified via PCR using an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 1 min at 95 °C, annealing at 54 °C for 1 min and primer extension at 72 °C for 1 min, a final extension at 72 °C for 10 min and cooled to 4 °C.

The PCR product was purified using a PCR purification kit (Qiagen, Germany). The purified PCR product was sent for sequencing to a service provider. The obtained partial 16S rRNA sequence was corrected manually and aligned using ClusterW Multiple alignment in BioEdit Sequence Alignment Editor version 7.0.5 Hall [25] to generate a consensus sequence. The consensus sequence of selected biosurfactant-producing actinomycete was then compared to those available in the GenBank database using Basic Local Alignment Search Tool to find the probable identity or nearest match of new sequence (<http://www.ncbi.nlm.nih.gov/>).

### Inoculum preparation and culture conditions

The inoculum was prepared by transferring a loopful of fresh single colonies of isolate from SCA into 50-mL nutrient broth. The inoculated medium was incubated for 48 h at 28 °C on a rotary shaker, agitated at 200 rpm (B. Braun Melsungen AG 886344/G, Germany). After 48 h of cultivation, 10% (v/v) of the 48-h-old seed culture (OD 0.2) was inoculated into 150 mL of production medium and incubated on a rotary shaker (B. Braun Melsungen AG 886344/G, Germany) at 28 °C, agitated at 200 rpm for 36 h. After 36 h of cultivation, the entire content of the flask was transferred into a stirred-tank bioreactor containing 1.5 L of fermentation medium. The fermentation medium was as follows: 6% (v/v) palm oil, 0.6% (w/v) yeast extract, 2% (v/v) Tween® 80, 0.7% (w/v) sodium chloride, 0.3 (g L<sup>-1</sup>) casein hydrolysate, 2.0 (g L<sup>-1</sup>) potassium nitrate, 2.0 (g L<sup>-1</sup>) potassium phosphate dibasic, 0.05 (g L<sup>-1</sup>) magnesium sulphate dehydrate, 0.01 (g L<sup>-1</sup>) ferrous sulphate dehydrate and 0.02 (g L<sup>-1</sup>) calcium carbonate. The initial pH of the medium was adjusted by adding 1 M NaOH or HCl to pH 6.

### Production of biosurfactant by *Streptomyces* sp. R1 in a benchtop stirred-tank bioreactor

Batch cultivations were conducted in a 3-L stirred-tank bioreactor with a working volume of 1.5 L equipped with a Rushton-type impeller (Bioflo 115, New Brunswick, USA). The reactor was equipped with a polarographic dissolved oxygen (DO) electrode (InPro6830/12/220, Mettler Toledo, Germany) and steam sterilisable pH probe (405-DPAS-SC-K8S/225, Mettler Toledo, Germany) to monitor the DO tension and pH in the culture medium, respectively. Operating parameters such as temperature, pH, DO tension, aeration and agitation were monitored and controlled by a computer using a program written in BioCommand OPC (New Brunswick Scientific Co., Inc., New Jersey). All cultivations were carried out at 28 °C and aerated at 0.5 vvm with compressed air. The pH was left uncontrolled during

the cultivation. Throughout the cultivation in the reactor, 30 mL samples of the culture were collected at 24-h intervals for analyses of biomass growth and biosurfactant production through measurement of surface tension.

The effect of impeller tip speeds on *Streptomyces* sp. R1 growth and its biosurfactant production were investigated by agitating the culture at different stirrer speeds (0.52, 1.05, 1.57 and 2.09 m s<sup>-1</sup>) corresponding to 200, 400, 600 and 800 impeller rpm.

### Determination of volumetric oxygen transfer coefficient ( $k_L a$ )

The determination of volumetric oxygen transfer coefficient ( $k_L a$ ) was conducted using the static gassing-out method [26]. The  $k_L a$  was evaluated in distilled water containing 6% (v/v) of palm oil with a DO probe. After calibrating the DO probe, the oxygen was stripped from the system by sparging with nitrogen gas. Then, air was introduced to the system at different stirrer speeds (0.52, 1.05, 1.57 and 2.09 m s<sup>-1</sup>) while aeration rate was held constant at 0.5 vvm. The increased DO tension was recorded every 30 s till reaching its saturation. The mass balance for dissolved oxygen is as follows:

$$\frac{dC_L}{dt} = k_L a (C^* - C_L) \quad (1)$$

$C_L$  represents DO tension in the broth at a specific time point,  $C^*$  means the saturated DO tension in the broth while  $\frac{dC_L}{dt}$  represents the DO change with time. The  $k_L a$  value (h<sup>-1</sup>) was calculated as the slope of  $\ln(C^* - C)$  versus time.

### Influence of impeller tip speed on morphology of *Streptomyces* sp. R1

The impact of impeller tip speeds on the morphology of *Streptomyces* sp. R1 was observed by taking 1 mL of sample at the end of the fermentation for every impeller tip speed. Two slides were prepared for every impeller tip speed. The slides were observed under compound light microscope (Olympus model BX41, Germany) at 4× magnification. At this magnification, ten images were captured for the morphological analysis. The diameter of pellet was measured using image analysis software CellB (Olympus, Germany).

## Analytical techniques

### Surface tension measurement

The surface tension of the cell-free culture broth was determined by the DuNouy ring method De Nevers and Grahn

[27] using a semi-automatic Du–Nouy Tensiometer, model 70535 (CSC Scientific Company, Inc, VA). This method measures the surface force between a liquid and air in liquid medium samples. Prior to measuring the surface tension of samples, the tensiometer was calibrated using distilled water and ethyl alcohol at 28 °C.

### Cell growth determination

In the interest of better measurement accuracy, especially for filamentous biomass grown in a hydrophobic substrate, the direct method of cell dry weight was chosen instead of an indirect method such as optical density. A known volume of a culture broth sample was filtered using a dry and pre-weighed filter paper (Whatman filter paper No. 1). The filtered sample was washed once with acetone and twice by distilled water to remove residual oil from cells. The sample was subsequently dried to a constant weight in an oven (UFE 600 Memmert, Germany) at 105 °C.

### Biosurfactant recovery

The biosurfactant was recovered from the cell-free broth by acid precipitation, followed by solvent extraction method [19]. The cell-free broth was acidified to pH 2 using 2 M HCl and incubated overnight at 4 °C for complete precipitation of the biosurfactant. The precipitate was then collected by centrifugation at 10,000×g, 4 °C for 30 min. The precipitate obtained was further purified three times using a mixture of chloroform:methanol (2:1 v/v) in a separatory funnel at 28 °C. The partially purified biosurfactant was concentrated using a rotary evaporator (EYELA Oil Bath OSB-2000, Japan), weighed and stored at -20 °C for further use.

### Biochemical composition of biosurfactant

The chemical composition of partially purified biosurfactant was determined using standard methods [9]. Carbohydrate content was determined by the phenol–sulfuric acid method Dubois et al. [28] using D-glucose as a standard. Protein content was determined according to the methods described by Lowry et al. [29], using bovine serum albumin as a standard and lipid content was determined using method adopted by Folch et al. [30].

### Effect of temperature, pH, and salinity on biosurfactant stability

The thermal stability of the biosurfactant at different temperatures was observed by heating the sample in a boiling water bath (20–100 °C) (WNB 22 Memmert, German) for 60 min. To study the stability of biosurfactant at 121 °C,

the extracted biosurfactant was autoclaved. All the samples were cooled at 28 °C prior to measurement of the surface tension.

The effect of the biosurfactant at different pH values was investigated by adjusting the pH of the extracted biosurfactant to pH 2, 5, 7, 10 and 12 using 1 M NaOH and 1 M HCl. The pH adjusted extracts were then subjected to surface tension measurements.

The effect of salinity on the biosurfactant stability was determined by manipulating NaCl concentration. The extracted biosurfactant was mixed evenly with the specific concentrations of NaCl [5, 10, 15 and 20% (w/v)] while keeping the biosurfactant concentration constant. The surface tension for each mixture was measured.

## Results and discussion

### Screening for biosurfactant-producing actinomycetes

A total of 33 isolates of actinomycetes have been screened for biosurfactant production in starch casein broth containing olive oil as the biosurfactant inducer. Olive oil was chosen as a sole carbon source in the screening process since it was proven in enhancing the production of biosurfactant by actinomycetes [31]. Table 2 summarizes the results of the screening experiment using two measurements; OST and  $E_{24}$ .

Out of the 33 isolates, 32 showed positive results in OST when compared to the control of uninoculated medium. These results highlight the reliability and sensitivity of this technique in detecting very low concentrations of biosurfactant. In a similar screening experiment, Youssef et al. [21] demonstrated that 16 strains that showed negative results in the drop collapse method, gave positive oil spreading result (0.5–0.9 cm), demonstrating the higher sensitivity of the oil spreading technique in detecting surfactants. The  $E_{24}$  test gave positive results for all isolates, with good  $E_{24}$  values ranging from 84.11 to 95.80%. Based on the OST and  $E_{24}$  values aforementioned (Table 2), isolate R1 was selected as the model biosurfactant producer for further studies.

### Identification and characterization of biosurfactant-producing actinomycete

The examination of the macroscopic characteristics for isolate R1 grown on SCA at 28 °C for 7 days showed that the outer surface of the colonies was perfectly round initially, but later developed aerial mycelium that appeared velvety white colour (Fig. 1a) whereas the substrate mycelium was pale brown (Fig. 1b). Isolate R1 showed concentric rings of colonies on the SCA plate as incubation time progressed.

This is one of the important diagnostic criteria for the genus *Streptomyces* [23]. Observation under the light microscope demonstrated that isolate R1 gave non-motile oval shape spores (Fig. 1c) and showed the smooth surface mycelia under scanning electron micrograph observation. On the basis of morphological characteristics, isolate R1 was presumptively assigned to the genus *Streptomyces*.

The 16S rRNA sequence data have been used in the modern *Streptomyces* identification system since they provide valuable information about streptomycetes systematic [32, 33]. Thus, further taxonomic characterization of selected biosurfactant-producing actinomycete was done by 16S rRNA gene sequence analysis. From the BLAST search, isolate R1 was identified as *Streptomyces* sp. R1 (KJ669362) with 92% similarity. Based on the consistency of results from morphological and molecular characterization, isolate R1 is likely a member of the genus of *Streptomyces*.

### Production of biosurfactant by *Streptomyces* sp. r1 in a stirred-tank bioreactor

#### *Influence of impeller tip speed on $k_La$ , growth and biosurfactant production*

As often highlighted, agitation or impeller tip speed is one of the important bioprocess parameters that provides effective oxygen transfer rate (OTR) during aerobic fermentation, thereby affecting the cell growth and its productivity. The volumetric oxygen transfer coefficient ( $k_La$ ) is often used to gauge OTR in the bioreactor system. It is important to have a good estimate of  $k_La$  value to ensure adequate transfer of oxygen in the bioreactor and for scaling up [26]. Therefore, the investigation on the influence of agitation speed on the  $k_La$  value, growth and biosurfactant production by *Streptomyces* sp. R1 in a batch cultivation was performed in this study.

Table 3 shows that as the speed of the impeller increased from 0.52 to 2.09 m s<sup>-1</sup>, the biomass density of *Streptomyces* sp. R1 also increased from 4.35 ± 0.01 to 8.47 ± 0.02 g L<sup>-1</sup>. This may be due to the fact that higher impeller tip speed facilitates oxygen transfer, consequently leading to better *Streptomyces* sp. R1 growth. However, the result presented here was contradictory to other reports. The increased agitation speed in cultivation of *Streptomyces clavuligerus*, *Streptomyces hygroscopicus*, and *Streptomyces flocculus* would result in a depletion in biomass density due to the shear stress that is introduced by the agitation intensity [34–36].

Although biomass density increased proportionally with stirrer speed, the highest biosurfactant production indicated by the lowest surface tension value for samples from each agitation did not follow a similar trend. It was



**Table 2** Screening of biosurfactant-producing actinomycetes using OST and  $E_{24}$ 

No.	Isolate	Diameter of clear zone (cm)	$E_{24}$ (%)
	Control	1.10±0.17	0.00±0.00
1	B1	4.40±0.17	90.40±5.71
2	B2	1.35±0.21	87.90±0.14
3	B3	1.27±0.12	86.84±3.72
4	B4	3.17±0.29	84.11±0.15
5	B5	1.17±0.15	90.00±4.71
6	I1	1.53±0.35	92.16±0.22
7	I2	3.70±0.14	87.25±0.35
8	I3	2.67±0.29	90.84±1.18
9	I4	1.90±0.14	90.63±4.42
10	I5	2.40±0.28	90.42±4.12
11	K1	1.25±0.07	89.18±0.41
12	K2	4.30±0.26	92.47±3.20
13	K3	2.47±0.15	88.05±0.07
14	K4	6.03±0.15	92.23±2.16
15	K5	2.53±0.06	89.24±0.33
16	K6	3.70±0.14	90.63±4.42
17	N1	4.10±0.14	85.17±0.77
18	P1	7.10±0.14	90.24±0.34
19	P2	6.46±0.24	93.46±3.60
20	P3	7.03±0.06	89.24±0.33
21	P4	5.65±0.07	91.95±4.32
22	P5	3.03±0.06	94.22±0.31
23	P6	6.15±0.21	88.74±1.04
24	P7	3.30±0.17	91.50±0.71
25	P8	3.07±0.12	89.69±1.12
26	P9	2.60±0.14	92.74±3.20
27	P12	0.97±0.06	88.94±2.79
28	P13	4.20±0.26	84.61±0.56
29	R1	7.45±0.07	95.80±0.28
30	R2	3.60±0.14	93.00±4.24
31	R3	5.95±0.21	85.36±0.50
32	R5	4.05±0.07	93.08±3.06
33	S1	6.35±0.07	84.26±1.32

found that the surface tension measurement correlated with  $k_L a$  value. In this study, the lowest surface tension measurement ( $40.50 \pm 0.50$  dynes  $\text{cm}^{-1}$ ) was obtained at highest  $k_L a$  value ( $50.94 \text{ h}^{-1}$ ) at  $1.57 \text{ m s}^{-1}$  stirrer speed (600 rpm). It seems that the higher  $k_L a$  value promoted better biosurfactant production by *Streptomyces* sp. R1 regardless of the agitation speed. This preliminary finding was supported by Ray [37], who published that the maximum biosurfactant production indicated by lowest surface tension reduction ( $22$  dynes  $\text{cm}^{-1}$ ) was attained at a high  $k_L a$  value ( $129.76 \text{ h}^{-1}$ ) in *Bacillus* sp. (m28) fermentation. In addition, Jokari et al. [38] also claimed that the high

biosurfactin production ( $0.0485 \text{ g}^{-1} \text{ L}^{-1} \text{ h}^{-1}$ ) by *Bacillus subtilis* ATCC 6633 was found at maximum OTR,  $0.01 \text{ mol}^{-1} \text{ L}^{-1} \text{ h}^{-1}$ .

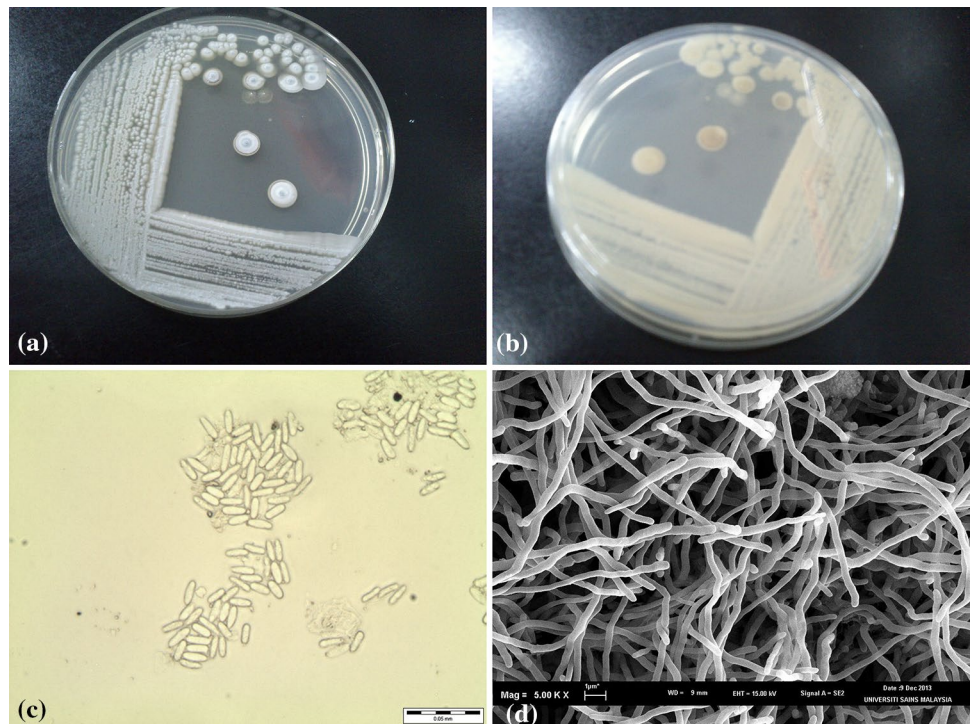
#### *Influence of impeller tip speed on morphological of Streptomyces sp. R1*

Unlike microbial fermentation, the cultivation of filamentous organisms is more complex due to the tendency of the culture to be non-homogeneous and greatly influenced by bioprocessing parameters such as impeller tip speed. These parameters affect the morphology of the cell, which consequently dictates the production of secondary metabolites. For instance, increasing impeller tip speed led to a lower production of avermectin B<sub>1a</sub> in *S. avermitilis* but a higher production of clavulanic acid in *S. clavuligerus* [36, 39]. Nevertheless, data on the effects of impeller tip speeds on the morphology of *Streptomyces* sp. and its biosurfactant production are yet to be reported. Hence, the influence of different impeller tip speed ( $0.52$ – $2.09 \text{ m s}^{-1}$ ) towards the cell morphology of *Streptomyces* sp. R1 and its correlation to the biosurfactant production were investigated in the present study (Table 4).

As is illustrated in Table 4 and Fig. 2, the impeller tip speeds gave a significant effect on the growth morphology of *Streptomyces* sp. R1 during batch cultivation. It was noted that at  $0.52 \text{ m s}^{-1}$  impeller tip speed, the pellets were uniform in shape in the form of 3–4 aggregates (Fig. 2a). This is probably because the mild agitation enabled the pellet to agglomerate [40]. In addition, the mean pellet diameter at this agitation speed was smaller ( $0.175 \text{ mm}$ ) than those seen at  $1.05 \text{ m s}^{-1}$  tip speed ( $0.366 \text{ mm}$ ), which are consistent with the results of biomass obtained in Table 3 ( $4.35 \pm 0.01 \text{ g L}^{-1}$  at  $0.52 \text{ m s}^{-1}$  and  $6.33 \pm 0.00 \text{ g L}^{-1}$  at  $1.05 \text{ m s}^{-1}$ ), possibly due to the low amount of dissolved oxygen,  $14.15 \text{ mmol O}_2 \text{ L}^{-1} \text{ h}^{-1}$  at  $0.52 \text{ m s}^{-1}$  compared to  $42.52 \text{ mmol O}_2 \text{ L}^{-1} \text{ h}^{-1}$  at  $1.05 \text{ m s}^{-1}$ , respectively, in the cultivation medium. The growth morphology of the culture at  $1.05 \text{ m s}^{-1}$  tip speed contained a mixture of big round pellet and filamentous form (Fig. 2b). However, increasing the speed of impeller from  $1.05$  to  $2.09 \text{ m s}^{-1}$  resulted in smaller mean pellet diameters ( $0.366$ – $0.094 \text{ mm}$ , respectively) with dispersed filamentous growth form (Fig. 2c, d). A similar finding was reported by Xia et al. [35], namely that the pellet diameter of *Streptomyces flocculus* decreases as the agitation speed increased from 400 to 800 rpm. This was probably due to the intense stirrer speed creating a higher shear stress, preventing filamentous organisms to grow in larger sized and dense pellets.

Upon closer inspection of Table 3 and Fig. 2, it appears that the maximum biosurfactant production, assumed as indicated by the lowest surface tension measurement ( $40.50 \pm 0.5$  dynes/cm) was obtained when the morphology

**Fig. 1** The morphology of isolate R1 after 7-day incubation on SCA at 28 °C. **a** Upper surface of isolate R1 showed white colour and **b** lower surface showed pale brown colour. Microscopic features of isolate R1 **(c)** oval-shaped spores (×40) **(d)** scanning electron micrograph showing smooth surface mycelium (6.27 ×K)



**Table 3** The  $k_La$  value, growth and biosurfactant production by *Streptomyces* sp. R1 at different impeller tip speed

Agitation rate (rpm)	Impeller tip speed (m s <sup>-1</sup> )	$k_La$ (h <sup>-1</sup> )	Maximum biomass (g L <sup>-1</sup> )	Surface tension (dynes/cm)
200	0.52	11.82	4.35 ± 0.01	47.17 ± 0.29
400	1.05	35.52	6.33 ± 0.00	56.80 ± 0.80
600	1.57	50.94	7.70 ± 0.03	40.50 ± 0.50
800	2.09	39.90	8.47 ± 0.02	50.20 ± 0.03

**Table 4** Effects of impeller tip speed on the morphology of *Streptomyces* sp. R1

Impeller tip speed (m s <sup>-1</sup> )	Morphology	Mean pellet diameter (mm)
0.52	Aggregate of 3–4 pellets of various size	0.175
1.05	Big rounded pellets and filamentous	0.366
1.57	Freely dispersed mycelia	0.106
2.09	Freely dispersed mycelia	0.094

of *Streptomyces* sp. R1 was in dispersed form of growth (Fig. 2c) at 1.57 m s<sup>-1</sup> tip speed. Consequently, it is tempting to speculate that the production of biosurfactant is also influenced by culture morphology. A similar finding was also reported by Ó Cléirigh [41] stating that the production of biosurfactant by *Streptomyces hygroscopicus* var. *geldanus* was not dependent on the biomass density but

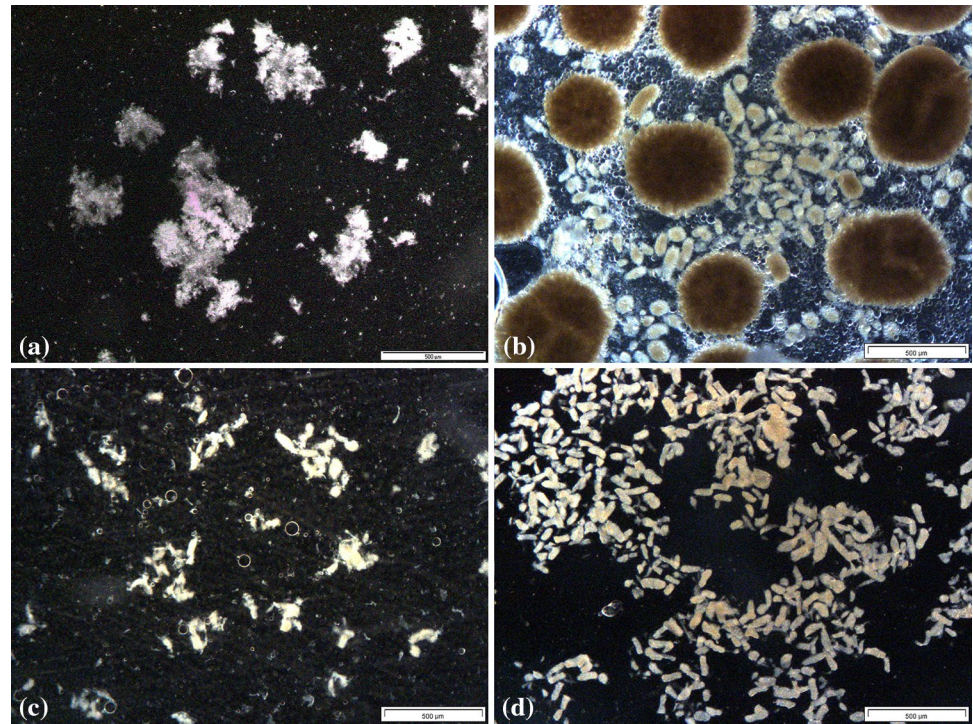
relied heavily on the culture morphology during submerged fermentation. The results clearly demonstrate that in the present study, the production of biosurfactant by *Streptomyces* sp. R1 may be a morphological auto-regulation mechanism.

### Biochemical composition of partially purified biosurfactant

The recovery techniques, namely acid precipitation, followed by the solvent extraction method used in this study, were successful in extracting biosurfactant from *Streptomyces* sp. R1 fermentation broth, giving a viscous light brown matter. These are the most common and effective recovery techniques that have been used by many researchers to extract biosurfactant from culture broth [42–44]. A similar result was obtained by Allada [12] where the yellowish, oily crude biosurfactant was successfully recovered from *Streptomyces Coelicoflavus* NBRC (15399<sup>T</sup>) fermentation broth using the same recovery techniques applied in this study. The concentration of the partially purified biosurfactant was 7.19 g L<sup>-1</sup>. This value is high compared to the concentration of biosurfactant reported by Bhuyan-Pawar et al. [19], 56.7 mg L<sup>-1</sup> from *Streptomyces* sp. V2. The chemical characterization revealed that the partially purified biosurfactant produced was of a lipopeptide nature, primarily consisting of lipid with the relative percent of 80.5% (w/w) and 11.11% (w/w) protein. Most studies reported that the biosurfactant produced by the genus *Streptomyces* sp. is



**Fig. 2** Pellet morphology of *Streptomyces* sp. R1 as a function of impeller tip speed under light microscope. **a**  $0.52 \text{ m s}^{-1}$ ; **b**  $1.05 \text{ m s}^{-1}$ ; **c**  $1.57 \text{ m s}^{-1}$ ; **d**  $2.09 \text{ m s}^{-1}$

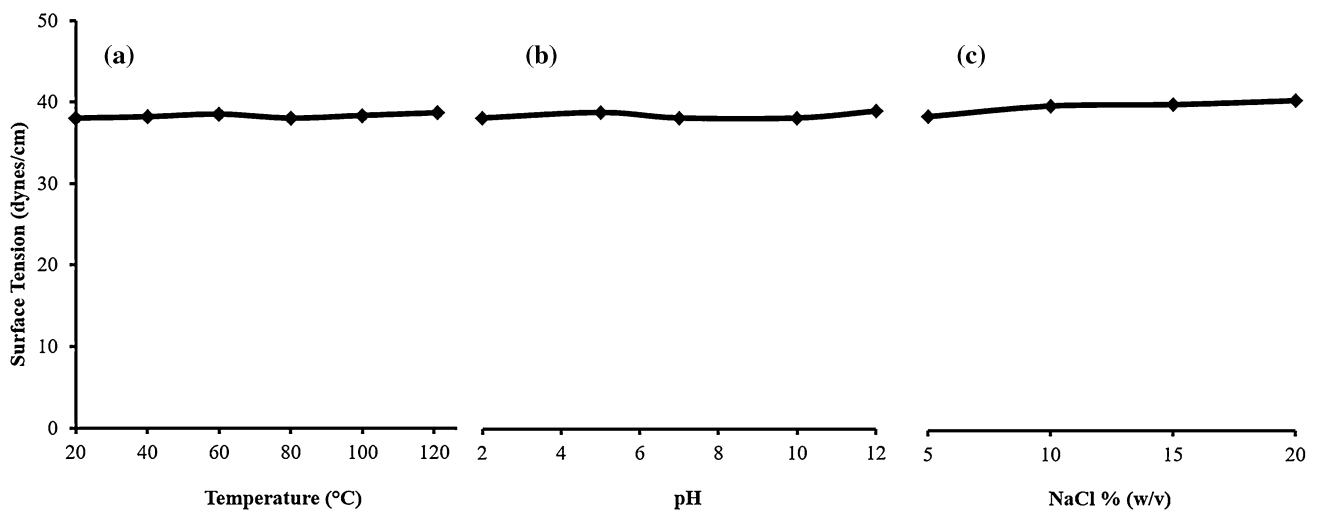


glycolipid [6, 9–12]. This is the first report of a lipopeptide biosurfactant produced by genus *Streptomyces*.

### Effect of temperature, pH, and salinity on biosurfactant stability

Figure 3 shows the effect of temperature, pH, and salinity on the stability of glycolipid biosurfactant produced by *Streptomyces* sp. R1. Figure 3a shows that the biosurfactant produced by *Streptomyces* sp. R1 was thermostable. Heating glycolipid biosurfactant from 20 to 121 °C

caused no significant effect on the biosurfactant performance indicated by the surface tension measurements ( $38.67 \pm 0.29 \text{ dynes/cm}$ ). A similar finding was reported by Khopade et al. [10] which claimed that the surface tension of glycolipid biosurfactant produced by *Streptomyces* sp. B3 was stable after heating at 100 °C. This meant that the biosurfactant produced can potentially be applied in food, pharmaceutical and cosmetic industries where heating to attain sterility is a common practice [45], pending safety considerations.



**Fig. 3** Effect of **a** temperature, **b** pH, and **c** salinity on the stability of glycolipid biosurfactant produced by *Streptomyces* sp. R1



Figure 3b shows the effect of different pHs within the range of 2–12 on the surface tension measurement of the glycolipid biosurfactant. The surface tension measurements ( $38.83 \pm 0.28$  dynes/cm) of the glycolipid biosurfactant remained relatively stable over a wide range of pH tested. The ability of the glycolipid biosurfactant to withstand a wide range of pH promotes its application in extreme conditions such as in acidophilic and alkalophilic environments [46]. Similar to temperature and pH, the surface tension of the glycolipid biosurfactant was unaffected with increased salinity from 5 to 20% (w/v) (Fig. 3c). This characteristic gives the biosurfactant an advantage to be used in the extreme salinity condition that is common in many oil reservoirs for MEOR applications and bioremediation of spills in the marine environment [45].

## Conclusion

The present study showed that, out of the 33 confirmed actinomycete isolates, 32 were able to produce extracellular biosurfactant when cultured in starch casein broth in the presence of olive oil as the biosurfactant inducer. From these isolates, isolate R1, characterized and identified as the genus *Streptomyces* based on morphological and molecular characterization was selected as a model biosurfactant producer for studies in a stirred-tank bioreactor. The results of batch cultivation in the stirred-tank bioreactor demonstrated that the production of biosurfactant was dependent on  $k_{La}$  value and cell morphology rather than biomass density alone. The partial characterization studies presented here suggests that the biosurfactant produced by *Streptomyces* sp. R1 belongs to lipopeptide type biosurfactants. This is the first report on lipopeptide biosurfactant produced by genus *Streptomyces*. Interestingly, this compound was stable across a wide range of pH, temperature, and salinity. Thus, *Streptomyces* sp. R1 presents an alternative to the current biosurfactant producers to produce viable industrial biosurfactant production.

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