Isolation of a novel thermophilic bacterium capable of producing high-yield bioemulsifier and its kinetic modelling aspects along with proposed metabolic pathway

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Abstract

Bioemulsifiers form stable emulsions and lower surface tension between two phases with potent anti-microbial activities. Some applications of bioemulsifier are performed at high temperatures and hence production of bioemulsifiers that are stable at high temperature is required. This study aimed at the production of bioemulsifier by an unexplored bacterial strain isolated from a local hot spring. The parameters tested for bioemulsifier production (emulsification ability, surface tension measurement and product formation) showed that 24 h is the optimal time for the production of bioemulsifier by strain S3 with yield of 1.4 g/l. The logistic growth curve of bacterial strain was analysed and kinetic constants for substrate utilisation and product formation were determined by Luedeking-Piret kinetic models. The bacterial strain S3 was Gram-positive and was classified as a strain of *Brevibacillus borstelensis*. The specific growth rate of the organism was 0.0096 h^{-1} with the kinetic rate constants as 11.246 (γ) and 10.626 (δ) for Luedeking-Piret substrate and 3.8423 (α) and -1.9075 (β) for Luedeking-Piret product. Knowledge of these values will help in estimating the substrate utilisation or bioemulsifier formed at any time point. These studies will also help in understanding internal metabolic fluxes hence rigorous analysis of metabolic pathway of bioemulsan is also performed in this study.

Keywords Thermophiles · *Brevibacillus* · Bioemulsifier · Kinetic model fitting

Introduction

Thermophilic microorganisms are economically important as they produce thermostable products which are suitable for various biotechnological processes requiring high temperature. The advantages of using thermophilic products as compared to mesophiles are the reduced risk of contamination by mesophiles, higher reaction yields due to increased diffusion of substrates and products, increased solubility of reaction substrates and decreased viscosity of the medium [1]. Thermophilic products offer the advantages of being thermostable, resistant to denaturing agents, tolerant to high solute concentrations and longer life [2]. The products from

thermophiles have applications in industries, and biomedical and agricultural sectors. Other than enzymes, the biomolecules produced by thermophiles which are of practical applications are lipids, proteins and small molecules.

One of the biomolecules produced by thermophiles, which is of great interest in biomedical and environmental sectors, is surface active agents. Surface active agents lower the surface tension between two phases. These phases can either be two liquids or a liquid and a solid. They are amphiphilic molecules, that is, these molecules consist of both hydrophobic (lipophilic) and hydrophilic groups. They are used as cleaning, dispersing, wetting, foaming and emulsifying agents and have applications in paints, shampoos, toothpaste, inks, adhesives, detergent, etc. Chemically, surface active agents are produced from petrochemicals which are non-renewable and recalcitrant. Microbial surface active agents, on the other hand, are synthesised by biodegradable and renewable feedstock, have low toxicity and are digestible and biocompatible. Microorganisms produce these compounds to increase the absorption of hydrophobic substrates from nutrients for their growth [3].

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Microbial surface active biomolecules can be categorised into low molecular weight biosurfactants and high molecular weight bioemulsifiers. Low molecular weight biosurfactants reduce surface and interfacial tension between two different phases and have emulsifying properties. They consist of amino acids, sugars, fatty acids and carboxylic acid as functional groups. Bioemulsifiers, on the other hand, are high molecular weight compounds that are composed of proteins, lipoproteins, polysaccharides, lipopolysaccharides or a mixture of these compounds. They have the ability to emulsify two immiscible liquids but do not reduce the surface tension between them. These compounds increase surface area between the two phases and allow bioavailability of hydrophobic water-insoluble substrates [4]. Bioemulsifiers help in the attachment of bacteria to surfaces which further lead to biofilm development. In microorganisms, they also have roles in microbial antagonism, microbial pathogenesis and quorum sensing [5].

Bioemulsifiers have the ability to form very stable emulsions and dispersions that do not mix together. They remain attached to the interfaces of droplets and can re-emulsify even by the addition or replacement of fresh solvent without getting diluted. Because of these advantages, bioemulsifiers are preferred over biosurfactants for their applications in cosmetics and food industries [6]. The bioemulsifiers are classified into four main groups depending on their chemical structure. Glycolipids, such as rhamnose, sophorose and trehalose, are bioemulsifiers containing carbohydrates complexed with lipid or fatty acids. This type of bioemulsifier is produced by *Pseudomonas aeruginosa*, *Aspergillus niger*, *Geotrichum* sp., *Trichosporon montevidense*, *T. mycotoxinivorans* and *Yarrowia lipolytica* [7, 8]. Rhamnolipids, produced by *P. aeruginosa* are made up of one or two rhamnose sugar moieties connected to β -hydroxy fatty acid [9]. Bioemulsifiers containing amino acids are another class of bioemulsifiers produced by *Methanobacterium thermoautotrophicum*, *Methylobacterium* sp. and *Solibacillus silvestris*. An example of this type of bioemulsifier is surfactin which consists of a seven amino acid ring linked to 3-hydroxy-13-methyl tetradecanoic acid [10]. A complex of polysaccharide with lipid is another type of bioemulsifier and is produced by *Acinetobacter*, *Pseudomonas*, *Streptomyces*, *Candida lipolytica* and *Saccharomyces cerevisiae* [11]. Some bioemulsifiers consist of carbohydrates linked to proteins and are produced by *A. calcoaceticus*, *Antarctobacter* sp., *Bacillus subtilis*, *Halomonas* sp., *Kluyveromyces marxianus* and *Pseudoalteromonas* sp. [5, 10].

Bioemulsifiers have diverse applications in cosmetics, biomedicine, biopharmaceutical, recovery of crude oil and environmental bioremediation and protection [12, 13]. Several studies have been reported for the production of bioemulsifiers from mesophilic bacteria [11, 14–18]. Many of the industrial processes utilizing bioemulsifiers require their

operation at high temperatures and hence there is a need for bioemulsifiers that can tolerate high temperatures and are active at these temperatures [13]. One method for the production of heat-stable bioemulsifier is by employing thermophiles. Few reports have suggested the isolation and use of thermophilic bacteria for bioemulsifier production from petroleum reservoirs or oil-contaminated soil. The earliest report was the study on *Methanobacterium thermoautotrophicum* for its emulsifying properties [19]. The bioemulsifier produced by this organism was effective even at a temperature of 80 °C. Another bioemulsifier-producing thermophilic bacteria isolated from oil-contaminated soil is *Geobacillus pallidus* XS2 and XS3 [20]. The bioemulsifier production was carried out at 60 °C and the organisms were effective in the removal of polycyclic aromatic hydrocarbons and crude oil. The bioemulsifier was stable over a broad range of temperature and salinity. *Bacillus licheniformis* ACO1, isolated from a petroleum reservoir, was capable of producing bioemulsifier at saline concentrations and temperature of 4% (w/v) and 45 °C, respectively, without the degradation of hydrocarbons [21].

The objective of this work was to study the production of bioemulsifier by a novel bacterial strain isolated from a local hot spring using glucose as the carbon source. The kinetics of the microorganism with respect to bioemulsifier production and substrate utilisation were studied by Luedeking-Piret kinetic models. Knowledge of the kinetic studies of the thermophilic strain will help to design processes utilizing agricultural wastes as inexpensive raw material for the production of bioemulsifier. Hence, a metabolic pathway for the production of bioemulsifier is also proposed in this work using the above-mentioned kinetic aspects. The monomeric units of bioemulsifier (trisaccharides of D-galactosamine, D-galactosaminouronic acid and a dideoxydiaminohexose) are linked by amide and ester bonds to fatty acids. The synthesis of emulsan (a type of bioemulsifier) in *Acinetobacter calcoaceticus* starts with the uptake of glucose within the bacterial cell membrane (Fig. 1). Glucose is converted to fructose 6-phosphate in a couple of enzyme-catalysed reactions. The molecule then initiates hexosamine biosynthetic pathway instead of entering in the glycolysis cycle. The reactions of hexosamine biosynthetic pathway lead to the production of UDP-N-acetylglucosamine in a series of reactions catalysed by enzymes. The UDP-N-acetylglucosamine is converted to the three monosaccharides required for the formation of a monomeric unit of emulsan with the help of *wee* gene clusters. *gale* (UDP-glucose 4-isomerase) interconverts UDP-N-acetylglucosamine to UDP-N-acetylglactosamine. *Wee K* catalyses the synthesis of UDP-4-keto 6-deoxy D-glucosamine from N-acetylglucosamine which is further converted to diamino 2,4-diamino-6-deoxy-D-glucosamine by *Wee J*. UDP-N-acetylglucosamine is converted to UDP-N-acetylglactosaminouronic acid by enzymes *Wee A*, *Wee B*,

Fig. 1 The metabolic pathway for the production of bioemulsan, a bioemulsifier

Wee E and Wee F. The three trisaccharides, UDP-N-acetylglactosamine, diamino 2,4-diamino-6-deoxy-D-glucosamine and UDP-N-acetylglactosaminouronic acid are acetylated and translocated to periplasm. The monomer units are polymerised and the polymer, emulsan, is transported to the bacterial outer membrane from where it is excreted outside with the help of Wee H, Wee D, Wee G, Wee C, Wee I, Wzx, Wzy and Wza [22, 23]. The main idea

behind the study of metabolic pathway of bioemulsan is to understand the relation between glucose consumption and metabolic flux rates and to observe the changes in the intracellular fluxes with respect to variation in substrates. This pathway can be explored further to elucidate metabolic fluxes at different time points by metabolic flux analysis and to develop dynamic models for bioemulsifiers.

Materials and methods

Isolation of thermophilic bacteria from a local hot spring

Water sample was collected from a hot spring in Ambikapur district of Chhattisgarh, India. The temperature of the hot spring was 92 °C. Pre-sterilised 250-ml glass bottles were used for sample collection and were stored at 4 °C until use. The sample was serially diluted in sterile distilled water, spread plated on nutrient agar (peptone, 5 g/l; NaCl, 5 g/l; beef extract, 1.5 g/l; yeast extract, 1.5 g/l; agar, 20 g/l) plates and incubated at 50 °C. After 16–18 h of incubation, the morphologically distinct colonies were isolated and sub-cultured multiple times on nutrient agar plates to obtain pure cultures. The isolated bacterial strains were screened using different biochemical tests, namely, Gram staining, catalase test, motility test, starch hydrolysis, gelatin hydrolysis, casein hydrolysis and citrate utilisation. They were cultured in bioemulsifier producing medium (McKeen medium) to test their ability to produce bioemulsifier. Finally, the strain capable of growing in McKeen medium was identified by 16S rRNA sequencing. The strains were preserved by freezing in glycerol stocks at –20 °C.

Growth of thermophilic bacteria under different pH and salinity conditions

The growth of the microorganism was also tested in varying pH and saline conditions. Nutrient broth with different salt concentrations was prepared by adding different volumes of NaCl to obtain the final concentrations of 1–5 M NaCl in addition to NaCl already present in nutrient broth. HCl and NaOH were used to adjust the pH of the medium from 1 to 9. The nutrient broth with different salt concentrations and pH was inoculated with the bacterial strain. The broth cultures were incubated at 50 °C and the cell growth was checked after 48 h by measuring the absorbance at 600 nm.

Fermentation conditions for bioemulsifier production

McKeen medium was used for the production of bioemulsifier with the following composition (g/l): glucose, 20; monosodium glutamate, 10; yeast extract, 3; MgSO₄·7H₂O, 1.02; K₂HPO₄, 1.0; KCl, 0.5 and trace element solution, 1 ml. The trace element solution was prepared by adding 0.5 g MgSO₄·7H₂O, 0.16 g CuSO₄·5H₂O and 0.015 g FeSO₄·7H₂O in 100 ml of distilled water, autoclaved separately and added to the McKeen medium before inoculation [24, 25]. The seed culture of the isolated bacteria was prepared in McKeen medium with 1 g/l glucose as the carbon source. This seed culture at a concentration of 1% was used to inoculate McKeen medium for bioemulsifier production. The culture flasks were

incubated at 50 °C for 120 h with a constant agitation of 150 rpm.

Kinetics of cell growth and glucose utilisation

Fifty millilitres of culture sample and control were withdrawn after every 24 h and the absorbance was recorded at 600 nm with control medium as blank. The samples were centrifuged at 8000 rpm for 10 min at 4 °C. The pellet was washed with distilled water and dried at 70 °C to calculate the dry cell weight until constant weight was obtained. To estimate the concentration of glucose utilised by biomass, the supernatant collected after every 24 h was suitably diluted and filtered using 0.22 µm Durapore GVWP01300 filter. The concentration of glucose was analysed by high-performance liquid chromatography (HPLC) with glucose as a standard. A total of 20 µl of the filtered samples were injected into Aminex HPX-87H (Bio-Rad) column with mobile phase of 0.005 M H₂SO₄ and flow rate of 0.6 ml/min. The temperature of the column was kept at 50 °C throughout the run and the output was detected using refractive index detector (RID).

Assessment of emulsification activity and surface active properties

The emulsification ability of the cells in the culture medium was tested by measuring the emulsification index. One millilitre of cell-free broth and equal volume of petrol were vigorously mixed using a vortex mixer for 2 min. The mixture was allowed to stand at room temperature for 24 h. After 24 h, the heights of the aqueous layer and emulsified layer were recorded. Emulsification index (E_{24}) was determined by measuring the ratio of height of emulsified layer to the total height of the liquid.

$$E_{24}(\%) = \frac{\text{Height of emulsified layer}}{\text{Total height of liquid}} \times 100$$

Isolation and purification of bioemulsifier

Thirty millilitres of cell-free supernatant was used for the isolation of bioemulsifier. Crude bioemulsifier was obtained by acidifying cell-free supernatant to pH 2.0 using 6 N HCl. The acidified solution was stored at 4 °C to precipitate the bioemulsifier. The precipitate was collected by centrifugation at 8000 rpm for 20 min at 4 °C. The pellet was neutralised with phosphate buffer of pH 7.0 and dried at 70 °C. The dried crude bioemulsifier was extracted using dichloromethane. The solvent was evaporated by placing the solution in hot air oven at 70 °C. The pale yellow solid obtained after drying was again extracted using dichloromethane and left for evaporation. The weight of the yellow solid was measured.

Kinetic model fitting for cell growth, substrate utilisation and product formation

The logistic differential equation of cell growth is calculated as

$$\frac{dC}{dt} = \mu C \left(1 - \frac{C}{C_m} \right)$$

where C is the biomass at time t , C_m is the maximum biomass and μ is the specific growth rate of bacteria.

Integration of the above equation will result in

$$\ln \left[\frac{C_m - C}{C} \right] = -\mu t - K$$

where K is a constant. The specific growth rate of bacterial culture can be estimated from the slope of $\ln \left[\frac{C_m - C}{C} \right]$ versus t graph.

The substrate utilisation kinetics can be derived by the following equation:

$$\frac{dGlu}{dt} = -\frac{1}{Y_{C/Glu}} \left(\frac{dC}{dt} \right) - \frac{1}{Y_{E/Glu}} \left(\frac{dE}{dt} \right) - m_s C$$

where Glu and E are the concentrations of glucose and bioemulsifier, respectively, at time t . $Y_{C/Glu}$ is the yield coefficient based on substrate for biomass and $Y_{E/Glu}$ is the yield coefficient based on substrate for product.

With the assumption that no product is formed at the initial stages of substrate utilisation (i.e. $E=0$) and the maintenance coefficient (m_s) for the cell growth is negligible, integrating the above equation for the initial biomass X_0 and substrate concentration S_0 at time $t=0$ and the final biomass and substrate concentration as X and S at time t , respectively gives

$$Glu - Glu_0 = \frac{e^{\mu_m t}}{1 - \frac{C_0}{C_m}(1 - e^{\mu_m t})} - C$$

The Luedeking-Piret substrate can be summarised as

$$q_s = \gamma \mu + \delta$$

From the plot of $\frac{e^{\mu_m t}}{1 - \frac{C_0}{C_m}(1 - e^{\mu_m t})} - C$ versus $Glu - Glu_0$, the substrate kinetic constants γ and δ can be deduced.

Similarly, the Luedeking-Piret product is represented as

$$q_p = \alpha \mu + \beta$$

The plot of $\frac{e^{\mu_m t}}{1 - \frac{C_0}{C_m}(1 - e^{\mu_m t})} - C$ versus $E - E_0$ gives the corresponding kinetic constants for product formation.

Results and discussion

Identification of bacteria using biochemical analysis

A total of six morphologically distinct bacterial strains were isolated from the hot spring. These strains were characterised physiologically by Gram staining, catalase test, motility test, starch hydrolysis, gelatin hydrolysis, casein hydrolysis and citrate utilisation and the results of characterisation are summarised in Table 1. Out of these isolates, only one strain showing emulsifying ability (designated as S3) was selected for the study. The isolate was found to be Gram-positive rod-shaped which grew as circular and off-white colonies on nutrient agar. The bacterial strain also exhibited negative catalase test, negative motility test, negative starch hydrolysis test, negative citrate utilisation test, positive gelatin hydrolysis and positive casein hydrolysis tests. 16S rRNA was performed for molecular identification of the isolate and the accession number of the sequence obtained by GenBank was MK878423. The similarity of the sequence obtained was searched using BLAST tool by considering the maximum score obtained, query coverage and percentage identity between the query and the subject sequences and a phylogenetic tree was constructed using MEGA-X (Fig. 2). The branches of phylogenetic tree were generated by neighbour-joining method. The results of BLAST revealed that the microbial strain S3 exhibits 99.92% similarity to *Brevibacillus borstelensis* NBRC 15714 and *Brevibacillus borstelensis* DSM 6347.

Table 1 Physiological characteristics of the six bacterial isolates

S. no.	Gram-staining	Morphology	Catalase	Motility	Starch hydrolysis	Citrate utilisation	Gelatin hydrolysis	Casein hydrolysis
1	Positive	Rods	+	–	–	–	+	+
2	Negative	Rods	+	–	–	+	+	+
3	Positive	Rods	–	–	–	–	+	+
4	Positive	Rods	–	–	–	–	+	+
5	Negative	Rods	+	–	–	+	+	+
6	Positive	Coccus	+	–	–	+	+	+

Fig. 2 Phylogenetic tree based on comparison of 16S rRNA gene of the bacterial isolate and constructed by neighbour joining method

The bacterial strain S3 was grown in nutrient broth with different pH and salt concentrations. The results indicate that the organism was unable to tolerate high salt concentrations and grew best without the presence of any additional salt. The growth of organism was slightly affected by 1 M salt concentration whereas increasing salt concentration exhibited detrimental effect on the cell growth (Fig. 3a). On the other hand, the microbial growth was almost negligible during the acidic pH ranges (pH 1–5) and increased significantly when the pH

was increased. The maximum growth was observed at pH 7.0 and further increase in pH led to the reduced growth (Fig. 3b).

Microbial growth during bioemulsifier production

The microorganism was cultured in Mckeen medium with 20 g/l glucose as the sole carbon source. The cell growth was measured during the entire duration of cell cultivation which is depicted in Fig. 4. The graph of cell growth represents that the microbial culture attained stationary phase after 24 h of growth and was in the same phase till 120 h.

Emulsification ability and surface tension measurements

A slight variation in the emulsification ability was seen throughout the growth of the microorganism (Fig. 4). The emulsification index was highest at 48 h with the value of 58.13% which was stable for more than 24 h (Fig. 5). Also, no significant difference in emulsification index values of samples drawn after every 24 h (except 96 h) was observed. As the cell growth data exhibits stationary phase after 24 h the results of emulsification ability indicate that the bioemulsifier is produced during the stationary phase of bacterial growth. These results show a trend similar to the one previously reported for *Aeromonas* sp. where the maximum emulsification of 56% for bioemulsifier was obtained at stationary phase

Fig. 3 The effect of growth of bacterial strain S3 at different NaCl concentrations (a) and pH conditions (b)

Fig. 4 A plot of the growth pattern of bacterial strain S3 and its emulsification ability over the duration of 120 h

Fig. 5 The emulsification layer produced by the bacteria at 0 h (a) and 24 h (b)

[26]. The data of surface tension measurements reveal that the organism is capable of reducing the surface tension from 72 mN/m to 35 mN/m in 24 h and was 48 mN/m at 120 h (Fig. 6). Bonilla et al. isolated *Pseudomonas putida* ML2 from polluted sediments and tested its efficiency for bioemulsifier production by measuring emulsification index and surface tension. The results demonstrate that the emulsification index of 68% was maximum at 96 h with the surface tension of 35 mN/m in tryptic soy broth [27]. The comparison of results of current study on thermophiles with the ones previously reported on mesophiles [26, 27] shows that there is no significant difference in terms of emulsification ability and surface tension reduction by bioemulsifier produced by both the groups of bacteria.

Glucose utilisation and product formation

The graph of substrate consumed over time (Fig. 7a) shows that the consumption of glucose by the bacterial strain S3 decreases for the first 24 h and then starts increasing until it reaches maximum at 72 h. Beyond this time, the glucose concentration in the medium starts to decline rapidly till 120 h.

Fig. 6 The reduction in surface tension values of the cell-free broth with respect to time indicating the presence of bioemulsifier

This suggests the presence of an additional glucose-rich product in the supernatant at 72 h which is degraded further. On the other hand, the formation of bioemulsifier reaches maximum at 24 h, declines till 72 h and then again increases after 72 h. These plots might indicate that due to the depletion of nutrients present in the medium after 24 h, the product, being a carbohydrate-rich macromolecule, was broken down by the microorganism as carbon source. This led to the release of glucose molecules in the medium. After 72 h, the formation of bioemulsifier starts again leading to decrease in glucose concentration. On contrary, the emulsification ability is almost constant after 24 h. This might be due to the formation of micelles which results in stable emulsification layer.

The partial purification of bioemulsifier by acid precipitation resulted in the formation of a pale yellow solid sticking to the surface of a glass plate and beaker (Fig. 7b). The difference in weights of the glass surface after and before the formation of precipitate gave the amount of the bioemulsifier produced. The maximum yield of bioemulsifier was 1.4 g/l after 24 h of incubation. The previous studies have reported the yields of bioemulsifier as 0.22 g/L by a Gram-negative bacterial strain [28] whereas bioemulsifier yields by yeast *Candida utilis* varied from 0.26 to 0.93 g/l [29]. This demonstrates that the current bacterial strain S3 is a high bioemulsifier-producing strain.

Kinetic model fitting for bioemulsifier production

Kinetic modelling helps in understanding the progress of reaction at any time point. Luedeking-Piret substrate considers the relationship of cell growth and substrate consumption whereas Luedeking-Piret product is the relationship of cell growth with respect to product formation. From the logistics growth curve of biomass, the specific growth rate (μ) of the bacterial species was found to be 0.0096 h^{-1} (Table 2). The kinetic rate constants for Luedeking-Piret substrate were calculated as 11.246 and 10.626 as the γ and δ values, respectively. Similarly, the kinetic rate constants for Luedeking-Piret products are 3.8423 and -1.9075 for α and β , respectively.

Fig. 7 The plot of substrate utilised and product formed by the bacterial strain S3 over the 120 h culture period (a) and the purified bioemulsifier (b)

Conclusion

In the current study, six bacterial isolates were isolated from a local hot spring and tested for the production of bioemulsifier. Out of the six isolates, one isolate, termed as S3, exhibited potential for bioemulsifier production. The bacterial strain S3 was Gram-positive, rod-shaped bacterium and was phylogenetically related to *Brevibacillus borstelensis* NBRC 15714 and *Brevibacillus borstelensis* DSM 6347. The microorganism showed maximum growth at 24 h with maximum emulsification ability (57.54%), maximum product formation (1.4 g/l) and maximum reduction in surface tension (35 mN/m). This suggests that 24 h is the ideal incubation period for the production of bioemulsifier by strain S3 using Mckeen medium. The growth pattern of the strain was analysed by logistic growth curve while Luedeking-Piret kinetic models were used to determine kinetic constants for substrate utilisation and product formation. The analysis shows that the specific growth rate of S3 was 0.0096 h^{-1} with kinetic rate constants as 11.246 (γ) and 10.626 (δ) for Luedeking-Piret substrate and 3.8423 (α) and -1.9075 (β) for Luedeking-Piret product. As the kinetic model fitting for bioemulsifier production has not been reported till date, this work provides new models for estimating the substrate utilisation or bioemulsifier formed at any time point throughout the bacterial growth. Understanding the kinetic models of substrate utilisation and product formation will be advantageous in

designing experiments in future for the production of bioemulsifier from agricultural residues and biowastes.

Future scope

In the current study, the metabolic pathway of the most common bioemulsifier, bioemulsan, is elucidated. The knowledge of metabolic pathway and flux analysis of bioemulsan will help to understand the changes in intracellular fluxes with respect to variation in substrates or environmental conditions. After the establishment of structure of bioemulsifier, the metabolic pathway can be extended for the product and flux analysis for the same can be studied. This will help in identifying the interaction between two or more substrates for the formation of a product which might be difficult and expensive to create in laboratory scale. This will also help in estimating the potential failures and limitations of the process.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Table 2 Specific growth rate and kinetic rate constants for Luedeking-Piret substrate and product

Specific growth rate (μ)	Kinetic rate constants for Luedeking-Piret substrate		Kinetic rate constants for Luedeking-Piret product	
	γ	Δ	α	β
0.0096 h^{-1}	11.246	10.626	3.8423	-1.9075

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