Optimization of Biosurfactant Production by *Bacillus licheniformis* STK 01 Grown Exclusively on *Beta vulgaris* Waste using Response Surface Methodology

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This work reports on the exploitation of *Beta vulgaris* for biosurfactant production by *Bacillus licheniformis* STK 01 and its optimization using statistical modeling of response surface methodology (RSM). Three variables were investigated: agro-waste concentration, pH, and temperature. The response and contour plots of the RSM showed perfect interaction among the variables, with the highest surface tension reduction of the culture medium to 30 mN/m observed at 42 °C, a pH of 8, and a substrate concentration of 4% (w/v). The biosurfactant produced demonstrated a high tendency for hydrocarbon emulsification. Furthermore, by numerical optimization techniques, the optimum conditions were found to be as follows: a pH of 6.72, an agro-waste concentration of 4% (w/v), and a temperature of 44.5 °C. The experiment conducted to validate the optimum conditions obtained showed a biosurfactant with remarkable surface activity, lowering the surface tension of the broth to 30 mN/m, when the organism was grown on *B. vulgaris*, and to 23.5 mN/m, when grown in glucose medium – the later representing one of the highest surface tension reductions ever reported for a biosurfactant. This study revealed, among others, that the exclusive utilization of cheap solid agro-waste without supplementation with a refined nutrient source is feasible and could ensure the economic sustainability of biosurfactant production.

Keywords: Agro-wastes; Biosurfactant; Central composite design; Emulsification; Surface tension

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INTRODUCTION

The term "biosurfactants" represents surface-active agents that are synthesized extra-cellularly by microorganisms. They are long-chain amphiphilic molecules with distinct structural and functional groups that give them a wide range of properties, such as the lowering of surface and interfacial tension of liquids and the formation of micelles and micro-emulsions between two different phases. These properties enhance the bioavailability of hydrophobic contaminants, thereby increasing their biodegradation (Pacwa-Płociniczak *et al.* 2011; Soberón-Chávez and Maier 2011). Typically, biosurfactants lower the Gibbs free energy of a two-phase system, leading to a decrease in the energy of the interface between the two phases (Makkar *et al.* 2011; Perfumo *et al.* 2010). In this way, biosurfactants enhance the mobilization of hydrophobic contaminants from solid matrices into the aqueous phase for microbial degradation. The critical micelle concentration (CMC) and surface tension are two important characteristics associated with

the application of biosurfactants. The formation of micelles leads to a significant increase in the apparent solubility of hydrophobic organic compounds, even above their water solubility limit, as these compounds can partition into the central core of a micelle. This effect enhances the mobilization of organic compounds and their dispersion into a solution (Perfumo *et al.* 2010). Efficient biosurfactants have low CMC values; consequently, minimal biosurfactant quantities are required to achieve the requisite reduction in the surface tension of aqueous media (Desai and Banat 1997).

Many microorganisms, including bacteria, fungi, and yeast, have been reported for biosurfactant production (Bodour *et al.* 2003; Sekhon *et al.* 2012). These microorganisms were isolated from different environmental samples such as oil-spill sites, petroleum reservoirs, water sediments, lignocellulosic wastes, *etc*. (Burgos-Diaz *et al.* 2011; Sobrinho *et al.* 2013; Sousa *et al.* 2012; Wang *et al.* 2011). Moreover, *Bacillus* spp., which appear to be the most profuse in environmental samples, have exhibited a higher ability for biosurfactant production compared with other bacteria that have been reported, producing cyclic lipopeptides and lipoproteins that include surfactins, fengycins, lichenysins, Bacillomycin, and iturins as major types of biosurfactant (Deleu *et al.* 1999; Mukherjee and Das 2005; Sekhon *et al.* 2012). The intended application of biosurfactant could, however, give a preference on where to sample when bioprospecting. For instance, isolates from an oil-spill site or a ligninocellulosic waste site will most likely degrade hydrocarbon contaminants, which are the most recalcitrant environmental contaminants.

In recent years, biosurfactants have been utilized in many unconventional fields, including in the bioremediation of water-insoluble pollutants; the enhancement of oil recovery to reduce environmental impact during extraction; the replacement of traditional synthetic surfactants in cosmetics, soap, healthcare, and paints; as well as in production and application in drug-delivery systems (Banat *et al.* 2000, 2010). Given their success in laboratory studies and some field applications, it has been speculated that the potential market growth for biosurfactants will soon surpass that of synthetic surfactants (Marketsandmarkets 2012). The reason for such speculation could also be because of regulatory constraints associated with the production of synthetic surfactants and the availability of a plethora of renewable substrates that can be used for biosurfactant synthesis. However, these advantages have not been fully exploited, owing to relatively high production costs primarily associated with biosurfactant recovery and purification. Application of crude biosurfactants has been reported for environmental remediation processes and enhanced oil recovery from storage tanks and capillaries (Amodu *et al.* 2013; Makkar *et al.* 2011; Mukherjee *et al.* 2008; Mutalik *et al.* 2008), implying that purification-related costs can be circumvented in such applications of the bioproduct. Furthermore, the process can become more economically viable when appropriate renewable resources are identified that will serve as exclusive sources of nutrients for microbial growth and synthesis of the requisite metabolites. Considering all cost-determining variables in biosurfactant production, the choice of suitable, low cost raw materials can account for 10 to 30% of the overall cost (Cameotra and Makkar 1998; Makkar and Cameotra 2002). Therefore, the utilization of agro-wastes, as well as suitable microorganisms under optimized conditions, can significantly increase biosurfactant yield, thereby enhancing the economic viability of the large-scale production of crude biosurfactants for the bioremediation of environmental contaminants.

Some agro-industrial waste substrates, such as olive oil mill effluent, plant oil extracts and wastes, distillery and whey wastes, potato peels, cassava waste-water, and rice straw, have been reported for biosurfactant production (Das and Mukherjee 2007; Makkar

et al. 2011; Nitschke and Pastore 2006; Zhu *et al.* 2013). Surface tension reductions in the range of 27 to 35 mN/m have been reported for the biosurfactants produced, as well as emulsification activity in the range of 20 to 75% for various hydrocarbon compounds (Barros *et al.* 2008; Fox and Bala 2000; Nitschke and Pastore 2006; Oliveira *et al.* 2013). Although the biosurfactants produced from these renewable substrates, often supplemented with refined nutrients, have been shown to be effective, identification of renewable substrates that can be used exclusively is expedient for the sustainability of the process. Yet, exclusive utilization of renewable resources, particularly solid wastes, for biosurfactant production, is rarely reported.

In addition, optimization of nutritional as well as cultivation parameters for enhanced biosurfactant production, either by direct quantification of biosurfactant produced or relative quantities such as the critical micelle dilution, is well reported in the literature (Ilori *et al.* 2005; Joshi *et al.* 2008; Najafi *et al.* 2010; Rodrigues *et al.* 2006; Zhu *et al.* 2013). These parameters include pH, temperature, agitation, inoculum size, cultivation time, oxygen availability, substrate concentration, and composition. Using design of experiment (DoE), the most influential parameters can be obtained or specified heuristically in order to reduce the number of variables. Interestingly, the response surface methodology (RSM) used in this study offers a statistical design of experiments to assess influential parameters that ultimately lead to peak process performance and the discovery of optimum conditions at a minimal cost. The use of a suitable substrate, in this case agro-waste, is inherently dependent on the amount of free and usable sugars, including trace elements, available for microbial growth and the expression of the biosurfactants. This study will most likely present the first report on the exclusive utilization of solid agro-waste as substrate for biosurfactant production.

Hence, the objectives of this study were as follows: (1) isolation and identification of a biosurfactant-producing strain capable of utilizing agro-wastes exclusively for the growth and expression of the requisite bioproduct, and (2) optimization of culture conditions (temperature, pH, and substrate concentration) to enhance the surface activity of the biosurfactant produced. The production of an effective biosurfactant from appropriate agro-wastes, such as those that do not require supplementation with refined substrates, could ensure the sustainable and economical production of biosurfactant, thus finding an application in the continuous bioremediation of environments polluted with hydrocarbon contaminants.

EXPERIMENTAL

Material and Methods

Microorganism

Bacillus licheniformis STK 01, a biosurfactant-producing bacterium isolated from rotting wood, was identified both morphologically and by a 16S ribosomal deoxyribonucleic acid (rDNA) sequencing analysis. It was maintained on nutrient agar slants at 4 °C and sub-cultured every three weeks.

Isolation of DNA and PCR amplification of 16S rDNA

Genomic DNA of the isolate used in this study was extracted using a pure gene kit (DNA Purification Kit, USA). The total genomic DNA of the strain was extracted for PCR analysis using the method described by Boot *et al.* (1993), with slight modifications. The

16S rDNA gene was amplified by PCR using the following two universal primers: (1) Forward: 5'- AGA GTT TGA TCI TGG CTC AG -3'; and (2) Reverse**:** 5'- ACG GIT ACC TTG TTA CGA CTT -3'. The PCR program was set for denaturation at 94 °C for 1 min, annealing at 46 °C for 1 min, and extension at 72 °C for 1 min, for a total of 30 cycles. The PCR products were analyzed by electrophoresis at 100 mV for 40 min on a 1% agarose gel (Sigma), using ethidium bromide (10 μg/mL) to ensure that fragments of the correct size were amplified. Then, 10 μL of the amplified product were added to 1 μL of the tracking dye, followed by loading onto the gel, which was visualized using UV trans-illumination (Wang *et al.* 1996). The forward and reverse overlapping sequencing primers were used to sequence the entire length of the double-stranded DNA, which was then blasted against the NCBI Genbank database and compared with known nucleotide sequences.

Inoculum preparation and culture conditions

An inoculum solution was prepared by transferring a loopful of the microbial cells from the slant agar into nutrient broth and incubating at 37 °C and 150 rpm for 24 h in an orbital shaking incubator. Serial dilutions were then prepared from the inoculum solution and cultured on nutrient agar at the same temperature of 37 °C for 24 h in a shaking incubator at 150 rpm. Isolated cells grew into colonies and were counted using a Quebec Darkfield Colony Counter (Reichert Scientific Instruments, USA). Typically, plates with fewer than 30 or more than 300 colonies were regarded as statistically unreliable (Benson 2001). The number of colonies counted multiplied by the dilution factor gave 10^8 CFU/mL, which represented the inoculum concentration used. Then, 250-mL Erlenmeyer flasks containing 100 mL of the culture media were inoculated with 8% (v/v) of the 10^8 CFU/mL, and incubated in an orbital shaking incubator at 150 rpm at various temperatures, and at concentrations specified for *B. vulgaris* waste in Table 1, for a maximum of 240 h. Samples were taken after 120 and 240 h to assay the surface activity of the biosurfactants produced. An uninoculated culture of *B. vulgaris* served as the control.

Screening and selection of a suitable agro-waste substrate

Several types of agro-waste, *i.e.*, *Pyrus communis* (pear, P), *Ananas comosus* (pineapple, PP), *Citrus sinensis* (orange, OR), and *Beta vulgaris* (beetroot, B) were screened as primary substrates, as was the combination of each of the wastes with *Saccharomyces cerevisiae* (spent Brewer's yeast, BY), for biosurfactant production. These prospective substrates were obtained as waste from a fruit and vegetable processing facility in close proximity to the Cape Peninsula University of Technology, Cape Town campus, with the exception of the spent brewer's yeast, which was obtained from a nearby brewery. The agro-waste was milled and oven-dried at 60 °C for 72 h, then pulverized into a particle size of less than 0.30 mm. Then, 250-mL Erlenmeyer flasks containing 100 mL of culture [5% (w/v) of each of the substrates] were prepared and autoclaved for 15 min at 110 °C. The cultures were allowed to cool to room temperature and then inoculated with a loopful of the microbial cells and incubated at 37 °C and 150 rpm for 96 h. The most suitable substrate, identified by testing the broth supernatant using the drop-collapse, oil displacement, and emulsification index methods, was then selected for further studies, using response surface methodology (RSM).

Surface tension determinations and FTIR analysis

The surface tension of the biosurfactant-containing culture broth was determined according to Podlogar *et al.* (2004). Bacterial cells were removed from the culture broth by

centrifugation at 15,000 rpm for 20 min at 4 °C. The surface tension of the cell-free supernatant was determined with a Kruss Processor Tensiometer (model K 100 Kruss, Germany) at 25 ± 0.5 °C, using the Wilhelmy plate method. The results presented were averages of duplicated measurements from two flasks. The biosurfactant produced from the most suitable substrate was partially purified by adjusting the pH to 2.0 using 1 M HCl and keeping it at 5 ± 1 °C overnight, followed by centrifugation at 15,000 rpm and 4 °C for 20 min. For further purification, the precipitate formed was dissolved in 5 mL of distilled water and extracted using three cycles with an equal volume of chloroform/methanol solution with a ratio of 2:1 (v/v). The organic layer was dialyzed and evaporated using a vacuum at ambient temperature; the dried extract obtained was used for Fourier Transform Infrared (FTIR) analysis.

Emulsification index

The emulsification index (E_{24}) was determined as reported by Cooper and Goldenberg (1987). Six milliliters of hydrocarbon were added to 4 mL of each of the cell-free supernatants containing the biosurfactant in test tubes, and homogenized by vortexing vigorously for 2 min. The mixtures were left to stand for 24 h, and the emulsification index was calculated as shown in Eq. 1 below. Tween 80 (1%, v/v) and pluronic F-68 (1%, w/v) are synthetic surfactants that were used as controls to assess the surface activity of the biosurfactant produced at optimized conditions.

$$E_{24} = \frac{\text{Total height of the emulsion}}{\text{Total height of aqueous phase + emulsion}} x\ 100 \quad (1)$$

Response surface methodology: Central composite design experiments

RSM offers a statistical design of experiments to assess influential parameters that ultimately lead to peak process performance and the discovery of optimum conditions at a minimal cost. Central composite experimental design (CCD) was applied in this study for evaluating three variables, substrate concentration, pH, and temperature, allowing a minimum number of experimental runs for determining the optimum fermentation conditions for maximizing biosurfactant production and thus the surface activity of the broth. The ranges of the variables were specified based on optimum values reported for most bacteria, considering the lowest and highest values possible (Abushady *et al.* 2005; Guerra-Santos *et al.* 1984; Powalla *et al.* 1989). The experimental design was generated using Design-Expert® software version 6.0.8 (Stat-Ease Inc., USA). Each variable was analyzed at five levels coded as −α, −1, 0, +1, and +α representing a core factorial, center, and axial points. A set of 20 runs was carried out consisting of the following: a 2^k complete factorial design (where k = 3, *i.e.*, the number of the test variables), six axial points representing two outlier points on the axis of each variable at a distance of + α from the high level (+1) and −α from the low level (−1), which equals $2^{k/4}$ (*i.e.*, 1.68 for k = 3) and six center points (level 0). Each sample was inoculated with 8% (v/v) of 10^8 CFU/mL, while the uninoculated broth served as a control at various specified temperatures. The pH of the samples was adjusted accordingly with the addition of 1 M NaOH or 1 M HCl. The results presented were three replicate measurements from two flasks.

Statistical analysis and modeling

Suitable statistical models were chosen to model the interactions among the different experimental variables and their effect on surface tension reduction by the

biosurfactants produced, based on the Sequential Model Sum of Squares and a Lack-of-Fit Test. The response, measured after 120 and 240-h incubation periods, was modeled with an overall mean and a response surface quadratic model, respectively. The results obtained after 240 h of incubation gave both the highest surface tension reduction and better statistical fitness, and were therefore subjected to further analysis by Analysis of Variance (ANOVA) to assess the significance of each variable on the surface activity of the biosurfactants produced. An empirical model that could relate the response measured to the independent variables was obtained using multiple regression analysis. The response (*Y*), after 240 h of the fermentation system, can be represented by the following quadratic model:

$$Y = \alpha_o + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X^2{}_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \alpha_{ij} X_i X_j + \varepsilon \ , \qquad (2)$$

where X_1, X_2, X_3, …, X_n are the independent coded variables, α_0 is the offset term, and α_i, α_{ii}, and α_{ij} account for the linear, squared, and interaction effects, respectively, and ε is the random error. However, a model reduction may be expedient if there are many redundant model terms, excluding those required to support hierarchy such as α_1, α_2, and α_3. Statistical properties of the model were further analyzed with the normal probability and the externally studentized plots to validate the normality of the residuals and the influential terms.

Table 1. The Various Media Components included in CCD Experiments and Their Corresponding High, Medium, and Low Concentration Levels

Variables	Code	Units	High levels (+1)	Medium levels (0)	Low levels (−1)
Substrates Conc.	*A*	% (w/v)	8.00	6.00	4.00
pH	*B*	-	8.00	7.25	6.50
Temperature	*C*	°C	42.00	37.00	32.00

Table 2. Central Composite Experimental Design for Three Variables and the Corresponding Responses

Run	*A*	*B*	*C*	Surface Tension (mN/m)		
				120 h	240 h (Experimental value)	240 h (Predicted value)
1	0	0	0	38.60	49.80	49.95
2	0	0	0	38.60	49.80	49.95
3	+1	−1	−1	42.58	42.70	39.54
4	0	0	0	38.60	49.80	49.95
5	+1	+1	+1	41.40	33.16	35.42
6	0	0	0	38.60	49.80	49.95
7	+1	−1	+1	35.30	34.00	33.17
8	0	0	+ α	33.82	31.62	28.62
9	0	0	−α	37.46	37.43	35.14
10	+ α	0	0	40.50	41.57	41.99
11	−α	0	0	40.27	45.20	39.44
12	0	+ α	0	47.27	48.90	40.16
13	0	− α	0	33.90	32.00	35.45
14	−1	−1	−1	34.32	33.48	34.00
15	0	0	0	38.60	49.80	49.95
16	−1	−1	+1	33.48	31.00	30.75
17	−1	+1	+1	32.11	30.00	36.91
18	+1	+1	−1	35.14	34.98	38.96
19	−1	+1	−1	34.26	33.73	38.31
20	0	0	0	38.60	49.80	49.95

A, *B*, and *C* represent the coded level of variables; α represents the axial point with a coded level of 1.682

RESULTS AND DISCUSSION

Microbial Identification

The morphological identification showed that the isolated bacterium strain was a gram-positive and a spore-forming *Bacillus* spp. with reddish-pink, rod-shaped colonies. The chain reaction of bacterial DNA using universal primers revealed that the gram-positive biosurfactant-producing isolate was closely related to three strains of *Bacillus licheniformis*, viz, strains ZML 1, SCCB 37, and 1 FTM8. Thus, it was identified as *Bacillus licheniformis* and designated strain STK 01.

Identification of a suitable agro-waste substrate

To select suitable renewable substrates for the production of biosurfactant by *B. licheniformis* STK 01, several agro-wastes/products were screened using various standard methods, including drop collapsing, oil displacement, and emulsification index. Of all these agro-wastes, *B. vulgaris* waste was determined to be the best substrate for use exclusively for microbial growth and the effective production of biosurfactant with a high propensity for hydrocarbon emulsification (Table 3).

The droplets of the biosurfactant produced from *B. vulgaris* collapsed immediately upon contact with the surface of the mineral oil used as the hydrocarbon and spread to cover 90% of the oil surface. Similar effective activity was noticed with the emulsification experiment, giving a 70% emulsification index. On the other hand, the droplets of the broth supernatant produced from *P. communis* displayed little or no effect on the oil surface, as shown in Table 3, maintaining an oval shape as observed for distilled water. Such observation was reported by other authors (Youssef *et al.* 2004; Batista *et al.* 2006; Chen *et al.* 2007; Satpute *et al.* 2008), which implied that the supernatant contained no surface-active agents. *Saccharomyces cerevisiae* is a rich source of protein and mineral elements that could enhance biosurfactant production and activity, but its supplementation with agro-wastes did not show any positive influence on biosurfactant production.

Table 3. Screening Methods of Various Agro-wastes for Biosurfactant Production by *B. licheniformis* STK 01

Agro-waste	Biosurfactant activity		
	Drop-collapse method	Oil displacement (%)	Emulsification index, E_{24} (%)
Citrus sinensis, OR	+	NA	50
Ananas comosus, PP	++	12.5	50
Beta vulgaris, B	+++	90	70
Pyrus communis, P	+	NA	30
OR + BY	+	NA	50
PP + BY	++	20	50
B +BY	+++	87.5	60
P + BY	NA	20	10

NA = no biosurfactant activity observed; +++ = complete collapse within 2 min; ++ = collapse after 2 min; + = collapse after 4 min of biosurfactant addition. Controls: distilled water = NA; Tween 20 = +++

The biosurfactants' ability to create emulsions of hydrocarbon contaminants and thereby increase their aqueous phase availability is best demonstrated by their surface and interfacial activities. The oil emulsification index recorded for biosurfactant from *B. vulgaris* waste here is comparable to those previously reported (Lai *et al.* 2009; Rocha *et al.* 2009; Oliveira *et al.* 2013). The nutritional content of *B. vulgaris* makes it suitable not only for human consumption, but also for microbial cultivation and biosurfactant production. It contains an average of 88% water, 1.2% protein, 9.3% carbohydrates, and 9% minerals, most of which are N, Na, K, Mg, Fe, Zn, Ca, Mn, P, I, Se, and Cl, as well as trace amounts of carotene, thiamin, riboflavin, niacin, biotin, and vitamins C, E, B1, B2, and B12 (Holland *et al.* 1991). A more detailed information on the nutritional composition

of *B. vulgaris* and other agro-waste used in this study was presented in our recent research (Amodu *et al.* 2014)

Central Composite Experimental Design

To determine the optimum components of culture medium to maximize effective biosurfactant production, three operational parameters were studied for their individual as well as their interactive effects, using RSM (Table 1). A total of 20 experimental runs were carried out as generated by the Design-Expert® software. The results presented in Table 2 showed stochastic variations in the responses measured, suggesting the effects of the various culture components on microbial activities. There was considerable surface tension reduction of broth to 30, 31, 31.62, and 32 mN/m, the lowest observed for runs 17, 16, 8, and 13, respectively. This corresponds to a reduction of 20 mN/m relative to the control (uninoculated broth). Comparing the responses measured after 120 h and 240 h of fermentation, the surface tension values were lower for some runs for the fermentation period of 240 h, which was expected since the solid residues of the substrate were not removed, and consequently the bound sugars were being released continuously to sustain microbial growth and biosurfactant production.

However, for some runs, the surface tension values measured for the 240 h fermentation were higher than those measured after 120 h. This scenario, where the surface activity of biosurfactant decreases with time owing to variations of the system parameters, has been observed previously (Das and Mukherjee 2007; Noudeh *et al.* 2010; Oliveira *et al.* 2013; Sahoo *et al.* 2011). It is akin to enzyme deactivation after long use or subjection to unfavorable conditions. Biosurfactants are produced extra-cellularly and the process has been reported to be growth dependent (Lin *et al.* 1998; Sahoo *et al.* 2011). Meanwhile, the surface activities are dependent on thermodynamic properties such as temperature and biosurfactant concentration. Deactivation of biosurfactants by stationary phase cultures was observed for *B. licheniformis* KGL 11 grown in a mineral salt medium supplemented with refined glucose (Lin *et al.* 1998). This could be a result of changes in metabolic activities as the biosurfactant production process goes through different stages of microbial growth. It can be explained that during cell lysis, which occurred due to prolonged incubation and probably after the CMC was reached, the concentration of biosurfactants increased considerably, forming more micelles, and thereby absorbing the surface-active molecules. Further work is under way on the kinetics of biosurfactant production from *B. vulgaris* by this novel strain, which will explicate this scenario.

A closer look at the results presented in Table 2 shows that the highest surface tension reduction (Runs 17, 16, and 8) occurred at temperatures of 42 and 45.4 °C and substrate concentrations of 4 and 6% w/v, suggesting that the isolated strain might be an extreme mesophile. Conversely, the supernatants produced from central points, a high extreme of pH, and a low extreme of substrate concentration (Runs 1, 11, and 12), did not show any biosurfactant activity. The interactive effects of the studied variables are better depicted with graphical representation of the response surface model discussed below.

Because the production of biosurfactant is growth dependent, often at the exponential growth phase (Lin *et al.* 1998; Reis *et al.* 2004; Sahoo *et al.* 2011), it can be deduced that the optimum effective biosurfactant production corresponds to the highest surfactant activity at the broth-air interface, just before the onset of CMC. This in turn corresponds to the highest surface tension reduction. The reduction in the surface tension of the culture broth to 30 mN/m showed the potency of the isolated strain in using unconventional substrates for biosurfactant production. Some researchers have tried to

benchmark an effective biosurfactant by its ability to lower the surface tension of water below 35 mN/m (Costa *et al.* 2006; Gudiña *et al.* 2010; Mulligan 2005). Several studies have demonstrated surface tension reduction similar to the results obtained in this study. Queiroga *et al.* (2003), investigating the ability of 13 microbial strains to produce biosurfactant using glycerol and glucose as carbon sources, observed a surface tension reduction of the medium to 30 mN/m. Recently, Oliveira *et al.* (2013) showed a surface tension reduction of the fermented broth medium to 30 mN/m by the biosurfactant produced by a *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice supplemented with $(NH_4)_2SO_4$ as a nitrogen source.

Furthermore, Barros *et al.* (2008) reported that the biosurfactant produced by *Bacillus subtilis* LB5, grown in cassava waste-water, reduced the surface tension of water from 72.31 to 27.01 mN/m. Nitschke and Pastore (2006), working with the same strain of *Bacillus subtilis* grown in cassava waste-water, reported a surface tension reduction of the medium to 26.6 mN/m by the biosurfactant produced. However, it may be inexpedient to compare surface tension reduction values as stated here, bearing in mind that the onus should be on producing effective biosurfactants, not only in their surface activities, but also in terms of cost and sustainability. The media containing the biosurfactants, the extent of purification involved, as well as the substrates used, whether refined sugars or renewable resources with or without supplementation, should form the basis of comparison. The agro-waste used as substrate in this study proved to be effective, giving results comparable to those obtained for refined substrates (Joshi *et al.* 2008; Nerurkar 2012; Sousa *et al.* 2012; Wang *et al.* 2011).

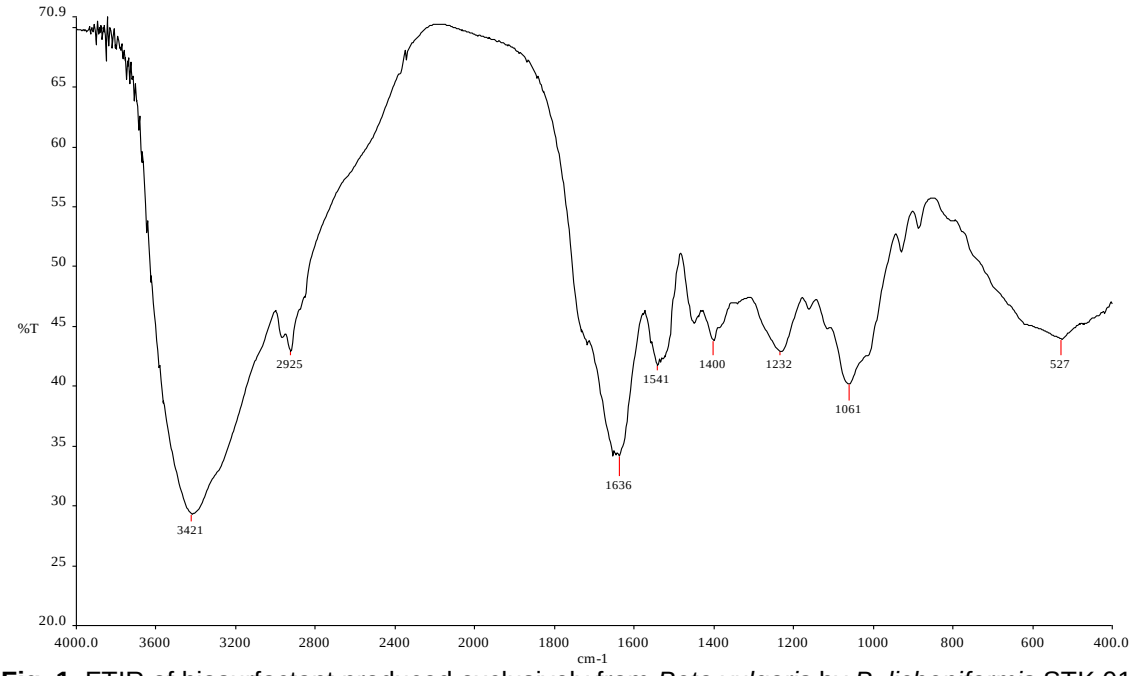

Fig. 1. FTIR of biosurfactant produced exclusively from *Beta vulgaris* by *B. licheniformis* STK 01

The IR spectrum of biosurfactant produced by *B. licheniformis* STK 01 showed strong absorption bands (Fig. 1), elucidating the presence of peptide components at wave number 3421 cm^{-1}, which emanated from the bond stretching of NH. The presence of CO amide stretch bonds is signified by wave numbers 1636 cm^{-1}. The presence of a C-H

aromatic ring is represented at 1000 to 1300 cm^{-1}. Also, the presence of an aliphatic group was observed at 3000 to 2850 cm^{-1} for CH_2 and CH_3. The identified bonds indicated that the biosurfactant produced might be a cyclic lipopeptide. Similar results were reported by previous studies (Das and Mukherjee 2007; Oliveira *et al.* 2013).

Statistical Model Analysis and Validation

The statistical model summary based on the Sequential Model Sum of Squares and Lack-of-Fit Test elucidated the fitness of mean and quadratic models for the responses measured after 120 and 240 h fermentation periods, respectively. Moreover, the data obtained for the 240 h fermentation were optimized, having given the highest surface tension reduction and better statistical model fitness. Using ANOVA to assess the significance of each variable in the model, an empirical quadratic model was obtained from Eq. 1 that could relate the surface tension of the biosurfactant measured to the independent variables.

Table 4. ANOVA for Response Surface Quadratic Model

Model Coeff.	Coeff. Estimate	DF	Standard Error	95% CI Low	95% CI High	F Value	Prob > F	Significance
α_0	49.95	1	1.97	45.51	54.39	4.47	0.0043	**
α_1	0.77	1	1.32	−2.18	3.72	0.34	0.5731	NS
α_2	1.40	1	1.32	−1.55	4.35	1.12	0.3150	NS
α_3	−1.94	1	1.32	−4.89	1.01	2.15	0.1732	NS
α_{11}	−3.26	1	1.29	−6.13	−0.39	6.40	0.0299	*
α_{22}	−4.30	1	1.29	−7.17	−1.43	11.13	0.0075	**
α_{33}	−6.39	1	1.29	−9.26	−3.52	24.63	0.0006	***
α_{12}	−0.98	1	1.73	−4.83	2.88	0.32	0.5847	NS
α_{13}	−0.54	1	1.73	−4.39	3.31	0.097	0.7617	NS
α_{23}	+0.70	1	1.73	−3.15	4.56	0.17	0.6925	NS

(***): significant at level 99.99%; (**): significant at level > 99%; (*): significant at level 95%
NS = Not significant; CL = Confidence level; DF = Degree of freedom
Values of "Prob > F" less than 0.05 indicate model terms are significant, while values greater than 0.1 indicate the model terms are not significant

The predicted response (Y) for the fermentation system was as follows:

$$Y = 49.95 + 0.77A + 1.40B - 1.94C - 3.26A^2 - 4.30B^2 - 6.39C^2 - 0.98AB - 0.54AC + 0.70BC \quad , \qquad (3)$$

where *A*, *B*, and *C* are the coded values for substrate concentration, pH, and temperature, respectively. Statistical analysis performed to determine and quantify the interactive effects of the coefficient in predicting the surface tension reduction of the biosurfactant showed a stochastic variation (Eq. 2). The interaction coefficients were estimated by taking the average of the two confidence levels (Table 3). By considering coefficients with significant effects, Eq. 3 can be reduced to the following:

$$Y = 50 - 2.71A^2 - 3.91B^2 - 6.72C^2. \quad (4)$$

Statistically, a model reduction may be appropriate for improving the model if there are more redundant model terms than the significant ones, excluding those required to support hierarchy. A model reduction was observed to enhance the fitness of the experimental data. The ANOVA of the quadratic regression model for the surface activity of the biosurfactant showed that the model was significant at the 99.8% level (Table 4), implying that the total variance in the response could be explained using this model. The Model F-value of 6.54 also enhances the significance of the model. There was only a 0.2% chance that a model F-value this large could occur because of noise. Adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable. Thus, the adequate precision ratio of 7.802 observed in this study indicated an adequate signal, further suggesting that this model can be applied to navigate the design space. The coefficient of variation value (CV% = 11.8) equally underscored the precision and reliability of the model.

Table 5. ANOVA for Surface Tension Reduction by Biosurfactant in CCD

Source of variation	Sum of squares	DF	Mean sq	F-value	Significance
Regression	961.19	9	106.80	4.47	**
Residual	239.02	10	23.90		
Lack of fit	239.02	5	47.80	0.000	***
Pure error	0.000	5	0.000		
Total	1200.00	19			

Std. Dev. = 4.89; C.V = 12%; R^2 = 0.8008; Adj R^2 = 0.6216; Pred R^2 = 0.5208; DF = Degree of freedom

The calculated value of the coefficient of determination (R^2 = 0.8044) implies that at least 80% of the variability in the actual and predicted values can be explained by the model. The non-significance of the F-value of the Lack-of-Fit Test (Table 5) also showed the strength of the model for the experimental data. The diagnostic details of the model, using studentized residual (Fig. 2), indicated normality in the error term, further justifying the fitness of the model. A high degree of correlation was observed between the experimental and predicted values that showed the accuracy and applicability of the model for predicting biosurfactant production. One unique aspect of this study is that it is possible to predict the optimum at which the biosurfactant produced will be most active in lowering the surface tension.

Graphical Representation of the Response Surface Model

The interactive effects of the independent variables on the system's response were investigated by plotting three-dimensional curves of the response against any two of the variables while keeping the third constant (Fig. 3). Such response surface plots allow for easy interpretation of experimental results and the prediction of optimal conditions. The 3-D and contour plots can be used to determine the level of interaction between the independent variables. An elliptical contour shape shows a perfect interaction between the two independent variable plots while a circular contour shows a non-interactive effect on the system response (Khuri and Cornell 1996; Montgomery 2008). The response contour plots showed ellipses for all the variable pairs plotted in Fig. 3, with Fig. 3b and Fig. 3f showing complete interactions.

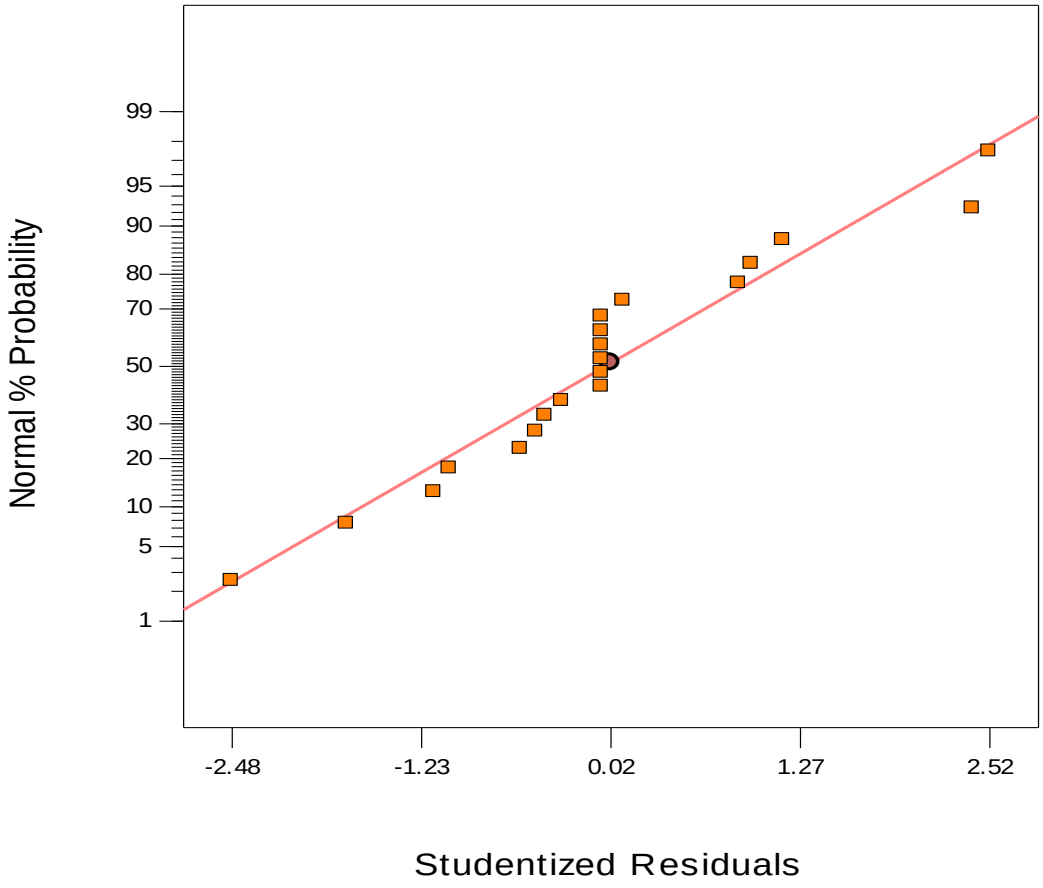

Fig. 2. Normal probability plot of the residuals

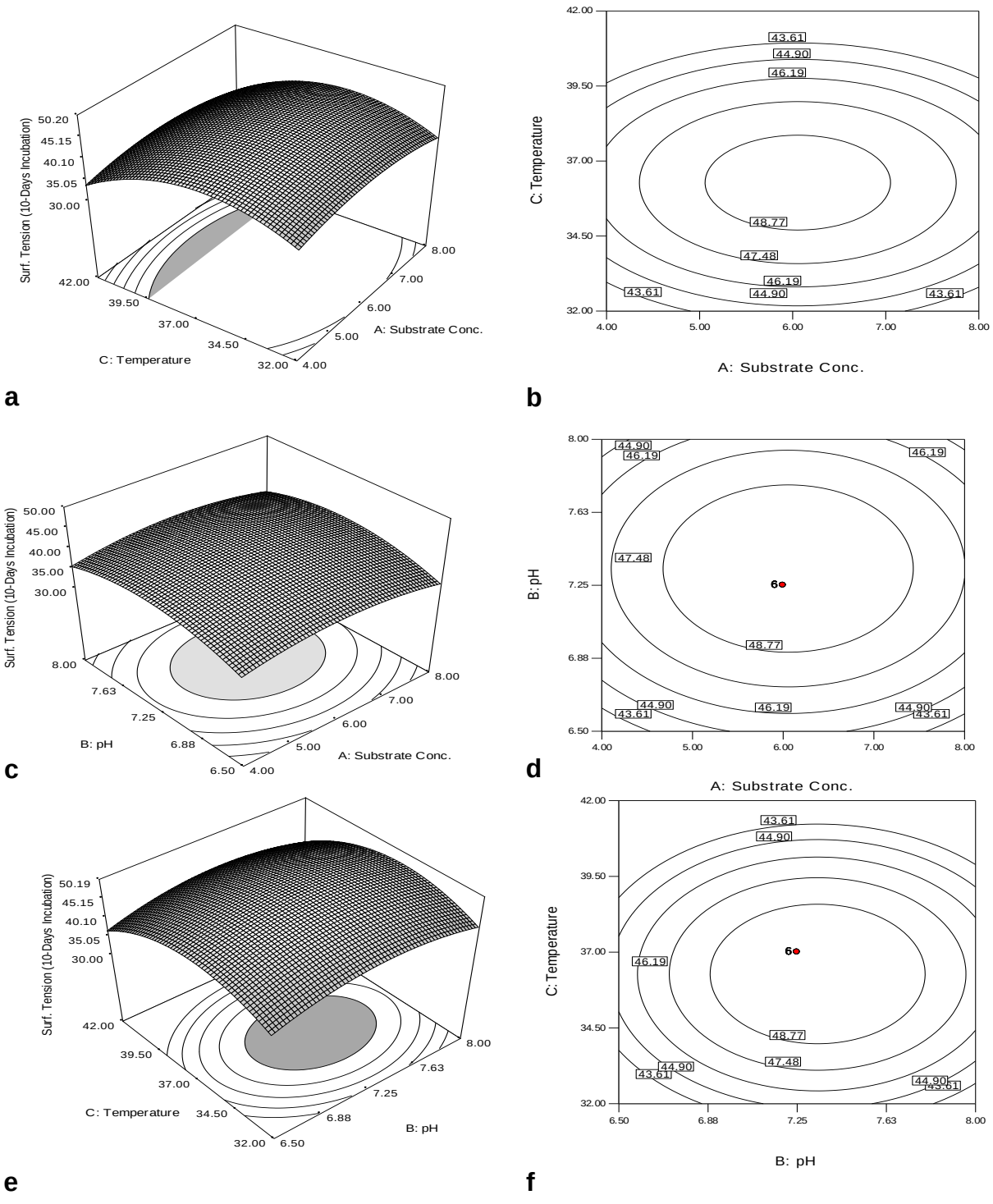

Fig. 3. 3-D plots a, c, and e and contour plots b, d, and f show the interactive effects of the independent variables on the effectiveness of the biosurfactant produced

It was equally observable from the responses measured that substrate concentration and temperature played more significant roles as seen in the experimental runs at temperatures 42 and 45 °C and at substrate concentrations of 2 and 4% (w/v). In Fig. 2d, the contour plot was not perfectly elliptical, indicating fewer interactions between the

independent variables. The composition and quantity of the substrate have been identified as the most important factors affecting the production of biosurfactant (Das *et al.* 2009; Joshi *et al.* 2007). The results of this study supported that observation.

Process Optimization

The optimization of the response in this study was carried out by the numerical option of the Design-Expert® software, in which the input factors were combined to achieve peak process performance. In the numerical optimization process, the desired goal for each process variable and response is selected. The weight or importance can give more or less emphasis on an individual goal relative to the others.

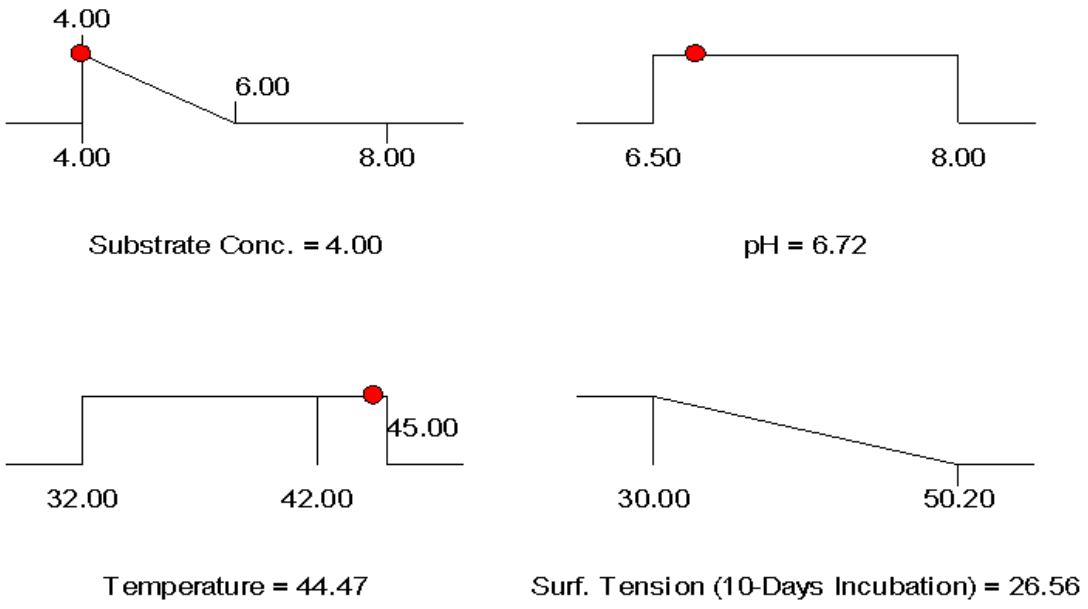

Fig. 4. Desirability ramp for the numerical optimization of three independent variables: substrate concentration, pH, and temperature

The input variables can be set to maximize, minimize, target, within range, or none, while the response is often set to minimum or maximum. In this analysis, substrate concentration was set at target; pH and temperature were set within range. The response was set at minimum, since the desirable optimum is the combination of the independent variables that will give the maximum reduction in surface tension. Design-Expert® software searches for and lists solutions to match the set criteria from the most to the least desirable – desirability ranges from zero (*i.e.* at least one goal was unachievable) to one (*i.e.* all goals were easily met). Fig. 4 shows the desirability ramp generated from 10 solutions via numerical optimization. The optimum point with the maximum desirability function was selected. Hence, the optimum condition for the maximum surface tension reduction of 26.56 mN/m was found to be at a pH of 6.72, a substrate concentration of 4% (w/v), and a temperature of 44.47 °C. The experiment conducted at these optimum conditions with the *B. licheniformis* STK 01 isolated, produced a biosurfactant which

lowered the surface tension of broth to 30 mN/m when the organism was grown on B. vulgaris, and 23.5 mN/m, when grown on refined substrate. To the best of our knowledge, this is the lowest ever reported for a biosurfactant, other than a study by Burgos-Diaz *et al*. (2011), where a surface tension reduction to 22.0 mN/m was reported, in which case, the authors suspected the activity of a consortium.

CONCLUSIONS

1. The surface tension reduction of culture broth to 30 mN/m reported in this study suggests the novelty of the microbial isolate in its ability to utilize solid agro-waste for growth and biosurfactant production without supplementation with refined nutrients, yielding results comparable to those reported for refined substrates. No work has thus far been reported on the utilization of *B. vulgaris*, and most likely this study is the first to report the exclusive utilization of solid agro-waste for biosurfactant production.
2. The analysis of response measured from the CCD using response surface plots identified substrate concentration and temperature as the most significant factors affecting biosurfactant production.
3. By numerical optimization, the optimum conditions were found to be a pH of 6.72, a substrate concentration of 4% (w/v), and a temperature of 44.47 °C, under which a surface tension reduction to 26.56 mN/m was predicted. The experiment conducted to validate the optimum conditions specified by the RSM in this study showed remarkable results. The biosurfactant produced on *B. vulgaris* within 96 h lowered the surface tension of the broth to 30 mN/m, while that which was produced on glucose, at the same optimum conditions, lowered the surface tension to 23.5 mN/m – this is one of the greatest surface tension reductions ever reported for biosurfactant.
4. The biosurfactant produced showed a high emulsification tendency for hydrocarbon, giving a 70% emulsification index for paraffin oil, which suggests its suitability for use as bioemulsifier.
5. The structural information provided by FTIR indicated that the biosurfactant might be a cyclic lipopeptide.
6. The biosurfactants' ability to lower surface and/or interfacial tension, thereby enhancing the emulsification of hydrophobic compounds rather than the quantity produced, is often the measure of their effectiveness. Thus, more attention should be focused on optimization of the surface activity of biosurfactants. Moreover, the cutting edge in this area of research appears to be the production of effective biosurfactants at a reasonable cost; the exclusive application of renewable substrates could also ensure the sustainability of the process, particularly for the enhanced bioremediation of environmental contaminants.
7. This study has provided a basis for further investigation on the kinetics of biosurfactant production from *B. vulgaris* and possible large-scale fermentation for *B. licheninformis* STK 01 lichenysin production.

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