Emulsification of Hydrocarbons by Biosurfactant: Exclusive Use of Agrowaste

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Novel biosurfactant-producing strains were isolated from hydrocarbon-contaminated environments that exclusively utilize agro-waste as their primary carbon source for the expression of biosurfactants. These were quantified using various standardized methods. Among the agro-waste screened, *Beta vulgaris* (Beetroot) proved to be the most suitable substrate, for which the biosurfactants produced by three bacterial isolates–*B. licheniformis* STK01, *B. subtilis* STK02, and *P. aeruginosa* STK03–lowered the surface tension of the culture media to 30.0, 32.98, and 30.37 mN/m, respectively. The biosurfactants achieved considerable emulsification activity, particularly for heavy hydrocarbons, with the highest emulsification indices being 65.5% and 95% for anthracene and lubricant oil, respectively. The emulsion formed with lubricant oil was thermally stable even up to 50 °C for 21 days. The results showed the proficiency of the novel bacterial isolates used, as well as the suitability of solid agro-waste for biosurfactant production, thus suggesting that exclusive utilization of solid agro-waste is a promising option for use in biosurfactant production for environmental remediation. The outstanding emulsification activity and thermal stability demonstrated by the biosurfactants produced showed their potential applications in enhancing bioavailability and bioremediation of recalcitrant and hydrophobic environmental contaminants.

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INTRODUCTION

The future commercialization of biosurfactants depends on research and development studies that can identify better, low-cost, renewable substrates to develop eco-friendly processes for the sustainable synthesis of suitable bioproducts. Compared to the traditional use of synthetic surface active agents in soaps, laundry detergent, and personal care products, biosurfactants have applications in many unconventional fields such as polymerization, foods/beverages, cosmetics, pharmaceuticals, petroleum recovery, and environmental remediation (Banat *et al.* 2000, 2010). Biosurfactants are unique organic compounds synthesized biologically from natural or renewable raw materials. Because of their amphiphilic structure and distinctive functional groups, they possess desirable properties, such as wettability, micellization, surface tension lowering, and formation of micro-emulsions between two different phases, which make them suitable for a variety of applications. For environmental bioremediation applications, these properties can enhance the bioavailability of hydrophobic contaminants, thereby

increasing their biodegradation (Pacwa-Płociniczak *et al.* 2011; Soberón-Chávez and Maier 2011).

Most environmental contaminants are hydrocarbon derivatives that are hydrophobic and recalcitrant, thus requiring surface active agents to mobilize them from their repositories–usually sediments and soil–into the aqueous phase for microbial degradation. Consequently, surface tension and emulsification are two important properties used to evaluate and screen surfactants for their capability to enhance the bioavailability of hydrophobic contaminants. An effective surface-active agent should be able to lower the surface tension of the medium enough to create emulsions of two phases and thus enhance the solubility of the hydrophobic compound. Although a surface tension reduction below 35 mN/m has been benchmarked for effective biosurfactants (Barros *et al.* 2008; Fox and Bala 2000; Nitschke and Pastore 2006), studies have shown some biosurfactants with a high capacity for emulsification of hydrophobic organic compounds whose medium surface tensions were above 35 mN/m (Oliveira *et al.* 2013; Lai *et al.* 2009; Rocha *et al.* 2009). Likewise, emulsion stability is an important consideration in environmental applications of biosurfactants. De-emulsification can occur due to acid stimulation and ionization of the constituents of interfacial films as a result of variations in soil temperature, pH, and salinity (Fortuny *et al.* 2007).

Advances in the utilization of agro-waste/agro-industrial waste materials for the production of biosurfactants have been on the rise, as more of these wastes are being identified as appropriate carbon and nitrogen sources (Amodu *et al.* 2014; Sobrinho *et al.* 2013; Sousa *et al.* 2012). Moreover, increasing environmental awareness has necessitated the study of eco-friendly feedstock and products. The possibility of replacing chemical surfactants with those produced biologically may be unrealistic in the near future, in spite of stringent environmental laws and relatively low availability and high cost of petroleum derivatives used for chemical surfactants' production, unless suitable biosurfactant-producing strains are identified. This quest also includes the availability of low-cost renewable resources such as solid agro-waste, agro-industrial waste, and effluent, which can be used exclusively (*i.e.*, without augmentation with refined sugar or any source of trace elements) for biosurfactant production. This could be one of the options to circumvent the low yield and high cost associated with the full-scale commercialization of biosurfactant production. Utilization of agro-wastes in this way will offer a concomitant advantage by reducing the pollution effects caused by these wastes and minimizing their disposal cost. In addition to these advantages, there is a plethora of suitable and easily accessible organic wastes that can be used for biosurfactant production, thus improving the sustainability of such processes. Furthermore, the application of crude surfactants can be as effective as their refined counterparts for certain applications, especially for bioremediation of environmental contaminants (Amodu *et al.* 2013; Kang *et al.* 2010; Kuyukina *et al.* 2005; Mulligan 2005; Nitschke and Pastore 2006).

Some agro-industrial waste/agro-wastes have been identified for biosurfactant production by certain microorganisms, depending on the nutritional composition required by specific microorganisms. They include olive oil mill effluent, biodiesel plant by-products, plant oil residue, distillery and whey waste, potato peels, and rice straw (Das and Mukherjee 2007; Makkar *et al.* 2011; Sobrinho *et al.* 2013; Zhu *et al.* 2013). In these studies, the renewable resources are often supplemented with refined glucose and other compounds, such as nitrogen sources and trace elements, required by the microorganisms. However, the exclusive application of agro-waste can offer considerable

cost-effective and sustainable systems for the production of biosurfactants and easy adaptation for *in-situ* bioremediation of environmental contaminants. Microorganisms differ in their requirements for carbon sources, including quantities, as well as for other requisite micronutrients, for their metabolic activities. This makes it necessary to identify suitable agro-waste for each isolate that has shown a tendency for biosurfactant production on refined substrates. Hence, the objectives of the present study were to isolate and identify biosurfactant-producing strains and suitable agricultural solid waste for exclusive use by the isolates for biosurfactant production; to investigate the capacity of the produced biosurfactants to emulsify hydrocarbons; and finally, the effects of pH and salinity on the stability of the emulsion formed were investigated.

EXPERIMENTAL

Microorganisms

Bacillus licheniformis STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03 are biosurfactant-producing strains from our Laboratory Culture Collection isolated from rotting wood, tar surfaces, and an oil spill site, respectively. They were identified by morphological as well as molecular-16S ribosomal deoxyribonucleic acid (rDNA) sequencing-analysis. The strains were 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 isolates was extracted using a Powersoil® DNA isolation kit (MOBIO laboratories; San Diego, USA). The total genomic DNA of the strains 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-Aldrich; USA), using ethidium bromide (10 μg/mL) to ensure that the fragments of the correct size were amplified. A 10-μL sample of the amplified product was added to 1 μL of the tracking dye, followed by loading onto the gel, which was visualized using a UV trans illumination procedure (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 compared with known nucleotide sequences, listed in the NCBI Genbank database.

Screening of Agro-waste for Biosurfactant Production

Several types of agro-waste, namely *Pyrus communis* (Pear, P), *Ananas comosus* (Pineapple, PP), *Citrus sinensis* (Orange, OR), and *Beta vulgaris* (Beetroot, B) were screened as primary substrates for biosurfactant production, as was the combination of each of the wastes with *Saccharomyces cerevisiae* (spent Brewers' yeast, BY). The nutritional compositions of these agro-wastes are listed in Table 1. These prospective substrates were obtained as waste from a fruit and vegetable processing facility within close proximity of Cape Peninsula University of Technology, Cape Town campus, with the exception of *S. cerevisiae*, which was obtained from a nearby brewery.

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Table 1. Nutritional Compositions per 100 g of Agro-Waste Screened for Biosurfactant Production

Nutritional composition	Agrowaste			
	Ananas comosus (Pineapple, PP)	*Beta vulgaris* (Beetroot, B)	*Citrus sinensis* (Orange, OR)	*Pyrus communis* (Pear, P)
Energy, kcal	50	43	47	58
Water, g	86	88	86.75	89.8
Protein, g	0.54	1.61	0.94	0.5
Carbohydrate, g	13.12	9.96	11.75	13.8
Total sugar, g	9.85	7.96	9.35	4.30
Ca ,mg	13	16	40	9
Fe, mg	0.29	0.8	0.10	0.17
Mg, mg	12	23	10	7
P, mg	8	64.6	14	11
K, mg	109	325	181	119
Na, mg	1.0	78	0	1.0
Zn, mg	o.12	0.35	0.07	0.1
Vitamin C, mg	47.8	8.1	53.2	6.1

Source: USDA National Nutrient data base (USDA 2011)

The agro-wastes were milled and oven-dried at 70 °C for 72 h and then pulverized into particles with diameters of less than 0.30 mm. Then, 250-mL Erlenmeyer flasks containing 100 mL of the culture media, *i.e.*, 5% (w/v) of each of the agro-wastes in distilled water, were prepared and autoclaved for 15 min at 110 °C. The cultures were allowed to cool to room temperature, inoculated with a 10% (v/v) inoculum of isolate cultures grown overnight subsequent to incubation at 37 °C and 180 rpm for 96 h. Each experiment was carried out in duplicate for the three isolates used, while uninoculated samples served as controls. Suitable substrates were identified by assaying the activity of the broth supernatants using the following standard methods: drop-collapse, oil displacement, emulsification index, and surface tension determination. Figure 1 demonstrates the procedure followed to select an appropriate agro-waste for biosurfactant production.

Biosurfactant Production and Extraction 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, obtaining a supernatant containing the biosurfactant, which was used for the screening methods. Crude biosurfactant was obtained from the cell-free supernatant by adjusting the pH to 2.0 using 1 M HCl, keeping it at 5 ± 1 °C overnight, followed by centrifugation at 15,000 rpm and 4 °C for 20 min to obtain the precipitate. For further purification, the crude surfactant was dissolved in 5 mL of distilled water and extracted using three cycles with an equal volume of a 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 functional group analysis using Fourier transform infrared spectroscopy (FTIR). Biosurfactant samples were prepared for FTIR assays by milling the extracts with KBr subsequent to pressing with an 8,000-kg load (Specac Bench-Top Hydraulic Presses) for 20 min to form translucent disks. IR spectra were monitored from 400 to 4000 wave numbers (cm^{-1}) using an FTIR spectrophotometer. Spectra showing the

functional groups were used to study the composition of the biosurfactant. Absorption spectra were plotted using a built-in plotter, while the KBr disk was used as a background reference. Pure biosurfactant obtained from Sigma-Aldrich (98% pure Surfactin) was used as a control.
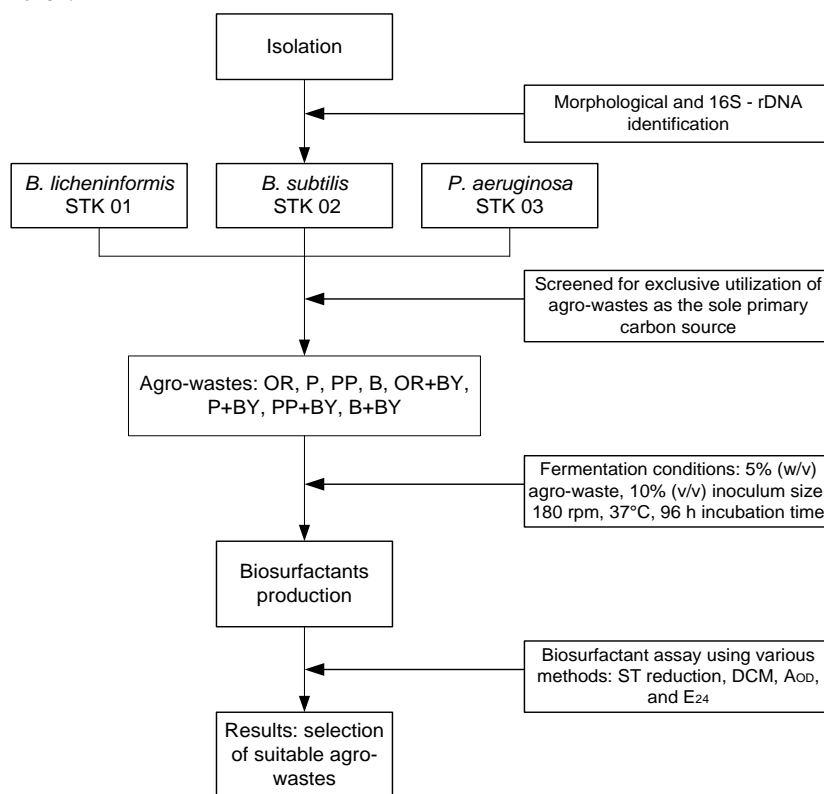

Fig. 1. A flow chart showing the summary of experimental procedure for the selection of suitable agro-waste for biosurfactant production. 'OR' – *Citrus sinensis*, 'PP'- *Ananas comosus*, 'P' – *Pyrus communis*, 'B'- *Beta vulgaris*, 'BY'- *Saccharomyces cerevisiae*, 'ST'- Surface tension, 'DC'- Drop collapse, 'A_{OD}'- Oil displacement activity, 'E_{24}'- Emulsification index

Biosurfactant Activity Assay

Drop collapse test

Drop collapse tests were carried out according to the method described by Jain *et al.* (1991) and Bodour and Miller-Maier (1998). Mineral oil (4 µL) was added into the well regions of a 96-well micro-plate and allowed to equilibrate for 24 h, which was followed by the addition of 5 µL of the cell free culture broth onto the oil-coated regions while the drop size was observed for 5 min with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the oil drop diameter was at least 1 mm larger than that produced by de-ionized water (control). A 0.5% (v/v) Tween® 20 solution was used for comparison.

Oil displacement assay

Oil displacement assays were performed according to the method described by Morikawa *et al.* (2000); 40 mL of distilled water was added to a Pyrex Petri dish followed by the addition of 20 µL of mineral oil to the surface of the water. Thereafter, 10 µL of cell-free supernatant from the culture broth was added to the oil surface. The presence of a biosurfactant was indicated by a clear zone on the oil surface, while the

diameter size of the cleared zone or displaced oil signified the biosurfactant activity. A negative control was maintained with distilled water (without biosurfactant), in which no oil displacement or clear zone was observed, while Tween® 20 (0.5% v/v) was used as a positive control. Oil displacement activity (A_{OD}) was determined as:

$$A_{OD} = \frac{\text{Diameter of cleared zone or displaced oil}}{\text{Diameter of oil surface}} \times 100 \qquad (1)$$

Surface Tension Determination

The surface tension of the biosurfactant-containing culture broth was determined according to Podlogar *et al.* (2004). The surface tension of the cell-free supernatant was determined with a Kruss Processor Tensiometer (model K 100, Germany) at 25 ± 0.5 °C, using the Wilhelmy plate method (Gannon and Faber 1978). The results presented were averages of duplicate measurements from two flasks.

Emulsification Index

The emulsification index (E_{24}) was determined as reported by Cooper and Goldenberg (1987). Six hydrocarbons, *i.e.*, mineral oil, kerosene, diesel, lubricant motor oil, anthracene, and phenanthrene, were added to a cell-free supernatant containing the biosurfactant (6 mL hydrocarbon:4 mL biosurfactant) in a test tube and homogenized by vortexing vigorously for 2 min. The mixtures were left to stand for 24 h, and the emulsion index (E_{24}) was calculated as indicated in Eq. 2. Tween® 20 (0.5% v/v) was used as the control.

$$E_{24} = \frac{\text{Total height of the emulsion}}{\text{Total height of aqueous phase + emulsion}} \times 100 \qquad (2)$$

Stability Assay

Stability assays were carried out using the cell-free supernatant containing the biosurfactant, obtained by centrifugation as described above. The pH of the biosurfactant was adjusted using 1 M HCl or 1 M NaOH in the range 2 to 12, after which the emulsification index of the samples was determined. Similarly, the effect of salinity on biosurfactant emulsification ability was investigated at varying concentrations of NaCl (4 to 10%, w/v). In both assays, the stability of the emulsion formed was assessed at different temperatures for 21 days.

RESULTS AND DISCUSSION

Microbial Identification

The morphological identification showed that two of the isolates were Gram-positive and spore-forming *Bacilli* spp. with reddish-pink rod-shaped colonies, while the third was a Gram-negative non-spore-forming strain, identified as *Pseudomonas* spp. The DNA sequence revealed that the Gram-positive biosurfactant-producing isolates were closely related to *Bacillus licheniformis* strains ZML-1 (96%), SCCB-37 (96%), and 1-FTM8 (96%) and *Bacillus subtilis* strains ZBSF-1 (98%) and SML-2 (98%). The isolates were thus identified as *Bacillus licheniformis* and *Bacillus subtilis*, respectively. *Bacillus licheniformis* was designated strain STK 01, while the *B. subtilis* strain was STK 02.

Similarly, the Gram-negative strain isolated belongs to the clad of *Pseudomonas aeruginosa*, sharing the highest similarity with strains AMBAS7 (97%) and SK9 (97%). Hence, it was identified as *Pseudomonas aeruginosa* and designated strain STK 03. It has been suggested that a bacterial strain can be regarded as novel when the genomic similarity to its closest neighbor is less than or equal to 97% (Stackebrandt and Goebel 1994). This correlation was, however, revised in 2005, recommending a nucleotide sequence similarity value of 98.5%, based on the DNA-DNA hybridization data used for delineating species (Stackebrandt and Ebers 2006; Stackebrant 2011). With respect to this recommendation, the bacterial strains isolated in this study were regarded as novel strains. The publication of their full genomic sequence is underway.

Identification of Suitable Agrowaste Substrates for Biosurfactant Production

The isolated strains *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 have shown biosurfactant-producing properties on refined substrates, reducing the surface tension of broth to 28.5, 30.2, and 32.0 mN/m, respectively. Thus, to identify appropriate renewable substrates that can be used by the microorganisms exclusively for growth and for biosurfactant production, several agro-waste/products were screened. The results obtained from the screening methods showed that the three bacterial isolates were able to utilize *Beta vulgaris* waste, as shown in Tables 2 and 3. The explanation for this observation could be adduced based on the nutritional composition of *B. vulgaris*. In addition to the sugar content of the agro-wastes (Table 1), which serves as carbon and hydrogen source, *B. vulgaris* is richer in essential macronutrients required for microbial cell structure and metabolism.

For the drop collapse test (Table 2), distilled water and Tween 20 were used as negative and positive controls, respectively.

Table 2. Drop Collapse Assay for the Screening of Various Agro-Wastes for Biosurfactant Production

Agro-waste	Biosurfactant activity from various agro-waste		
	B. licheniformis STK 01	*B. subtilis* STK 02	*P. aeruginosa* STK 03
Citrus sinensis, OR	+	NA	+++
Ananas comosus, PP	++	++	NA
Beta vulgaris, B	+++	+++	++
Pyrus communis, P	+	NA	NA
OR + BY	+	+	++
PP + BY	++	++	NA
B +BY	+++	+++	++
P + BY	NA	NA	NA

'NA' – no drop collapse, '+++'- complete collapse within 2 min, '++'- collapse after 2 min, '+' – collapse after 4 min of biosurfactant addition, 'BY' - *Saccharomyces cerevisiae*. Controls: distilled water: 'NA', Tween 20: '+++'

The effectiveness of the biosurfactant produced was displayed by a rapid and complete collapse of its droplets on oil surfaces, as observed for *B. vulgaris*, and also when it was supplemented with *Saccharomyces cerevisiae*. However, some of the agro-wastes used were found to be unsuitable for biosurfactant production by the microorganisms, as the droplets of their supernatants maintained an oval shape on oil surfaces, similar to the experiments in which water droplets were used. For instance, the broth obtained after 96 h of fermentation of *Pyrus communis* with the three microorganisms did not seem to demonstrate any surface-active properties. Meanwhile, *Citrus sinensis* was shown to be a suitable nutrient source only for *P. aeruginosa* among the three microorganisms. This microbial selectivity of agrowaste substrate has been reported (Kumar *et al.* 2011; Singh *et al.* 2007).

Similar results and trends were observed for the surface tension studies. Considerable surface tension reduction was achieved by the crude biosurfactant produced from *B. licheniformis* and *B. subtilis* on *Beta vulgaris*. These strains reduced the surface tension of broth to 30.2 and 32.98 mN/m, respectively. Nonetheless, *P. aeruginosa* seemed to thrive more on *C. sinensis*, giving a surface tension reduction of 29.06 mN/m (Table 2). Comparable surface tension reductions have been reported for some agro-wastes/agro-industrial wastes, such as oil refining extracts and waste, distillery and whey waste, potato peels, cassava wastewater, and rice straw (Amodu *et al.* 2013; Makkar *et al.* 2011; Nitschke and Pastore 2006).

Table 2. Surface Tension Determination of Biosurfactants Produced from Various Agro-Wastes

Agro-waste	Surface tension (mN/m)			
	B. licheniformis	*B. subtilis*	*P. aeruginosa*	Control
Citrus sinensis, OR	39.64 ± 0.01	40.15 ± 0.02	29.06 ± 0.04	43.05 ± 0.01
Ananas comosus, PP	37.66 ± 0.02	38.91 ± 0.01	39.86 ± 0.01	45.07 ± 0.01
Beta vulgaris, B	30.20 ± 0.03	32.98 ± 0.05	30.37 ± 0.01	45.30 ± 0.02
Pyrus communis, P	46.81 ± 0.01	47.68 ± 0.02	45.05 ± 0.04	47.42 ± 0.02
OR + BY	43.05 ± 0.02	43.20 ± 0.01	35.04 ± 0.03	45.50 ± 0.03
PP + BY	41.62 ± 0.01	42.04 ± 0.01	41.52 ± 0.01	47.20 ± 0.02
B + BY	31.53 ± 0.01	44.09 ± 0.02	41.08 ± 0.01	41.35 ± 0.01
P + BY	48.00 ± 0.03	48.25 ± 0.01	46.80 ± 0.02	53.8 ± 0.01

'BY': *Saccharomyces cerevisiae*

Additionally, *S. cerevisiae*, even though a good protein source, did not enhance biosurfactant production when used to supplement agrowaste in this study.

Biosurfactants produced from the various agro-wastes were further screened using the oil displacement method (Fig. 2), which showed the spreading and wettability effects of the produced biosurfactants. These are essential properties required for surface active

agents used for industrial cleaning, bioremediation of hydrophobic contaminants, and oil recovery from reservoirs (Banat *et al.* 2010). Again, the results obtained were similar to those observed for the surface tension and drop collapse methods. A 95% oil displacement was produced by biosurfactants from *B. subtilis* and *P. aeruginosa*, both microorganisms grown on *Beta vulgaris* waste, as compared with 85% observed for 0.5% Tween 20 used as the control. The assessment also showed that none of the microorganisms could use *P. communis* waste as a nutrient source for biosurfactant production.
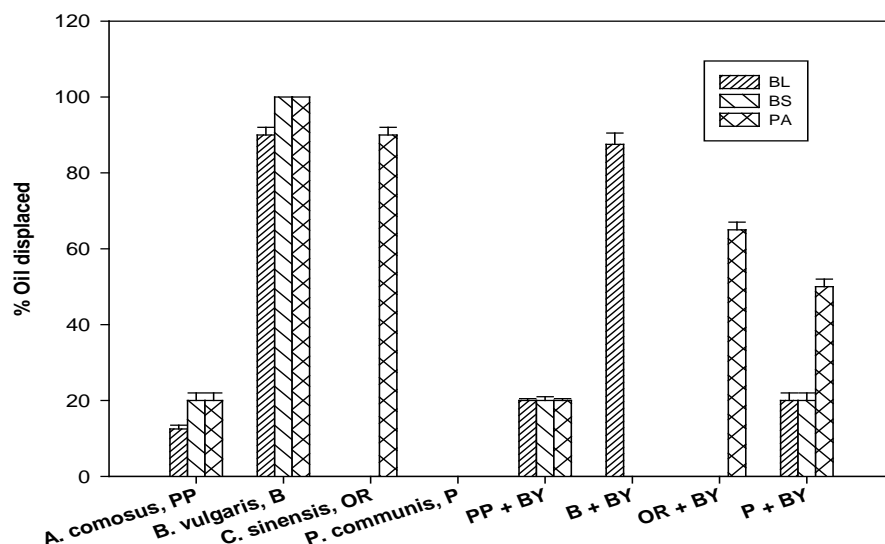
Fig. 2. Oil displacement activity of biosurfactants produced exclusively from solid agro-waste. 'BY'- *Saccharomyces cerevisiae*, 'BL'- *Bacillus licheniformis* STK01, 'BS'- *Bacillus subtilis* STK02, 'PA'- *Pseudomonas aeruginosa* STK03; Controls: Distilled water and 0.5% Tween 20. Tween 20 gave 85% oil displacement

Microorganisms differ in their nutrient requirements, compositions, and fermentation conditions, which could influence their metabolic activities (Coulon *et al.* 2005). Hence, in prospecting for suitable renewable substrates for microbial growth and biosurfactant production, it is expedient to perform a screening test for surface activity on the fermented broth rather than screening based on nutritional and chemical compositions. This study shows the possibility of effective biosurfactant production from solid agro-waste without supplementation with refined nutrient sources.

Biosurfactant Activity Assay

Furthermore, the activity of biosurfactants produced by the microorganisms was assayed against different hydrocarbon compounds by the emulsification method, as shown in Figs. 3 and 4. The ability of biosurfactants to create emulsions of hydrocarbon compounds, and thereby increase their bioavailability, is often used as a basis for determining their effectiveness in environmental bioremediation of hydrophobic contaminants. Emulsions are formed when a liquid phase is dispersed as microscopic droplets in another liquid phase. The biosurfactant produced showed a high hydrocarbon emulsification index, particularly for heavy hydrocarbons (Fig. 3). The highest emulsification values recorded for biosurfactants produced by *B. licheniformis* STK 01 were 49, 65.5, and 95% for phenanthrene, anthracene, and lubricant oil, respectively. The

biosurfactant from *P. aeruginosa* STK 03 gave a 66.7% emulsification index for phenanthrene, while a 70% index was recorded for kerosene. Similarly, the biosurfactant produced by *B. subtilis* STK 02 showed a 90% emulsification index for lubricant oil, but failed to emulsify phenanthrene and anthracene. The results obtained in this study were similar to those reported by Sumiardi *et al.* (2012), whereby the highest emulsification index of 93.7% was achieved for a hydrocarbon compound by a bacterial consortium. Emulsification indices in the range of 69 to 71% were also reported for diesel and kerosene by a biosurfactant expressed by *Agrobacterium* spp. QS-6 (Lai *et al.* 2009). Oil emulsification using biosurfactants can be influenced by some thermodynamic and rheological properties of the system, including aqueous phase composition (salinity and pH), organic phase composition, emulsion-stabilizing nature of the biosurfactants, the presence of fine particulates, and temperature (Kosaric 1992). This result shows the stability of these isolates for biosurfactant production, particularly for applications in environmental bioremediation of heavy hydrophobic contaminants, whereby the bioprocesses used are supported by cheap and easily accessible agro-waste substrates.

The surface activity of biosurfactants produced by *B. licheniformis* STK01 and *P. aeruginosa* STK03, grown on the same agro-waste, was compared in this study. The two microorganisms produced biosurfactants that both lowered the surface tension of the fermented broth to 30 mN/m. However, Fig. 3 shows a disparity in their emulsification activity. The biosurfactant produced by *B. licheniformis* STK01 exhibited a better emulsification tendency for all the hydrocarbons used, except for kerosene. This shows that surface tension reduction only may not be appropriate to assess the emulsification capacity of biosurfactants.
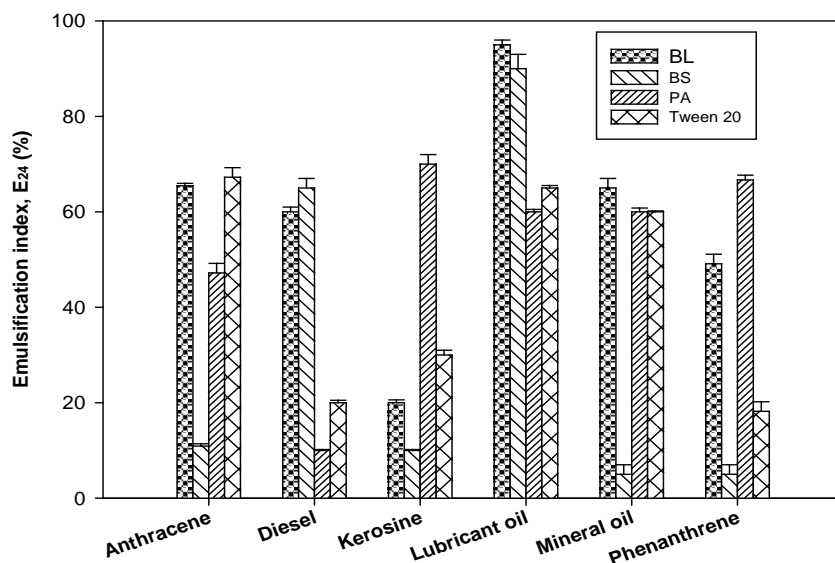

Fig. 3. Emulsification activity of biosurfactants produced by *Bacillus licheniformis* STK 01(BL), *Bacillus subtilis* STK 02(BS), and *Pseudomonas aeruginosa* STK 03(PA) exclusively from *Beta vulgaris*. 0.5% Tween 20 was used as a positive control while uncultured broth of *B. vulgaris* was used as a negative control which showed 20 and 10% E24 for lubricant oil and mineral oil respectively, and zero for the other hydrocarbons

The two microorganisms, even though they were grown on the same solid agrowaste, had different metabolic pathways and thus produced biosurfactants with different functional groups. This in turn affects the formation and stability of the

hydrocarbon emulsion. A similar scenario has been reported (Oliveira *et al.* 2013; Lai *et al.* 2009; Rocha *et al.* 2009), whereby it was hypothesized that the different metabolic activities of biosurfactant-producing microorganisms affect the chemical structure and functional groups of the biosurfactant produced, and thus the emulsification index.

Figure 4 allows a comparison to be made between the emulsification activities of biosurfactants produced from two different agro-wastes - *A. comosus* and *B. vulgaris* – but by the same microorganism. The biosurfactant produced by *B. subtilis* STK02 from *A. comosus* waste lowered the surface tension of broth to 38.91 mN/m, while that which was produced from *Beta vulgaris* reduced the surface tension to 32.98 mN/m, as shown in Table 2; the latter demonstrated a higher emulsification tendency, as expected. The results obtained showed that emulsification activity of biosurfactants produced by the same microorganisms, for particular hydrocarbons, is proportional to the extent to which they can lower the media surface tension, irrespective of the nutrient sources used. This is thus important when assessing the emulsification tendency of biosurfactants based on their capacity to lower surface/interfacial tension.
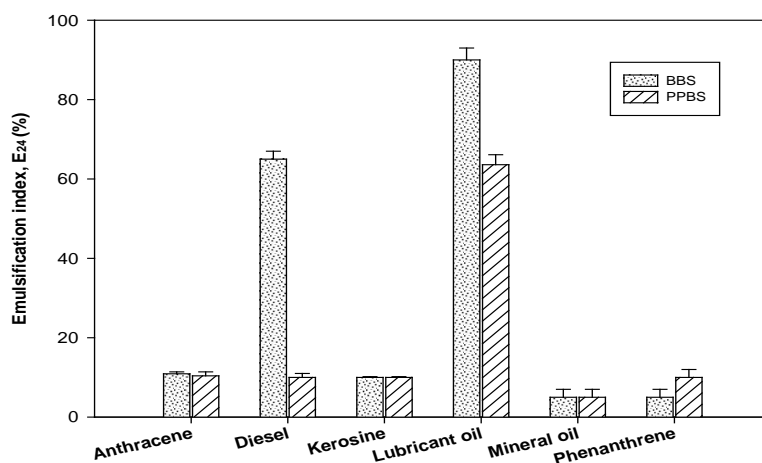

Fig. 4. Comparison of the emulsification index of biosurfactants produced by *Bacillus subtilis* STK 02 grown exclusively on *B. vulgaris* (BBS) and *A. comosus* (PPBS)

Emulsion Stability

Biosurfactant emulsion stability under varying conditions is of great importance, as it can impair their applicability. De-emulsification may not be desirable in most environmental applications, whether oil-in-water or water-in-oil emulsion is being considered. The former is encountered during mobilization of hydrophobic contaminants from their sinks, usually sediments or soil matrices, to become available for microbial degradation. The stability of the emulsion formed by the biosurfactant produced by *B. licheniformis* STK 01 while using *Beta vulgaris* waste was investigated at various pH levels, salinities, and temperatures (Fig. 5).

The variation in hydrocarbon emulsification with respect to pH shown in Fig. 5a indicates how much effect the environmental pH can have on the continuous bioavailability of hydrocarbon contaminants. The emulsification index (E_{24}) rose gradually to a maximum at a pH between 5 and 8, with optimum at 6 to 7, and thereafter showed exponential de-emulsification. In the same vein, the highest E_{24} was observed at a salt concentration range of 6 to 7% w/v (Fig. 5b). The hydrocarbon representatives used were lubricant oil and n-hexane, for pH and salinity studies, respectively. This made it

possible to test the stability of the emulsion formed with light as well as heavy hydrocarbons. The changes in the pH of biosurfactant solutions can affect the physicochemical properties of the hydrocarbon and the formation and rigidity of the interfacial films, thus causing the emulsions formed to coalesce. Emulsion stability can be severely upset due to acid stimulation and ionization of interfacial films' constituents (Fortuny *et al.* 2007). Salinity can also have an important effect on hydrocarbon emulsification and stability due to ionization caused by the interaction of ions present in the salt solution with the asphaltenes-resins, aromatic, and saturated hydrocarbons.
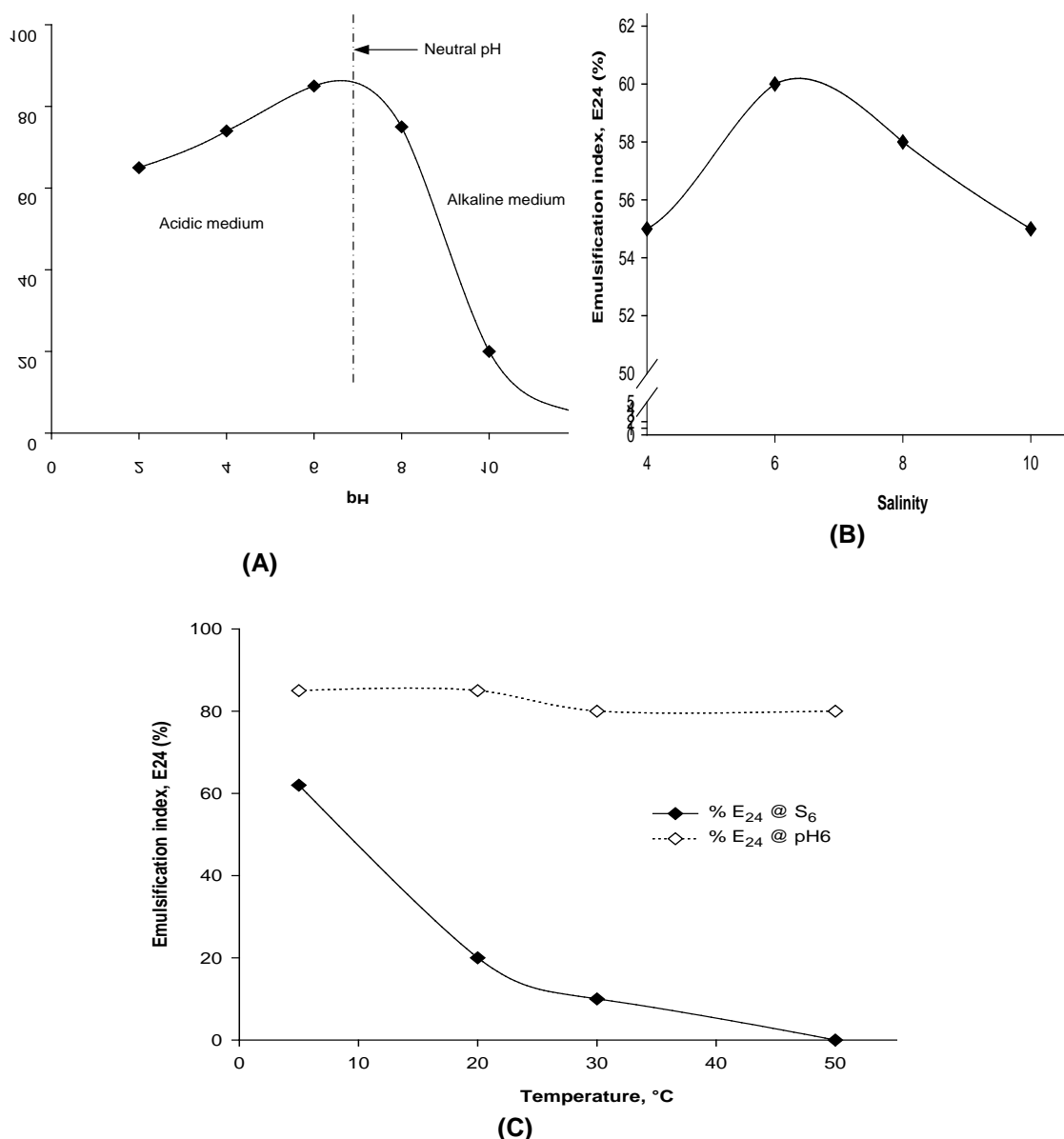

(A)

(B)

(C)

Fig. 5. (a) Hydrocarbon emulsification as a function of pH - demonstrated with lubricant oil; (b) effect of salinity on emulsification- illustrated with n-hexane; and (c) thermal stability of emulsion formed at 6 %(w/v) salt concentration (% E24@S6) and at pH 6 (% E24@pH6)

According to the USDA Natural Resources Conservation Service (USDA 2001) and Natural Resources Management of Queensland (QNRM 2006), most soils have pH

values between 3.5 and 10. Typically, this range contracts to between 5 and 7 during rainfall, whereas in the dry season, the range is from 6 to 9. It has been reported that most soil microbes thrive in a slightly acidic pH range (6 to 7) because of the high bioavailability of nutrients in that pH range (Das *et al.* 2007; Sylvia *et al.* 2005). Seasonal variations in soil moisture, temperature, and plant growth usually cause changes in soil pH and salinity as well as microbial activities, such as, in this case, biosurfactant synthesis and continuous emulsification of environmental contaminants.

Figure 5c shows the variability and the stability of emulsions. The emulsions formed at normal pH (*i.e.*, pH 6) and salinity (*i.e.*, 6% w/v) were subjected to various temperatures. It was observed that temperature plays a major role in emulsion stabilization. Emulsion stability decreased at higher temperatures by affecting the physical properties of oil, water, interfacial films, and surfactant solubility in the oil and water phases. For the salinity stability test, which was demonstrated by n-hexane emulsification, the E24 value decreased significantly and approached zero as the temperature increased. This was due to the high volatility of n-hexane; the interfacial films around the n-hexane droplets coalesced, leading to the de-emulsification of hexane. In fact, about 33% of the hexane evaporated at 50 °C. Lubricant oil emulsion, on the other hand, was relatively stable due to the low volatility of the oil, but could also decrease significantly if the temperature was increased further. According to the USDA (2001), typical soil temperature ranges from 20 to 50 °C throughout the year. This suggests one of the reasons for seasonal variation in the accumulation and bioavailability of environmental contaminants and their biodegradation (Coulon *et al.* 2005; Nedwell 1999). The effect of salinity on emulsion stability may not be severe, except in sites that are prone to erosion, leachate sinks, or areas that are erosion products' repositories, such as sediments. Such environments have higher saline content, but typical soils generally have a neutral salinity (QNRM 2006; USDA 2001).

FTIR Analysis of Biosurfactant Produced by *B. licheniformis* STK 01 Grown on *Beta vulgaris*

The biosurfactant produced by *B. licheniformis* STK 01 *Beta vulgaris* was subjected to further characterization by FTIR, having demonstrated highest surface tension reduction and emulsification tendency for the hydrocarbons used in this study. The IR spectrum of the biosurfactant showed strong absorption bands, elucidating the presence of peptide components at 3368 cm^{-1} for the biosurfactant produced, compared to 3309 cm^{-1} for commercial Surfactin, which emanated from the bond stretching of NH. Figures 6 and 7 show the translucent disks and the chromatograms of the biosurfactant produced, respectively.

Fig. 6. Pictures of translucent disks used for FTIR analysis: A- potassium bromide pellet (used as blank); B- biosurfactant pellet from *Beta vulgaris*; C- pellet of standard surfactin from Sigma Aldrich
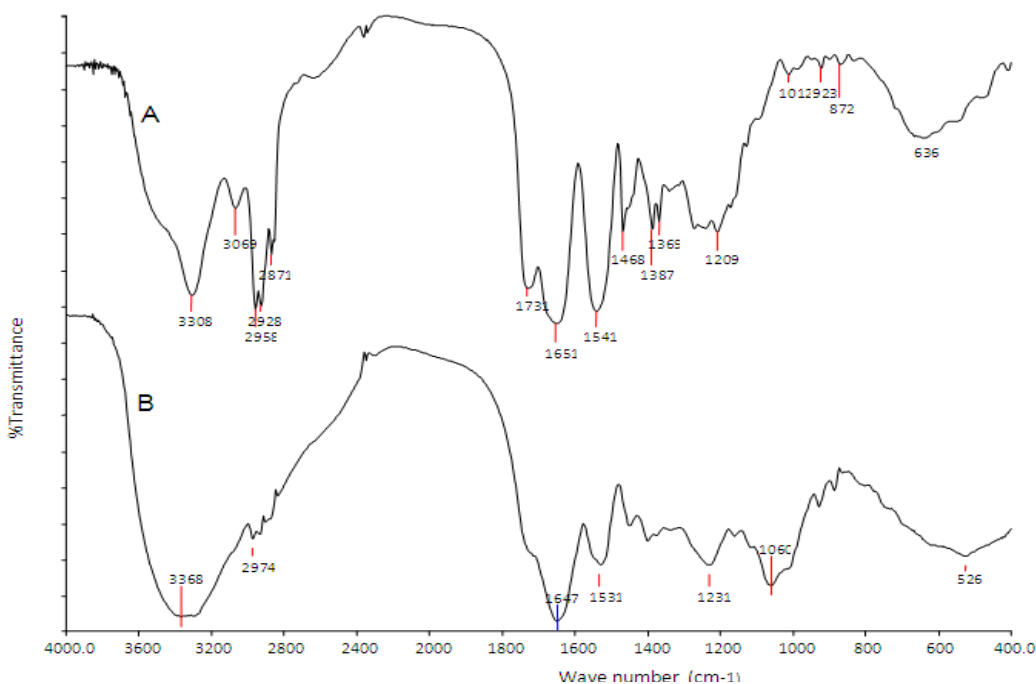
Fig. 7. FTIR of commercial surfactin (A) and crude biosurfactant (B) produced by *Bacillus licheniformis* STK 01, grown exclusively on *Beta vulgaris*

The presence of CO and CN bonds in the two samples is signified by wave numbers 1651 to 1531 cm^{-1}. Also, the presence of an aliphatic group was observed at 3000 to 2850 cm^{-1} for CH$_2$ and CH$_3$, indicating that the biosurfactant produced is a lipopeptide. A carbonyl moiety at 1731 cm^{-1} was observed in the commercial Surfactin, but was not conspicuous in the produced biosurfactant. Similar results have been reported in previous studies (Das and Mukherjee 2007; Oliveira *et al.* 2013). The commercial Surfactin used (98% pure) was obtained from Sigma-Aldrich.

CONCLUSIONS

1. This study revealed that exclusive utilization of solid agro-waste for microbial growth and effective biosurfactant production is feasible and has promising application with a view to enhancing the bioavailability and bioremediation of recalcitrant environmental contaminants.

2. Among the agro-wastes screened for biosurfactant production in this study, *Beta vulgaris* proved to be the most suitable substrate; the biosurfactants produced by the three bacterial isolates–*B. licheniformis* STK01, *B. subtilis* STK02, and *P. aeruginosa* STK03–were able to lower the surface tension of the culture medium to 30.0, 32.98, and 30.37 mN/m, respectively. These surface tension reductions exemplified the suitability of using microbial isolates supported exclusively on agro-waste for biosurfactant production.

3. The FTIR analysis of the biosurfactant produced by *B. licheniformis* STK 01, which demonstrated highest surface tension reduction and emulsification tendency for the

hydrocarbons used in this study, indicated that the biosurfactant produced might be a lipopeptide.

4. The emulsification of heavy hydrocarbons and environmental contaminants by the produced biosurfactants suggests the potential application of the isolates in utilizing cheap agrowaste for biosurfactant production, as well as their application for bioremediation of hydrophobic contaminants in the environment.

5. Investigation of the emulsion formation and stability showed that the highest emulsification occurred at a pH range of 6 to 7 and 6 to 7 % w/v salt concentration, which have been reported to be the ranges of these parameters in typical environmental soils (USDA 2001). This further suggests the suitability of the agrowaste with the isolated microorganisms for continuous bioavailability of environmental contaminants for *in-situ* bioremediation.

6. The study also showed how severe temperature variation can upset emulsion stability, particularly for hydrocarbons with relatively high volatility, thus explaining one of the reasons for seasonal variation in the accumulation, bioavailability, and biodegradation of hydrocarbon contaminants in the environment.

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