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Research Letter

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Biosurfactant production by halophilic yeasts isolated from extreme environments in Botswana

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One sentence summary: Halophilic yeasts from the biggest saltpans in the whole world produce potential antimicrobial agents important in the era of increasing global antimicrobial drug resistance crisis.

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ABSTRACT

Nine morphologically distinct halophilic yeasts were isolated from Makgadikgadi and Sua pans, as pristine and extreme environments in Botswana. Screening for biosurfactant production showed that *Rhodotorula mucilaginosa* SP6 and *Debaryomyces hansenii* MK9 exhibited the highest biosurfactant activity using *Xanthocercis zambesiaca* seed powder as a novel and alternative inexpensive carbon substrate. Chemical characterization of the purified biosurfactants by Fourier Transform Infra-Red spectroscopy suggested that the biosurfactant from *R. mucilaginosa* SP6 was a rhamnolipid-type whereas the biosurfactant from *D. hansenii* MK9 was a sophorolipid-type. The two biosurfactants exhibited antimicrobial activities against eight pathogenic bacteria and fungal strains (*Proteus vulgaris*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Micrococcus luteus*, *Cryptococcus neoformans*, *Candida albicans* and *Aspergilus niger*). The sophorolipid-type biosurfactant was found to be the most potent among the antimicrobial drug resistant strains tested. The findings open up prospects for the development of environmentally friendly antimicrobial drugs that use an inexpensive source of carbon to reduce the costs associated with the production of biosurfactants.

Keywords: biosurfactants; *Xanthocercis zambesiaca*; *Rhodotorula mucilaginosa*; *Debaryomyces hansenii*; antimicrobial activity

INTRODUCTION

The use of biosurfactants as antimicrobial agents in the medical field as an alternative to conventional antimicrobial therapies is becoming more attractive. The current alarming global crisis of antimicrobial resistance further increases their potential (Rodrigues *et al.* 2006; Gomaa 2013; Gardiner *et al.* 2019; Haque 2020; Waghmode *et al.* 2020). These surface-active compounds from biological sources, usually of microbial origin, have hydrophilic and hydrophobic moieties, a property similar to their chemical counterparts produced from petroleum, which allows them to reduce surface tension by preferentially partitioning at the interphase between fluids of different polarity. Over the past few decades, the use of biosurfactants has gained

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more interest compared to their petroleum counterparts. This is due to the increasing global environmental awareness (Georgiou, Lin and Sharma 1992). Their environmental compatibility and acceptability is due to their unique properties such as biodegradability (Razafindralambo *et al.* 1996), high selectivity, and thus low toxicity (Yu *et al.* 2014) as well as the capacity to be produced using renewable raw materials (Schultz and Rosado 2020). Despite all these advantages, there are significant disadvantages that reduce the economic feasibility of biosurfactant production, such as low yields and high costs of production associated with the use of robust strains, as well as the use of costly substrates (Rodrigues *et al.* 2006; Ferreira 2008). In this context, there is a need to identify efficient biosurfactant producers and inexpensive or cost effective raw materials for economic feasibility of biosurfactant production.

The extreme environments of Botswana, such as the Makgadikgadi and Sua saltpans, which are characterised by extremity havens with high pH, high salinity, high radiation, and high temperatures which can harbour microorganisms whose adaptations and survival strategies to remain metabolically active merits exploration for potential biosurfactant producers. The information on novel and versatile biosurfactants from halophilic environments is well documented (Cameotra and Makkar 1998; Schultz and Rosado 2020). The Makgadikgadi and Sua solar saltens harbour a variety of rich novel microorganisms (Gareeb and Setati 2009; Baloi 2011) whose potential for the discovery of surfactants has not been explored. It is noteworthy that microorganisms that produce industrially relevant molecules such as enzymes (Govender, Naidoo and Setati 2009) and antimicrobials (Lebogang and Taylor 2009) have also been described.

In the context of the foregoing, the study is an investigation of halophilic yeast isolates from the Makgadikgadi and Sua salt pans for the production of biosurfactants using the fruit pulps from indigenous wild *Xanthocercis zambesiaca* (Baker) Dumasle-Grand (known colloquially in Botswana as *nyala, motlha* or *mashatu* tree) belonging to the *Fabaceae* family, as a low cost carbon and nitrogen source. *Xanthocercis zambesiaca* is an abundant tree whose fresh fruits are often eaten by livestock resulting in bulky defecated seeds running off with surface water after rains, thus causing associated waste disposal and environmental problems. The fruits/seeds have also been found to be rich in essential oils and amino acids (Ketshajwang, Holmback and Yeboah 1998), attributes that engrossed this particular species to be investigated in the present study as inexpensive sources of carbon and nitrogen in the production of biosurfactants. We present the isolation and characterisation of extremophilic yeasts for their potential for production of biosurfactants using several screening methods. The purified biosurfactants were partially characterised using FTIR, and they were evaluated for their antimicrobial activity against selected human pathogenic fungi and bacteria.

MATERIALS AND METHODS

Sample collection and enrichment of halophilic yeasts

Soil samples (10 from each location) were collected from different regions of the Sua (20.8406°S | 25.9941°E) and Makgadikgadi (20.7914°S | 25.4734°E) saltpans in Botswana. 1 g of each of the soil samples was then inoculated into 100 mL sterile mineral salt medium (MSM) of the composition (w/v); 0.2% KH_2PO_4, 0.2% Na_2CO_3, 0.2% Mg_2SO_4, 0.2% KCl, supplemented with 0.2% yeast extract, 2% mannitol, 0.02% chloramphenicol and NaCl at a pH of 7 (Saimmai *et al.* 2012) and incubated in a continuous orbital shaker at 25°C and 160 rpm for 7 days. Different concentrations of NaCl (5%, 10%, 15% and 20%) were used to determine the optimal conditions of growth for the halophilic yeasts.

Isolation of halophilic yeasts

After 7 days of incubation, 1 mL of the actively growing culture in broth was spread-plated on corn meal agar (CMA) (Merck, Darmstadt, Germany) supplemented with the corresponding concentrations (5%-20%) of NaCl as stated in 'Sample collection and enrichment of halophilic yeasts' section. The plates were incubated at 25°C for 3 to 5 days. Yeast colonies were sub-cultured on fresh CMA plates and observed under the microscope before preserving at –86 °C in 15% glycerol until used for screening assays.

Screening assays for potential biosurfactant producing yeasts

Prior to screening, the actively growing yeast cultures were first adjusted to 1.8 McFaclarland standards, using a DensiCHEK® (bioMeriéux, Hazelwood, Missouri, USA). Yeast isolates selected from Sua pan (SP4, SP6, SP11, SP17 and SP22) and from Makgadikgadi pans (MK1, MK9, MK10, and MK19) were subjected to three screening tests to identify the strains with the most promising biosurfactant activity.

Haemolysis assay

The haemolytic activity was determined as a preliminary test for the production of biosurfactants. Yeast isolates were streaked on 5% (w/v) human blood agar and incubated at 25 °C and 37 °C for 3 days. They were observed daily for alpha-, beta- and gamma-haemolysis, which was recorded, based on zones of clearance in the agar around the colonies (Carrillo *et al.* 1996).

Drop collapsing assay

This was done according to a method described by Youssef *et al.* (2004). Five microlitres of the culture was added to the surface of 2 μL mineral oil in each of the wells of 96-well microtiter plates. After incubation for 1 h at room temperature, the plates were examined for biosurfactant activity. A positive result was recorded when the drop collapsed and recorded as negative when the drop remained beaded up.

CTAB-methylene blue agar test

CTAB-methylene blue agar test was evaluated by an agar well diffusion assay as described by Siegmund and Wagner (1991). The MSB medium was supplemented with Cetyltrimethylammonium bromide (CTAB) (0.2 g/L), methylene blue (0.005 g/L) and agar (17 g/L). Wells with an 8 mm diameter were punched at the center of CTAB-methylene blue agar using a sterile cork borer. Thereafter, 100 μL of standardized yeast cultures were added to each well. The plates were incubated at 25 °C for 7–8 days. Positive results were recorded as a dark blue halo and a yellow zone of clearance around the colony.

Production of biosurfactant using *Xanthocercis zambesiaca* seeds

After confirmatory tests of biosurfactant activity, the strains with the most promising biosurfactant activity were selected for

production of biosurfactant using *Xanthocercis zambesiaca* seeds powder as a potential inexpensive substrate.

Preparation of Xanthocercis zambesiaca seeds powder and chemical analyses

Xanthocercis zambesiaca seeds were obtained from Mokokwana village (22.6429°S | 27.6345°E) in the Central District of Botswana. The seeds were peeled off and then cut into thin slices before drying at 60°C for 12 h. The dried *X. zambesiaca* seed slices were ground into powder (XSP). Chemical analyses of the XSP was conducted according to the Association of Official Analytical Chemists (AOAC 1990). The following specific methods were used for proximate analyses; total fat (method 7.056), total ash (method 14:006), crude fibre (method 14.020), and total protein (method 2.057). The carbohydrate content was determined using the sulphuric acid-UV spectrophotometry method previously described by Albalasmeh et al., (2013). About 20 g/L XSP was used as the only carbon source added to the MSB agar for subsequent experimental procedures.

Production of biosurfactant

The fermentations for biosurfactant production were performed in a Mineral Salt Medium (MSM) (Saimmai *et al.* 2012) supplemented with XSP as the only source of carbon. As positive controls, glucose and olive oil were also examined as the only carbon sources. For optimization, the carbon source, salinity, and temperature were varied in order to investigate the conditions for maximum production of biosurfactant. The culture broths were inoculated into Erlenmeyer flasks with 50 mL of media containing various carbon sources (XSP, glucose and olive oil) supplemented with different concentrations of NaCl (5%, 10% and 15%) and incubated at different temperatures (25°C, 30°C and 37 °C) on an orbital shaker at 180 rpm for 7 days.

Investigation of properties of biosurfactants

Production of biosurfactant was detected by centrifuging the fermented culture broth at 12 500 rpm for 10 min and examining the surface tension and emulsification activities of the cell free supernatant.

Surface tension

The surface tension of the biosurfactants produced by yeast isolates under different conditions (temperature, carbon source and NaCl concentration) was determined using the Du Nuoy ring method in a Kruss K9 (New Canada, USA) digital surface tensiometer as described by Rodrigues *et al.* (2006). Each experiment was run in triplicate and average values were recorded.

Emulsification index

The emulsification index of the biosurfactants produced by nine yeast isolates under different conditions (*temperature*, carbon source and NaCl concentration) was determined using a method described by Cooper (1986). Yeast isolates were grown in 4 mL of Yeast Extract Mannitol Broth (Merck, Darmstadt, Germany) for 7 days. About 2 mL of XSP was then added to the test tubes. The mixture was vortexed at a high speed for approximately 1 minute and then allowed to settle at room temperature overnight. The emulsification index (E_{24}) was then calculated using the following formula;

$$\text{Emulsification index } (E_{24}) = \frac{\text{height of emulsion layer}}{\text{total height}} \times 100$$

Liquid-liquid extraction of biosurfactants

After the yeast had grown in MSM Broth supplemented with Yeast Extract and Mannitol (as described in the 'Sample collection and enrichment of halophilic yeasts' section), the biosurfactants were obtained using the solvent extraction methods described by Saravanan and Vijayakumar (2012). Cell-free supernatant was obtained by centrifugation at 12 500 rpm for 15 min, acidified with concentrated hydrochloric acid to pH 2.0, and it was kept at 4°C overnight, and extracted three times with ethyl acetate in a volumetric ratio of 1:1 leading to the formation of two phases; an upper organic phase, which contains biosurfactant, and a lower phase, which contains residual culture broth. The upper phase containing biosurfactant was then transferred to a clean vial and the solvent was removed using a rotary evaporator (Buchi Rotavapor R-210) (Rose Scientific Ltd, Alberta, Canada) while the lower phase was discarded. The resulting solid was used to partially characterize the biosurfactant using FTIR analysis as described below.

Fourier Transform Infra-Red (FTIR) Analysis

The crude biosurfactants obtained from the liquid-liquid extractions were dried and partially characterised using a Spectrum Two PerkinElmer FTIR (PerkinElmer Inc., Massachusetts, USA) spectrophotometer to determine their functional groups. The sample (1 mg) was dried and pelleted with 100 mg KBr. The infrared spectra were collected 400 to 4000 cm^{-1} and were then analyzed.

Molecular identification of biosurfactant producing yeast isolates

Yeast isolates producing biosurfactants were identified by ITS-PCR sequencing to the species level. DNA was extracted using *Quick*-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) and then amplified with universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) using the following thermocycling conditions: initial denaturation at 94°C for 8 min; followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 12 min in a Techne thermocycler (Cole-Parmer, Staffordshire, UK). Three microliters of each of the amplicons were stained with ethidium bromide and analysed for their quality using 1% agarose gel electrophoresis, before being visualized in a gel documentation system (Bio-Rad, California, USA). The rest of the PCR products were then purified using DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) and then sequenced in both directions with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in an automated ABI 3500XL sequencer (Applied Biosystems).

Sequence analyses

The raw sequencing data was edited using BioEdit (BioEdit. [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]) and examined with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences of halophiles and cognate species downloaded from GeneBank were trimmed, and aligned using MAFFT version 7.307 (Katoh and Standley 2013). A phylogenetic tree was constructed based on the Kimura-2 model parameter using Neighbour-Joining method in MEGA6 software version 6.06 (Tamura *et al*. 2013). Bootstrap analysis with 1000 replications was performed to statistically support the phylogenetic tree.

Antimicrobial activity of biosurfactants

The antimicrobial activity of the biosurfactants was evaluated using the agar-well diffusion method as described by Martínez-Vázquez *et al*. (1999) with modifications. The biosurfactants were tested against eight pathogenic bacteria and fungi (*Proteus vulgaris* ATCC 13315, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 10240, *Cryptococcus neoformans* ATCC 208821, *Candida albicans* ATCC 10231 and *Aspergilus niger* ATCC 1015). The actively growing bacterial and fungal cultures were first adjusted to 0.6 and 1.8 McFaclarland standards, using a DensiCHEK® (bioMeriéux, Hazelwood, Missouri, USA). Inocula (1000 μL) were mixed with Luria Bertani (LB) agar (Merck) for bacteria and potato dextrose agar (Merck) for fungi and allowed to set in 90 mm Petri dishes. Wells of 8 mm diameter were then punched at the center of the media in Petri dishes once the media had hardened using a sterile cork borer. Test solutions (100 μL) containing three different concentrations (0.5, 1 and 2 g/L) of biosurfactant were added to each well for each test microorganism. In addition, a control Petri plate was prepared for each microorganism containing a biosurfactant-free solution. The plates were then incubated for 24 h at 37 °C for bacteria and 72 h at 25 °C for fungi. After incubation, the diameter of inhibition of microbial growth (clear zones around the wells) was measured in millimeters using a ruler. All experiments were performed in triplicate and an average of the results was recorded.

Statistical analyses

In order to compare the effect of the carbon source on the production of biosurfactants by both isolates, the differences in surface tension and the emulsification activity and the effect of the biosurfactants on selected bacteria and fungi, a one-way ANOVA was conducted using STATISTICA, version 13.2 (Statsoft Inc., Tulsa, Oklahoma). We implemented a post-hoc Turkey HSD to test whether the same attributes of the biosurfactant significantly differed between the two isolates. The significance level was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS AND DISCUSSION

Biosurfactant-producing yeasts

Nine yeast colonies of different morphology were isolated from the saltpans. Five of the isolates (SP4, SP6, SP11, SP17 and SP22) were from the Sua pan and four yeast isolates (MK1, MK9, MK10 and MK19) from the Makgadikgadi pans. These isolates were then tested for their potential to produce biosurfactants using a haemolytic blood assay, the drop collapse method, and the CTAB-methylene blue agar test. Screening for the potential to produce biosurfactants using several primary-screening methods was consistent with similar studies (Ariech and Guechi 2015; Yalçın, Ergin-Tepebaşı and Uyar 2018). All isolates showed positive results in the drop collapse test and the CTAB methylene blue tests (Table 1), indicating the presence of biosurfactants in the media. However, only six of these isolates showed positive results in all three tests. This inconsistency is not surprising because of the heterogeneous nature of secondary metabolites as biosurfactants (Youssef *et al*. 2004; Satpute *et al*. 2008). The haemolytic activity by biosurfactant producing microbes, especially in saline environments, is also known to be inconsistent. (Youssef *et al*. 2004; Satpute *et al*. 2008). Of these six isolates, only two, SP6 and MK9, obtained from the Sua Pan and the Makgadikgadi pans produced the best results based on these screening tests. Therefore, the two yeast strains were used in the subsequent tests.

Biosurfactant-producing microorganisms have been isolated from a wide variety of environments (Bodour, Drees and Maier 2003). The potential in the discovery of novel biosurfactants in extremophiles is attractive because of their ability to thrive under such growth-limiting conditions. The current study expanded the search for biosurfactant-producing halophilic yeasts from the Sua and Makgadikgadi saltpans in Botswana. The saltpans in the Kalahari desert are the largest in the world, covering 12 000 km^2 (Cowen and Lindquist 2005). These saltpans are extremely harsh, pristine environments characterised by hot desert summer temperatures of around 45°C along with a very arid ecosystem whose immense potential for the discovery of novel biosurfactant producing yeasts remained underexploited. Extremophilic yeasts from such an environment have the potential to harbour diverse metabolic gene clusters that encode the synthesis of compounds to thrive in such extreme conditions (Bose, Rodrigo-moreno and Shabala 2014). The economic feasibility of biosurfactants requires robust production strains (Santos *et al*. 2016; Manure *et al*. 2020) and use of inexpensive substrates as carbon sources (Cameotra and Makkar 1998). Despite the remarkable opportunity in diversity and extremophilic nature of yeasts from Makgadikgadi and Sua saltpans of Botswana, no cases of industrial exploitation have been reported. Therefore, the objective of the study is to investigate the potential of halophilic yeasts in biosurfactant production.

Identity of the best biosurfactant producers

The sequencing of the ITS1/ITS4 amplicons followed by a Basic Local Alignment Search Tool (BLAST) homology search, showed that the isolate SP6 shared 98% similarity to the *Rhodotorula mucilaginosa* reference strains while isolate MK9 had 99% sequence identity with the *Debaryomyces hansenii* reference strains. This is in accordance with the previous studies, since the two species are halophilic yeasts known to produce biosurfactants (Yalçın, Ergin-Tepebaşı and Uyar 2018). The inference of the evolutionary relationships of the biosurfactant producers and their close relatives based on ITS1/ITS4 amplicons is shown using the Neighbour-Joining method (Fig. 1).

Biosurfactant production using inexpensive *Xanthocercis zambesiaca seeds*

One of the major drawbacks of the feasibility of the biosurfactant as a replacement of its chemical counterparts is the

Table 1. Screening tests for biosurfactant production by yeasts isolated from extreme environments in Botswana.

Sampling location	Yeast isolate	Drop collapse test*	CTAB methylene blue agar	Haemolysis
Sua Pan	SP6	++	++	+
Sua Pan	SP4	++	+	+
Sua Pan	SP22	+	+	+
Sua Pan	SP11	+	+	-
Sua Pan	SP17	+	+	-
Makgadikgadi pans	MK9	++	++	+
Makgadikgadi pans	MK1	+	+	+
Makgadikgadi pans	MK10	+	+	-
Makgadikgadi pans	MK19	++	+	+

*-, no activity; +, moderate activity; ++, good activity

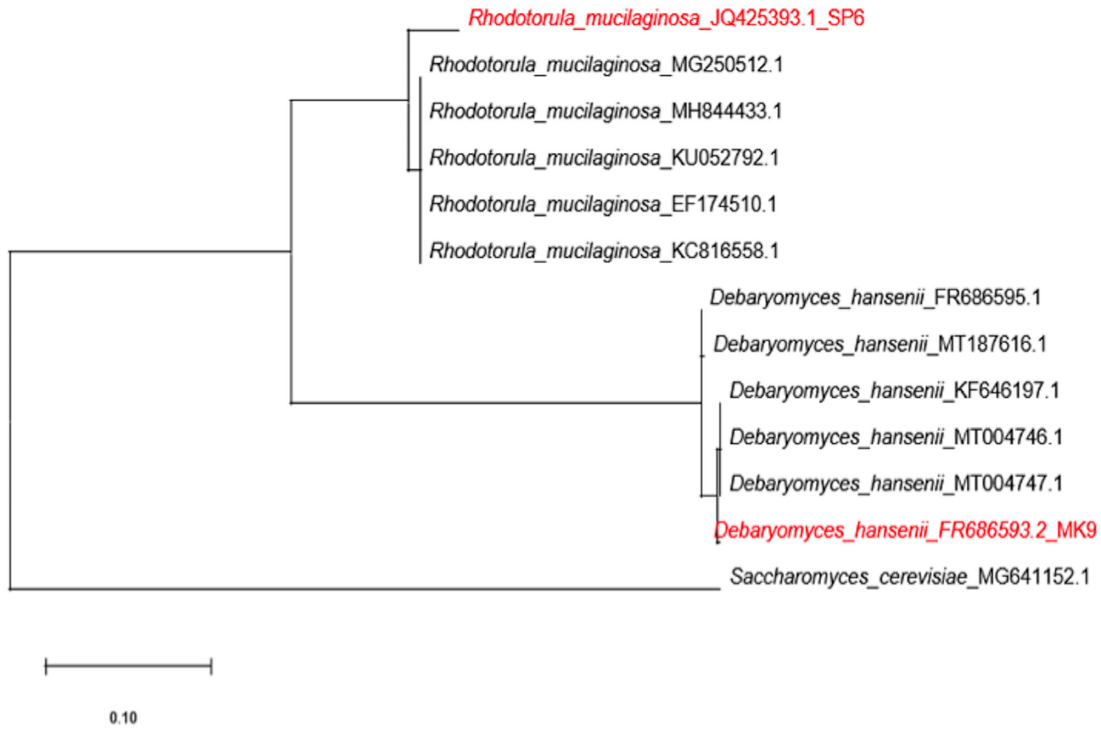

Figure 1. Phylogenetic relationships of the halophilic yeast strains. *Rhodotorula mucilaginosa* SP6 and *Debaromyces hansenii* MK9 ITS sequences with reference to closest strains from National Centre for Biotechnology Information (NCBI) database were used to construct a Neighbour-Joining phylogenetic tree based on the Kimura-2 model using MEGA6 software version 6.06 (Tamura *et al.* 2013). Reference sequences of closely related species used in this analysis were retrieved from GeneBank database.

high production costs (Henkel *et al.* 2012). Using inexpensive substrates for production is an attractive strategy since the cost of substrates generally contributes 50% to 70% of the final production cost of bioproducts (Rufino, Sarubbo and Campos-Takaki 2007). This study evaluated the use of an abundant and inexpensive *X. zambesiaca* seeds as a low-cost carbon substrate in terms of the economic feasibility of biosurfactants over their chemical counterparts. Chemical analyses of *Xanthocercis zambesiaca* seed powder shows that the powder is rich in carbohydrates (42.3 g/100 mL), fats (0.91 g/100 mL and proteins (9.42 g/100 mL). XSP also had 1.88 g/100 mL of ash and 2.31 g/100 mL fibre, suggesting its ability to sustain microbial growth. These results are in agreement with Ketshajwang, Holmback and Yeboah (1998) who reported the presence of various fatty acids and essential oils in *X. zambesiaca* seeds. Other studies have made similar observations using various seed extracts and have obtained results with promising yields and biosurfactant activities (Dumont and Narine 2007; Chooklin *et al.* 2014).

Surface tension activity of the biosurfactant

According to previous studies, good microbial candidates for surfactant production are expected to reduce surface tension to 40 mNm^{-1} or less (Cooper 1986; Olivera *et al.* 2003). The biosurfactant produced by *R. mucilaginosa* SP6 reduced the surface tension of distilled water (32 mN/m) to an average of 26.1 mN/m when XSP was used as the carbon source (Fig. 2 A). In comparison, the biosurfactant was made from glucose and olive oil as carbon sources and it reduced surface tension to an average of 31.2 mN/m and 36.2 mN/m, respectively, thus slightly higher than that of XSP as a carbon source. There was a statistically

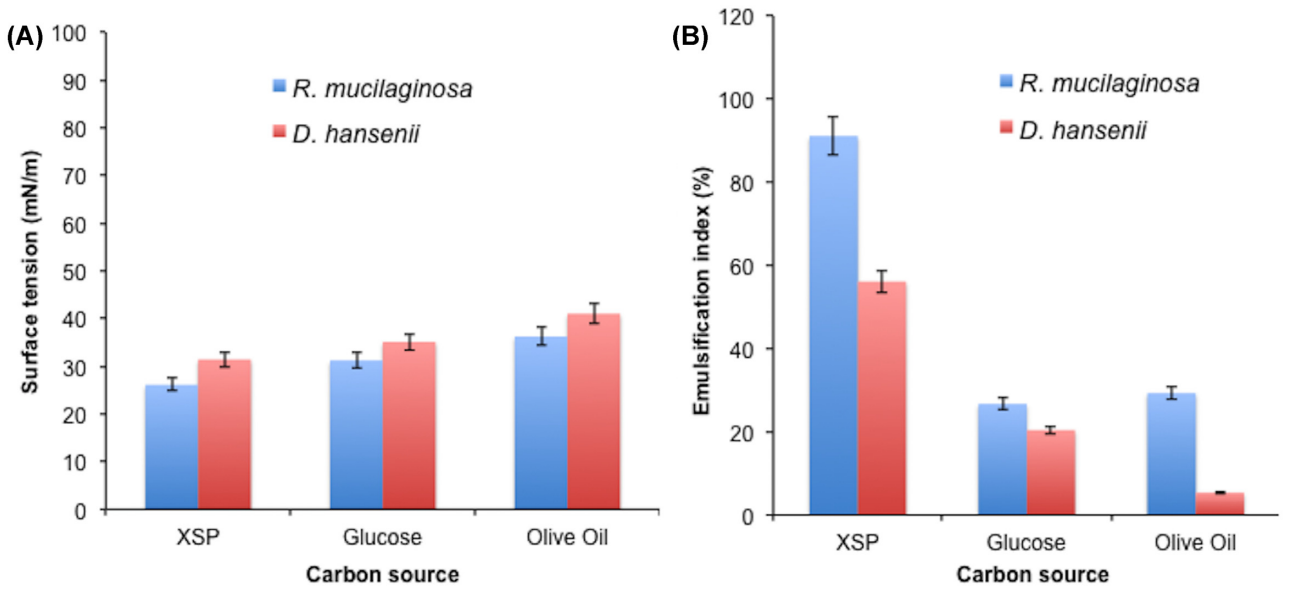

Figure 2. Biosurfactant activity of the two isolates. **A** Surface tension activity **B** Emulsification activity.

significant difference between the surface tension of the biosurfactant produced and the type of carbon source used (ANOVA, $p < 0.01$) Tables S5-S9). On the other hand, *Debaryomyces hansenii* MK9 produced a biosurfactant, which only reduced the surface tension to 31.4 mN/m at an increased NaCl concentration of 10%. There was no statistical difference between the surface tension of the biosurfactant produced and the type of carbon source used (ANOVA, $p < 0.01$). Both isolates reduced the surface tension of the culture medium below the threshold value. Although *D. hansenii* MK9 reduced the surface tension less than in the *R. mucilaginosa* SP6, it was in the range of other yeasts such as *Candida rugosa* (Chandran and Das 2011) and *Candida lipolytica* (Rufino, Sarubbo and Campos-Takaki 2007). This implies that biosurfactant production depends not only on the producer-species, but also on the environmental conditions. This suggests that XSP is one of the attractive alternatives for the economic feasibility of biosurfactant production in Botswana.

Emulsification activity of the biosurfactants

In addition to the surface tension, the emulsification activity of the biosurfactants produced by *R. mucilaginosa* SP6 and *D. hansenii* MK9 when grown on XSP, glucose and olive oil was evaluated. The results suggest that there was a significant difference between the emulsification index of the biosurfactants produced by both yeasts and the substrates used (ANOVA, $P < 0.0001$) (Table S11, Supporting Information). There were significantly higher indices recorded when XSP was used as a carbon source, as compared to when both strains were grown in glucose and olive oil (Fig. 2 B) (ANOVA, $p < 0.0001$)(Supplementary material Tables 7 and 9) suggesting that XSP is a better substrate. *R. mucilaginosa* exhibits the best credentials as a biosurfactant producer as it produced a biosurfactant with a significantly higher emulsification index using XSP as compared to *D. hansenii* (Tukey HSD Test, $P < 0.001$) (Table S12, Supporting Information). Bosch et al. (1988) reported that a promising biosurfactant should have an emulsification index (E24) of more than 50%. Emulsification indices of 91.1% (*R. mucilaginosa*) and 56.1% (*D. hansenii*) were recorded (Fig. 2B).

Functional groups characterisation

The functional groups present in the purified biosurfactants were determined using infrared FTIR spectroscopy (Fig. 3). Figure 3A shows the FTIR spectra produced by the biosurfactant recovered from the *R. mucilaginosa* SP6 isolate. The spectra show broad peaks at the wavenumber 3358.5 cm^{-1} that can be assigned as –OH stretching vibrations of hydroxyl groups. The peak displayed at 1635.8cm^{-1} indicates the presence of a –C = O characteristic of an ester carbonyl group. In addition, the peak at 1390 cm^{-1} is characteristic of C–N characteristic of amide bonds. The peak of 1277.5 cm^{-1} can also be assigned to the C–O and C–O–C bonds of carboxylic acids characteristic of the ester functional group in the structure of the biosurfactant. The details of the biosurfactant spectra are characteristic of the rhamnolipid (Guo *et al.* 2009; Abbasi *et al.* 2012, Elazzazy, Abdelmoneim and Almaghrabi 2015).

On the other hand, the FTIR spectra in Fig. 3B is from the biosurfactant produced by *D. hansenii* MK9. A broad band at 3367cm^{-1} characteristics of hydroxyl groups (–OH), indicates the presence of polysaccharides. The spectrum also showed sharp peaks at 2924.9 and 2852 cm^{-1} indicating that the hydrocarbon chain contains C–H bands from methylene groups (CH_2–CH_2). In addition, the sharp bands at 1742.8 cm^{-1} followed by a weak peak at 1644.5 indicate carbonyl esters, carbonyl lactones or carboxylic carbonyls and non-conjugated olefins (C = C). All of these structural details were characteristic of the sophorolipid spectra reported in the literature (Hu and Ju 2001; Mousavi, Beheshti-Maal and Massah 2015, Claus and Van Bogaert 2017). Other bands could be associated with either group in the sophorolipids structure of the biosurfactant. However, further analysis is required to reveal the detailed structure of these two biosurfactants. Although sophorolipid production by yeasts has been described elsewhere (Claus and Van Bogaert 2017), this study gives the first report on its characterization from *D. hansenii* MK9.

Antimicrobial activity of the biosurfactants

The antimicrobial activity of the partially purified biosurfactants from *R. mucilaginosa* SP6 and *D. hansenii* MK9 was eval-

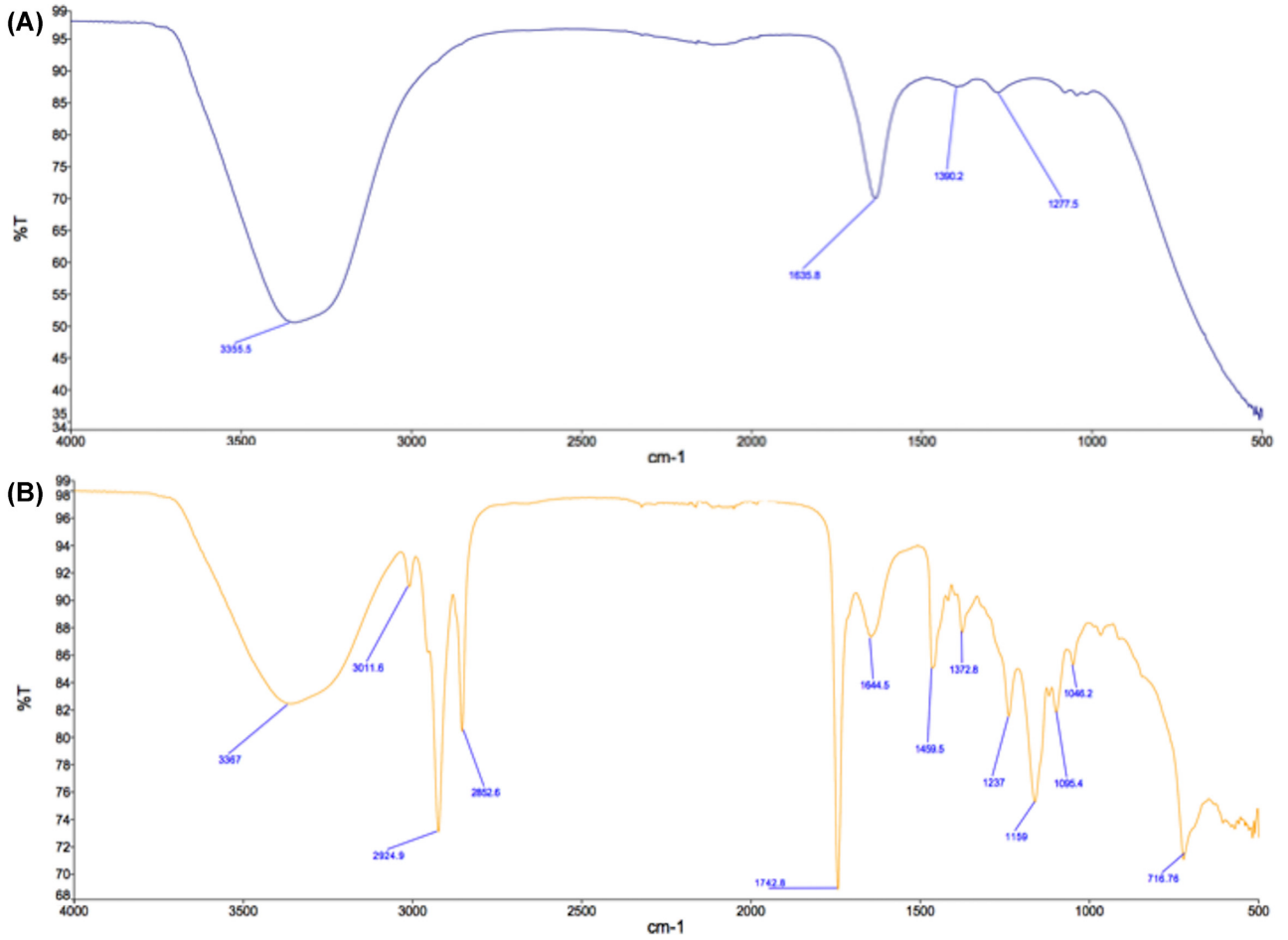

Figure 3. Fourier transform infrared spectra (FTIR) of the biosurfactant. **(A)** produced by *Rhodotorula mucilaginosa* SP6, **(B)** produced by *Debaromyces hanseii* MK9

uated. The results showed that the biosurfactants were active against both bacterial and fungal pathogens (Fig. 4). There was a correlation between the concentration of biosurfactants and the diameter of the zone of inhibition with higher zones of inhibition being observed at a biosurfactant concentration of 2 g/L. In general, both extracts showed antimicrobial activity against all the strains tested in this study. In addition, it was observed that both biosurfactants were more effective against Gram-positive than Gram-negative bacteria at concentrations of 0.5g/L and 1 g/L (ANOVA, $P < 0.001$, Tukey HSD $P < 0.05$) (Tables S16–21, Supporting Information). The reasons for the enhanced antimicrobial activity in the Gram-positive than in the Gram-negative bacteria are not clear and were not investigated in the present study, but may be related to differences in cell wall architecture. Among the Gram-negative bacteria, *Klebsiella pneumoniae* showed the highest resistance, a finding that was not surprising since this species is a multidrug resistant pathogen (Nordmann, Naas and Poirel 2011). The efficacy of biosurfactants on fungal pathogens was significantly lower when compared to the effects on bacterial pathogens (ANOVA, $P < 0.001$; Tukey HSD, $P < 0.05$) (Supplementary material Tables 28 –36). Since the cell walls of fungi differ with those of bacteria, differences in the penetration of the cell walls by biosurfactants probably account for the observed results. A detailed understanding of biosurfactants mechanisms of action is required. *Aspergilus niger* was the most resistant among all the microbes tested. 1 g/L and 2 g/L of biosurfactants from *D. hansenii* were effective against *A. niger*, while a lower concentration (0.5 g/L) was not affective. In contrast, only the highest concentration (2 g/L) of the biosurfactant from *R. mucilaginosa* was effective against *A. niger* (Fig. 4). *Aspergillus niger*, an emerging fungal pathogen, especially in immunocompromised patients is multi-drug resistant (Chowdhary *et al.* 2014) which may indicate the resistance observed. Among the fungi, *C. albicans* was more susceptible to the biosurfactants than *C. neoformans* (ANOVA, $P < 0.001$; Tukey HSD, $P < 0.01$) (Tables S23–S27, Supporting Information). In general, the latter is more drug resistant than the former (Hsueh *et al.* 2005). The biosurfactant extracts from *D. hansenii* showed a higher antimicrobial activity (at 2 g/L) as compared to the biosurfactant produced from the *R. mucilaginosa* (ANOVA, $P < 0.001$) (Tables S21 and S27). *Debaryomyces hansenii* is an established biocontrol agent, and it has been reported to produce various antimicrobial metabolites (Medina-Córdova *et al.* 2018) that could explain the comparatively higher efficacy of its biosurfactant. Overal, the antimicrobial activities of the two biosurfactants, even at low concentrations, suggest that they can be used as alternatives to conventional antimicrobial therapy and help stop the increase in antibiotic resistance. The antimicrobial activity of biosurfactants from yeasts is a significant finding in the era of increasing antimicrobial resistance in pathogenic microorganisms (Luna *et al.* 2011; Sen *et al.* 2017). This is consistent with several studies in which the antimicrobial activity

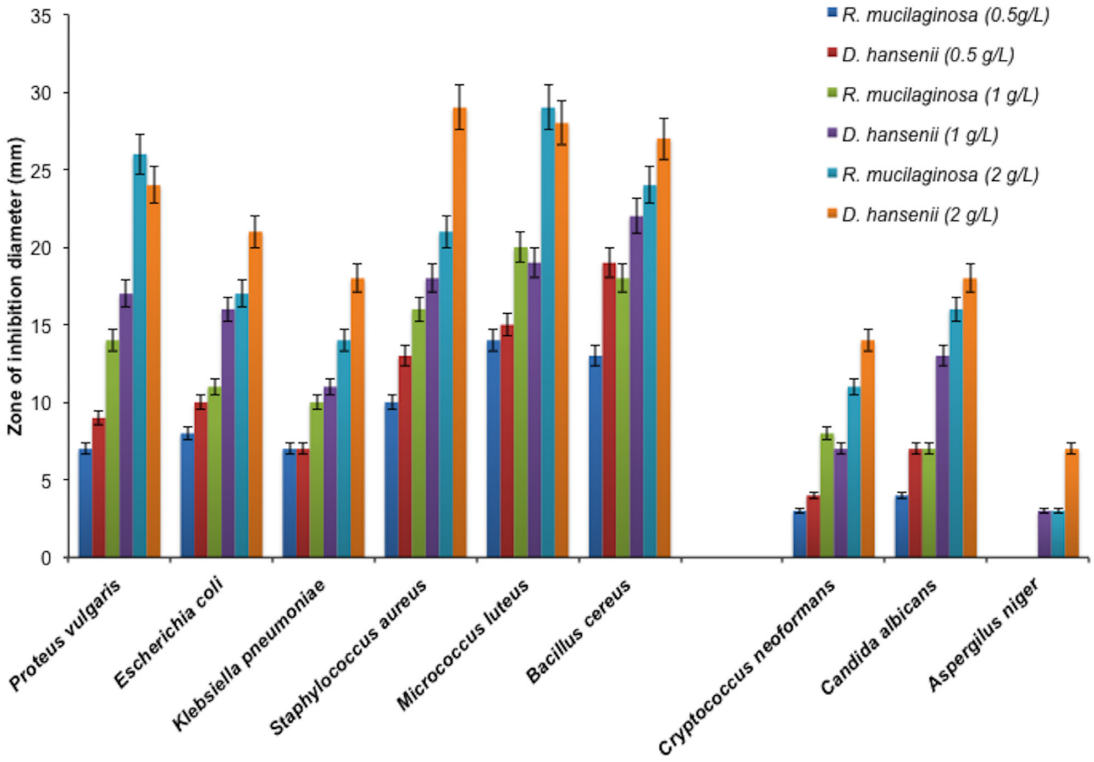

Figure 4. Antimicrobial activity of biosurfactants produced by two isolates based on agar well diffusion method. The experiment was repeated twice. The error bars show the results were within 5% of the mean.

is a property of biosurfactants (Kim et al. 1988, Lotfabad et al. 2010). This finding is consistent with the antimicrobial properties of rhamnolipids and sophorolipids. Rhamnolipids and sophrolipids disrupt cell plasma membrane, alter hydrophobicity and cell surface charge (Glover *et al.* 1999; Kaczorek *et al.* 2012; Díaz De Rienzo *et al.* 2015). The use of biosurfactants when compared to their petroleum counterparts produced from non-renewable resources is environmentally friendly due to their biodegradability, renewable production processes and lower toxicity (Das and Mukherjee 2007).

CONCLUSIONS

The present study successfully isolated nine halophilic yeasts from saltpans in Botswana and characterised the potential antimicrobial activity of the best biosurfactant producers using an inexpensive and renewable feedstock, seeds from an indigenous tree, *Xanthocercis zambesiaca*, which is important in the production of low cost biosurfactants. The biosurfactants produced by *R. mucilaginosa* SP6 and *D. hansenii* MK9 were characteristic of rhamnolipids and sophorolipids. The antimicrobial activity against the pathogenic microorganisms discussed in this work, suggests a great potential for the use of these biosurfactants as antimicrobial agents in the biomedical field in view of the antimicrobial resistance crisis. This study is the first to report on sophorolipid (biosurfactant) production by *D. hansenii*.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. The authors declare that there is no conflict of interest. We certify that the submission is original work and is not under review at any other publication. We confirm that no part of this manuscript is duplicated, reproduced or violates the copyright of others.

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