RSC Advances

PAPER

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Cite this: *RSC Adv.*, 2025, **15**, 42968

Lactonic sophorolipids by *Saccharomyces cerevisiae*: production, characterization and their evaluation for combating clinically important microbial pathogens

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The purpose of this study was to produce sophorolipids (SLs) through microbial conversion of *Lepidium sativum* (LS) oil cake, an agro-industrial waste, using locally isolated yeast (*Saccharomyces cerevisiae*), and to examine their physicochemical characteristics. Furthermore, anti-proliferative activity was investigated against many drug-resistant microbial pathogens. Some mechanistic parameters of SLs on the treated bacterial cells were also evaluated *via* oxidative stress activities. The SLs yield was 7.2 g per 100 g of substrate using methanol extraction and 28.6 g per 100 g of substrate through ethyl acetate re-extraction from the same medium, resulting in a total yield of 35.8 g per 100 g of substrate. Our findings indicated that the SLs have an effectiveness in lowering the surface tension (ST) to 48 mN m^{-1} at a critical micelle concentration (CMC) level of 30 mg L^{-1}. The produced SLs were identified by FTIR, LC-MS/MS, ^{1}H NMR spectroscopy, and GC/MS chromatography, confirming the existence of a lactonic form of SLs with the majority of total unsaturated fatty acids (81.98%). The produced SLs demonstrated strong antimicrobial properties, exhibiting MIC values lower than 100 μg mL^{-1} against various clinically important pathogens. Efficiently, SLs have shown significant activity in inducing oxidative stress through lipid peroxidation in bacterial cell membranes and increasing the release of reactive oxygen species within some treated bacterial pathogens, such as *Bacillus cereus* and *Klebsiella pneumonia*.

Received 2nd September 2025
Accepted 29th October 2025

DOI: 10.1039/d5ra06594a

rsc.li/rsc-advances

Introduction

The depletion of finite resources necessitates a circular economy by focusing on agro-industrial wastes, especially with overpopulation. Complex carbohydrates, lipids, proteins, antioxidants, minerals, vitamins, and fibers are all abundant in these wastes. They also contribute to manufacturing raw materials for several industrial processes, including synthesizing enzymes, bioactive compounds, biofuels, biodegradable polymers, and biosurfactants. With a simple, cheap, and sustainable biotechnological process, wastes support the global trend towards recycling for maximizing the benefits of agricultural and agro-industrial processes.[1,2] Microbial conversion of such wastes provides an environmentally friendly alternative as it reduces the accumulation of these wastes, diminishes climate change, and exploits them to produce value-added products, with low-cost raw materials, and higher productivity.[3,4] *Lepidium sativum* (LS) is an oily medicinal plant, grown for its high-value seeds and extracted oil. Yet it is not economically fully utilized despite its numerous applications and has recently received much attention in many reports.[5–9] LS has origins in ancient Egyptian and Indian civilizations and is cultivated mainly in temperate climates. Major producers include India (60%), Ethiopia (20%), and Pakistan (15%). The global annual production is estimated to be about 15 000 tons. LS is significant for global health due to its nutrient density and adaptability.[10] The LS meal, also known as oil cake, is a by-product left after

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extracting oil from seeds. Protein is the most abundant nutrient in this product, containing 34.15% protein, 1.86% crude oil, 9.85% crude fiber, 5.89% ash, and 48.25% nitrogen-free extract on a dry weight basis.[11] According to ref. 12 and 13, the range of LS oil content in the oil cake potentially leaves 9% oil unextracted after cold pressing. The LS oil mostly comprises 42% polyunsaturated fatty acids (PUFAs) and 39% monounsaturated fatty acids. Previously, LS oil cake was used as a substrate to produce sophorolipid compounds by growing *Yarrowia lipolytica* and investigating their potential as an antidiabetic agent in rats.[9] Based on the above, it becomes clear that this waste, with its components, is a suitable and promising substrate for the growth of many microorganisms to produce economically valuable compounds derived from LS oil cake. Recently, attention has been drawn to the exploitation of various oil cakes through biotechnological utilization to produce valuable materials such as antibiotics, biosurfactants, biofuels, and polyunsaturated fatty acids.[14,15] Sophorolipids (SLs), a member of the biosurfactant family, are less environmentally harmful than synthetic ones and can freely dissolve in water and oil. These unique characteristics allow SLs to play roles as anti-food spoilage, anti-corrosive, anti-hypercholesterolemic, emulsifying, anti-cancer, and antimicrobial agents.[16–19] According to ref. 20, SLs' treatment of *Bacillus subtilis* enhanced the intracellular enzyme malate dehydrogenase leakage, suggesting that sophorolipids may interact with a cellular membrane. Later,[21] indicated the SLs' capacity to alter the permeability of bacterial membranes and harm the biofilms that microorganisms develop, leading to their potential use as natural antimicrobial agents. Notwithstanding their benefits over synthetic surfactants, SLs' expensive production is the largest barrier to broad use. As a result, recent research has focused on several factors, such as raw materials, which contribute between 10% and 50% of total costs. Using agro-industrial wastes as production process substrates would be both cost-effective and environmentally advantageous.[4] Therefore, different agro-industrial residues have been used for the production of SLs, such as potato, orange, cassava, and banana peels, and the residues of the oil production industries, for example, coconut, soybean, moringa, and jojoba oil cakes.[4,9,22,23] To our knowledge, there are no reports discussing the production of SLs from *Saccharomyces cerevisiae* utilizing *Lepidium sativum* oil cake as a nutrient substrate. The ability of microorganisms to resist many traditional antibiotics has led to an increase in their pathogenicity and virulence. Microbial infections rank among the top priorities for the World Health Organization and are a significant clinical challenge in infectious diseases today. This challenge arises from the inherently high resistance of these pathogens and their natural ability to develop multidrug resistance. This occurs due to mutations in chromosomal genes or through the horizontal transfer of broad-spectrum resistance mechanisms. The drug-resistant bacteria have commonly developed resistance towards the traditional antibiotics, which become more virulent and difficult to treat effectively.[24] Therefore, discovering new natural bioactive compounds is worth exploring this phenomenon. SLs produced by *Candida parapsilosis* grown on potato peel and frying oil wastes were reported by ref. 23, which had a high ability to inhibit the growth of all Mucorales fungal strains that cause mucormycosis. To our knowledge, SLs have not been previously produced by the *Saccharomyces cerevisiae* cultivated on LS oil cake as a substrate. Also, there are no reports investigating the SLs role against the utilized clinically drug-resistant microbial pathogens. In light of the aforementioned, the goal of this study is to produce SLs through microbial conversion of LS oil cake, by locally isolated yeast (*Saccharomyces cerevisiae*), and assess the chemical and physical properties of the final product. The antimicrobial activity of SLs will also be evaluated against some drug-resistant microbial pathogens. Some mechanisms will also be investigated, such as lipid peroxidation (LPO) activity and the production of Reactive Oxygen Species (ROS), to predict the mode of action for the SLs' inhibition activity.

Results and discussion

Production of SLs

A shift to a circular economy is necessary to tackle the current global problems caused by human activity and overpopulation, which include resource misuse and mismanagement, climate change, and environmental pollution. Considering oil cakes are by-products of the oil extraction process, which transforms waste into useful resources, they have a high sustainability rating. They can be utilized in a sustainable manner as feedstock for biofuels and biogas, soil enhancers, animal feed, and biofertilizers. Nonetheless, controlling anti-nutritional substances in some cakes, the effects of large-scale oilseed production on the environment, and making sure that the cakes are handled properly to avoid deterioration are possible difficulties. However, appropriate management of wastes as raw materials for fermentation, and the employment of robust microorganisms, may be reasonable solutions for the economic production of SLs.[24] LS oil cake, an agro-industrial waste, served as the low-cost substrate in this study. The solid-state fermentation (SSF) technique was used for the production process because it maximizes production yield while using minimal amounts of water.[25] The utilized strain (*Saccharomyces cerevisiae*), however, was previously identified at the molecular level in the GenBank after being isolated from another waste, namely castor oil cake.[15] SLs have never been produced before by growing *Saccharomyces cerevisiae* on LS oil cake as a substrate. These processes were able to produce a yield of 7.2 g methanol and 28.6 g ethyl acetate re-extracts from the same medium, with a combined yield of 35.8 g/100 g substrate. Comparable yields were reported by ref. 4 for the production of SLs (32.1 g/100 g substrate) from *Yarrowia lipolytica* grown on *Moringa oleifera* oil cake, and[22] for *Saccharomyces cerevisiae* SLs produced from banana peels (30.6 g/100 g substrate). Lower yields were obtained for SLs (18.4, 18.6, 20, and 24.4 g/100 g substrate) by solid state fermentation from different agro-industrial wastes cultivated by *S. cerevisiae*, *Y. lipolytica*, *Starmerella bombicola*, and *Candida bombicola*, respectively. While higher SLs productivities from different yeast strains grown on various agro-industrial wastes ranging from 39–55.3 g/100 g substrate were reported by ref. 9, 17, 22 and 26.

Critical micelle concentration and surface tension of the produced SLs

The ring technique was used to assess the reduction in distilled water ST caused by the produced SLs and CMC. The data showed that the SLs were effective in lowering the ST from 72 to 48 mN m^{-1} at a CMC level of 30 mg/100 mL (Fig. 1). A lower ST level of 32.6 mN m^{-1} was revealed by ref. 27, for SLs generated by *Rhodotorula babjevae* with a CMC value of 200 mg L^{-1}. Also, a lower level (35 mN m^{-1}) was reported for the SLs produced by *Metschnikowia churdharensis* at a CMC value of 12 mg mL^{-1} from synthetic media.[28] In the same context, ST level of 38 mN m^{-1} for SLs produced by *S. cerevisiae* cultivated on moringa oil cake at a CMC point of 60 mg L^{-1} was indicated by ref. 9. Similar results, however, (48 mN m^{-1}) were reported at the CMC of 125 mg L^{-1} by growing *Candida parapsilosis* on the wastes of potato and frying oil.[23]

SLs chemical structure estimation by FTIR spectroscopy

FTIR spectroscopy analysis was used to evaluate the chemical structure of the produced SLs (Fig. 2) (raw data are provided in the SI). Asymmetrical and symmetrical stretching ($_{vas}CH_2$) of the methylene group was detected by FTIR analysis of SLs, which revealed absorption peaks at 2923.7 cm^{-1} and 2853.6 cm^{-1}. The peak at 1741 cm^{-1} corresponds to the C=O stretching of ester/lactone.[29] Additionally, bands observed at 1231.23 cm^{-1} and

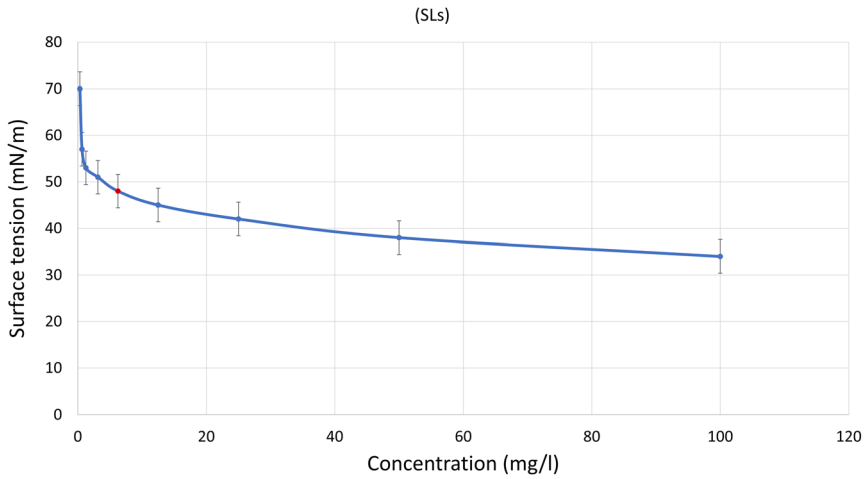

Fig. 1 Critical micelle concentration (CMC) and surface tension of the SLs produced by *S. cerevisiae* grown on LS oil cake (data were represented as mean ± S. E. of 3 experiments).

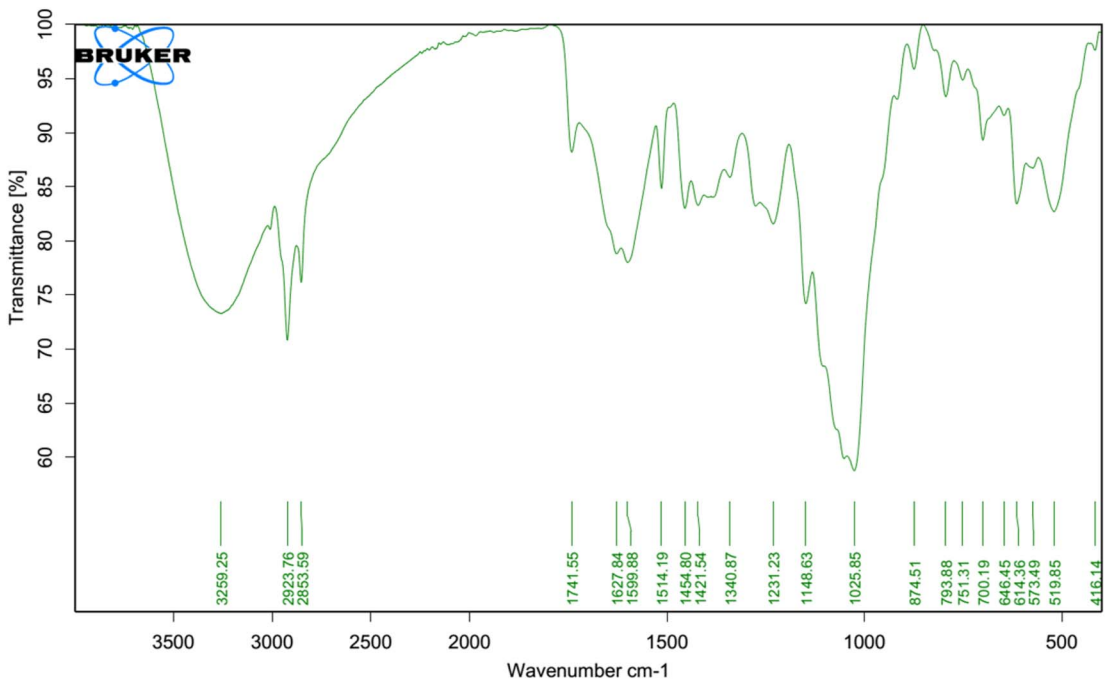

Fig. 2 FT-IR spectrum of the produced SLs from *S. cerevisiae* cultivated on LS oil cake.

1148.6 cm^{-1} corresponded to the C(=O)–O–C bond characteristic of lactone groups. The O–H stretching band at 3259 cm^{-1} belongs to the expected free chain of fatty acids that could be extracted with the methanol.[9] Lastly, the presence of sophorose sugar was indicated by the absorption at 1025 cm^{-1}, linked to C–O stretching in C–O–H groups.[4,27,28] This FTIR analysis confirms the presence of surface-active glycolipid structures compatible with sophorolipids and similar biosurfactants. The essential characteristics support this identification.

Proton nuclear magnetic resonance (^{1}H NMR) analysis

The hydrogen-1 proton nuclear magnetic resonance (^{1}H NMR) spectrum was used to evaluate the structure of SLs produced from *S. cerevisiae* grown on LS oil cake (Fig. 3) (raw data are provided in the SI). The presence of the –$COCH_3$ group in its structure was confirmed by the signal at 2.07 ppm in the spectra, while the presence of the –CH=CH– group of the fatty acid moiety was established by several peaks at 5.32–5.36 ppm. Additionally, according to 30, who revealed the structure of SLs lactonic form, the presence of a fatty acid chain moiety in the SLs structure was indicated by the numerous indications of protons between 1.23 and 1.42 ppm.[15] Two protons of glucose resonated at 3.46–4.28 ppm. Additionally, methyl groups from the acetylated hydroxyl (1.98 ppm) and lactone methylene (2.28 ppm) were detected.[31].

LC-MS/MS spectroscopic analysis of the prepared SLs

The LC-ESI-MS/MS spectrum of SLs analysis is shown in Fig. 4. Where lactonic SLs peaks and their fragments appeared at *m*/*z* 688.26 and at the retention time of 24.8 min. Prominent peaks at 688.26, 531.24, and 440.6 *m*/*z* indicate the presence of different derivatives within the sophorolipid complex due to variations in lipid chain length, where the 531.24 and 440.6 *m*/*z* are attached to C15 : 3 and C10 : 1 fatty acids, respectively.[32,33] However, the peak at *m*/*z* 432.77 indicates the presence of the fragment containing the sophorose with a part of the fatty-acyl moiety in the lactonic sophorolipid.[34] Also, a peak at *m*/*z* 408.33 representing the sophorose molecule fragment was noticed.[35] At the same retention time, a peak at *m*/*z* 359.16 appeared, indicating the existence of C22 hydroxy fatty acid.[4] Furthermore, a peak of oleic acid was presented at *m*/*z* 281.96 as described by ref. 36. Finally, the existence of the octadecanoic acid fragment was observed at *m*/*z* 271.59 at the same retention time.[4] From the FT-IT, LC-MS, and ^{1}H NMR spectroscopy analyses, it can be concluded that the produced compound was in the lactonic form of sophorolipids.

Fatty acid composition of the produced SLs

After confirming the existence of SL compounds in the extract through spectroscopic analyses, GC/MS chromatography was performed to identify the types of fatty acids linked to the sophorose molecules. Table 1 displays the fatty acid content of the produced SLs. The findings showed that the two most common unsaturated fatty acids were linoleic (48.69%) and oleic (22.4%) fatty acids, which combined made up nearly 71.09% of total fatty acids. However, unsaturated fatty acids (oleic, linoleic, linolenic, and *cis*-11-eicosenoic) constitute

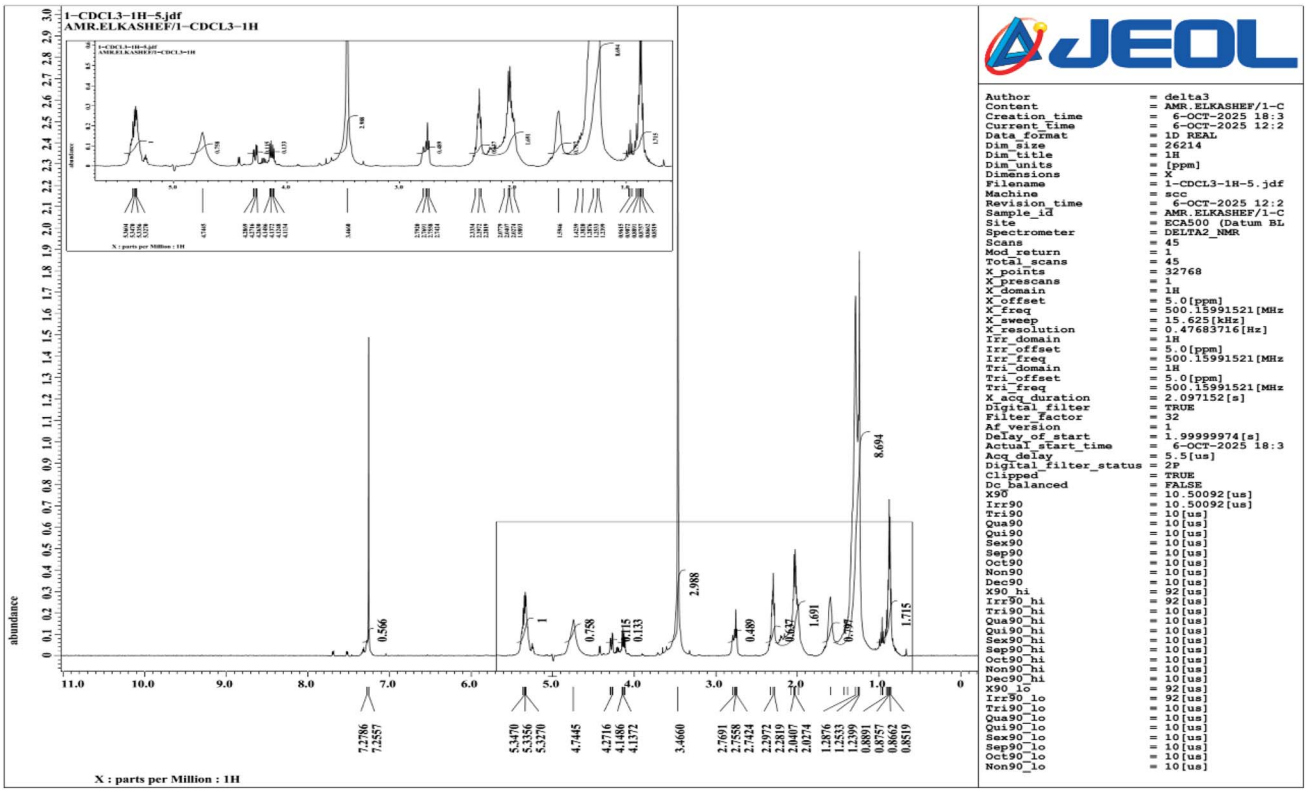

Fig. 3 ^{1}H NMR spectrum of the produced SLs by *S. cerevisiae* grown on LS oil cake.

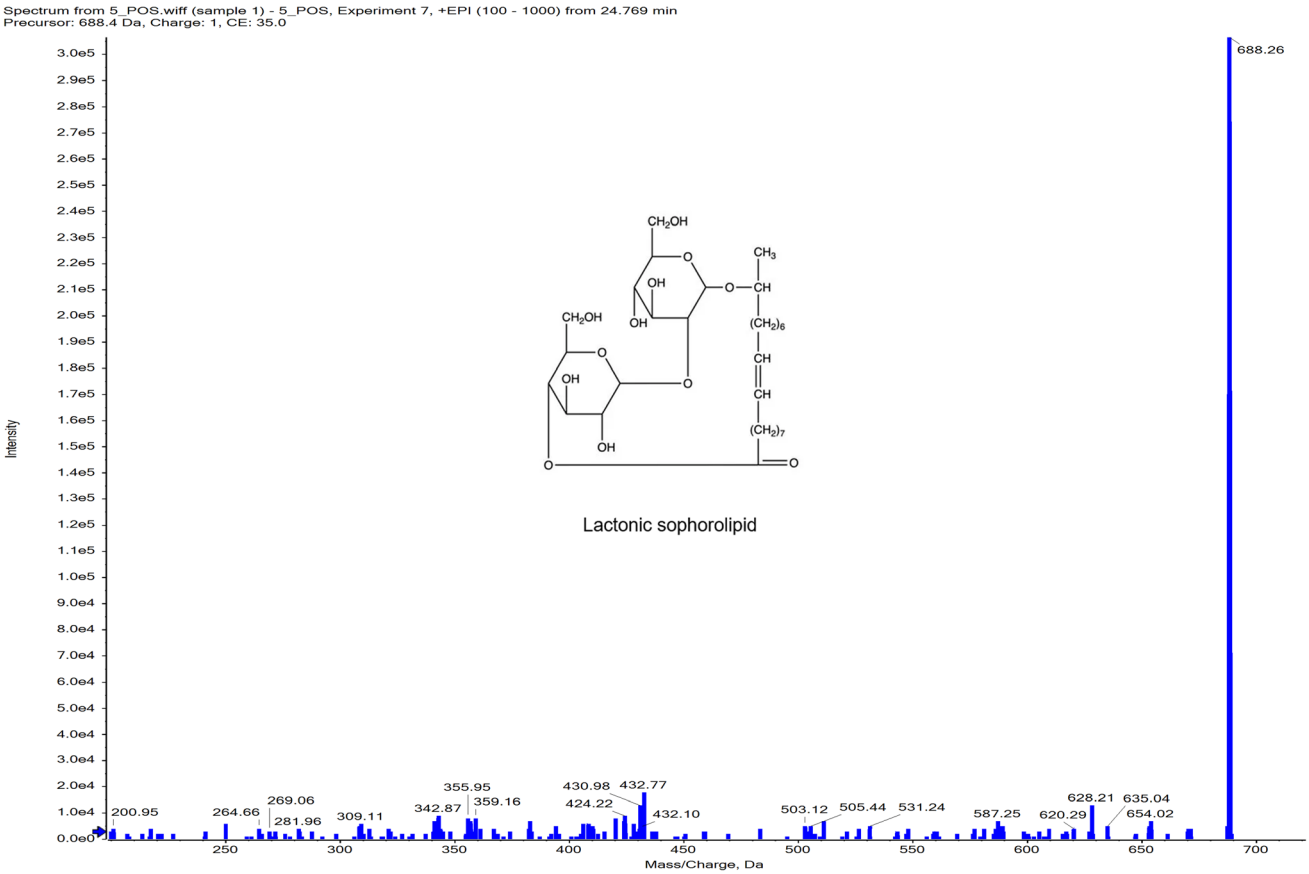

Fig. 4 LC-ESI-MS/MS analyses of the produced SLs by *S. cerevisiae* grown on LS oil cake presented as lactonic type of SLs.

Table 1 Fatty acid composition of the produced SLs

Fatty acids	Formula	Area%
Palmitic acid	$C_{17}H_{34}O_2$	11.34
Stearic acid	$C_{19}H_{38}O_2$	5.3
Oleic acid	$C_{19}H_{36}O_2$	22.4
Linoleic acid	$C_{19}H_{34}O_2$	48.69
Linolenic acid	$C_{19}H_{32}O_2$	9.34
Arachidic acid	$C_{20}H_{40}O_2$	0.79
Cis-11-Eicosenoic acid	$C_{20}H_{38}O_2$	1.55
TSFAs		16.64
TUSFAs		81.98
PUFAs		59.6

81.98% of the total fatty acids in the produced SLs. Based on the data collected, it is clear that the resulting analysis of the PUFAs constituting the produced SLs extract indicates a high-quality content of essential fatty acids (raw data are provided in the SI).

Antimicrobial activity of the produced SLs

From the same nutritional media, sophorolipids-producing yeast can produce a variety of SLs chemical structures, such as lactonic and acidic forms with varying lengths of attached fatty acids.[4,9,22,23] The capacity to use the same group of extracted SLs in a variety of industrial and pharmacological applications is greatly enhanced by this diversity. It may also give it the capacity to function as a potent antimicrobial agent.

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens pose significant challenges in healthcare settings, particularly in intensive care units (ICUs), leading to increased morbidity and mortality. These pathogens have developed resistance through genetic mutations and biofilm formation, enhancing their defense against multiple antibiotic classes. The rapid spread of such resistant strains has rendered traditional antibiotic therapies less effective, necessitating advanced treatment strategies for swift eradication. Therefore, antimicrobial resistance requires an advanced treatment that can cause short-time eradication. Herein, we investigate the killing activity of SLs against some clinical MDR pathogens. The antibiotic profile for each bacterial pathogen indicated a significant resistance towards classical antibiotic groups, such as Cephalosporin (Cephradine), Aminopenicillin (Ampicillin), Macrolide (Erythromycin), Aminoglycoside (Kanamycin) and Polypeptide (Polymyxin B) families. In contrast, *Pseudomonas aeruginosa* was slightly affected by Polymyxin B, and *Bacillus cereus* was also moderately inhibited by Kanamycin. Interestingly, a fluoroquinolone family (Ciprofloxacin) can inhibit all bacterial pathogens with more than a 3 mm inhibition zone. Therefore, the capability of the produced SLs to prevent microbial growth was examined primarily under a standard antibacterial procedure using the agar-well diffusion method.[37]

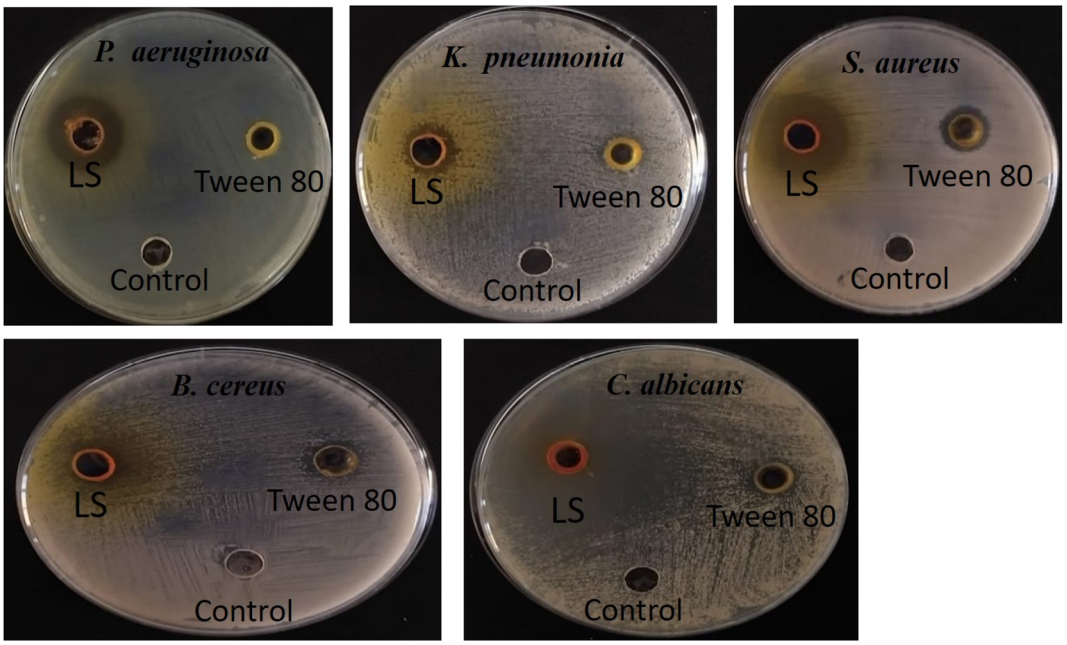

Fig. 5 Antibacterial activity of the targeted molecules using the agar-well diffusion method.

As shown in (Fig. 5), SLs activity was proved to be suitable for causing an appreciable inhibition toward bacterial and fungal growth. Efficiently, SLs had greater activity against MDR than XDR pathogens, whereas two-fold inhibition activity was observed compared to the standard antibiotic drugs (Table 2). Likewise, the potent activity of SLs against *Candida albicans* was notably increased by threefold that obtained by the most-active antifungal agent, Amphotericin B. On the other hand, the activity of SLs against XDR was also noted, but lower than that observed by the standard antibacterial agents (*i.e.* Ciprofloxacin).

Recent studies have explored alternative approaches to combat these resistant pathogens. One promising strategy is novel antimicrobial peptides. For instance, an amphipathic peptide has been identified with potent and rapid antimicrobial activity against various MDR and XDR Gram-negative pathogens. This peptide exhibits broad-spectrum efficacy and rapid bactericidal action, making it a potential candidate for treating infections caused by resistant bacteria.[38] Another promising strategy against MDR and XDR pathogens is glycolipid biosurfactants produced by certain non-pathogenic yeast species, such as *Candida apicola* and *Starmerella bombicola*. These

Table 2 The antimicrobial properties of SLs using the agar well diffusion method[d]

	Inhibition zone (mm)				
Compound code	*Staphylococcus aureus* (MDR)	*Bacillus cereus* (MDR)	*Pseudomonas aeruginosa* (XDR)	*Klebsiella pneumonia* (XDR)	*Candida albicans* (MDR)
SL (1)	8 ± 1.08	6 ± 0.22	5 ± 0.71	4 ± 0.71	9 ± 0.02
Tween 80 (2)	ND	ND	ND	ND	ND
Fluconazole[a]	—			—	ND
Amphotericin B	—			—	4 ± 1.02
Nizoral	—			—	ND
Cephradine[b]	ND[c]	ND	ND	ND	—
Ciprofloxacin	5 ± 0.55	4 ± 0.61	6 ± 1.02	5 ± 0.83	—
Ampicillin	ND	ND	ND	ND	—
Polymyxin B	2 ± 0.04	3 ± 0.33	3 ± 0.06	4 ± 0.51	—
Erythromycin	2 ± 0.23	2 ± 0.06	ND	ND	—
Kanamycin	3 ± 0.91	2 ± 0.22	ND	ND	—
Sulfadiazine	ND	ND	4 ± 0.88	ND	—

[a] Fluconazole, Amphotericin B and Nizoral were used as standard antifungal agents. [b] Kanamycin, Ciprofloxacin, Ampicillin, Polymyxin B, Erythromycin, Sulfadiazine and Cephradine were used as standard antibacterial agents at 20 μg mL^{-1}. [c] ND: not determined. The SLs was used in 80 μg mL^{-1} in the screening test. [d] Values are expressed as mean ± SD ($n = 3$).

compounds have garnered attention for their antimicrobial properties, particularly against MDR pathogens.[27,39] Furthermore, the application of SLs in the food preservation was also examined due to its capability in the prevent the food-borne pathogens to proliferation.[40]

Determination of the MIC value

The response of SLs with different concentrations toward the tested microbial pathogens must be justified *via* Minimum Inhibition Concentration (MIC), which may induce further inactivation sensitivity for the bacterial proliferation. Therefore, the utilization of different concentrations of SLs in the culture medium of the microbial pathogen was investigated using Mueller–Hinton Broth (MHB) medium. As shown in Table 3, a potential suppression effect was relatively taken place based on the SLs dose; the lowest MIC values were obtained in the case of *Candida albicans* (45 μg mL^{-1}) and *Bacillus cereus* (65 μg mL^{-1}).

The clear resistance of *Pseudomonas aeruginosa* and *Staphylococcus aureus* towards SLs was also indicated, even over 100 μg mL^{-1}. However, the ability of SLs to cause the lowest growth of microbial strains with different ratios could reflect their potency when compared to the β-lactam antibiotic drugs. Collectively, the greater effect of SLs at low concentrations to inhibit the maximum microbial growth could imply their potential to be promising candidates as antimicrobial agents. A variety of SLs producers from yeast species confirmed their capability to serve as bactericidal and fungicidal agents. This fact could open an alternative route to controlling the virulence of several microbial pathogens, particularly those that are susceptible to resist different antibiotic drugs.[41] Several beneficial properties of SLs make it a good bioactive agent, such as low toxicity with high biodegradability. In this respect, the addition of SLs to the preservative food materials proved to increase the antimicrobial activity against food-borne pathogens.[40] The combination of SLs with low-active materials demonstrated a strong activity against many microbial pathogens. For instance, the incorporation of SLs into polylactic acid (PLA) films increases their antimicrobial activity due to the higher water-soluble that results from the hydrophilic sugar moiety of SLs. Furthermore, the combination of lactic acid with SLs causes a severe suppression of some bacterial pathogens such as *Clostridium perfringens* and *Campylobacter jejuni*.[40] Furthermore, the synergistic effect of SLs when used in conjunction with antibiotics has been observed, allowing for the use of lower antibiotic concentrations while maintaining inhibitory effects. This synergy is believed to enhance drug action by facilitating antibiotic entry across cell membranes, thereby achieving the necessary intracellular concentrations at reduced dosages.[42]

Lipid peroxidation activity of the potent molecules (LPO)

The effectiveness of SLs on the bacterial cell may be carried out by targeting cell membrane disruption *via* oxidation of their lipid contents. Lipid peroxidation (LPO) activity is a significant parameter that can help to predict the mode of action for the targeting molecule. Oxidative stress of the tested molecule on the bacterial cells is one of the important pathways for easily penetrating the bacterial cell membrane. Therefore, the LPO activity of SLs was determined using concentrations around the MIC value. As seen in (Table 4), the lipid oxidation activity of SLs was notably increased as the dose increased. The oxidation potential of bacterial cell membrane by SLs was dramatically increases, in particular against *B. cereus* followed by *S. aureus* and *K. pneumonia*. Obviously, there was a difference between the LPO response between the two types of bacterial pathogens, which could be related to the differences of cell membrane composition. Sophorolipids (SLs), biosurfactants produced by non-pathogenic yeasts, have demonstrated notable antimicrobial properties, particularly against Gram-positive bacteria such as *Bacillus cereus* and *Staphylococcus aureus*. This heightened efficacy is attributed to their ability to disrupt the lipid membranes of these bacteria.[42] In contrast, SLs exhibit reduced activity against Gram-negative bacteria. This diminished efficacy is likely due to the presence of an additional outer lipid membrane and lipopolysaccharide layer in Gram-negative bacteria, which impedes the interaction between SLs and the bacterial cell membrane.[43] The antimicrobial mechanism of SLs

Table 3 Determination of the MIC value for the targeted SL using different concentrations[a]

	Minimum inhibitory concentration (MIC, μg mL^{-1})				
Compound	*Staphylococcus aureus* (MDR)	*Bacillus cereus* (MDR)	*Pseudomonas aeruginosa* (XDR)	*Klebsiella pneumonia* (XDR)	*Candida albicans* (MDR)
SL	122.1 ± 2.15	83.7 ± 1.24	181.9 ± 3.17	64.2 ± 0.72	43.8 ± 2.33
Fluconazole a	—				>100
Amphotericin B	—				12.6 ± 1.99
Cephradine	>100	>100	>100	>100	—
Ampicillin	>100	>100	>100	>100	—
Erythromycin	22.2 ± 2.84	13.8 ± 1.77	>100	>100	—
Kanamycin	9.7 ± 0.45	44.1 ± 0.85	>100	22.8 ± 0.06	—
Polymyxin B	18.7 ± 0.88	9.1 ± 0.66	14.7 ± 0.57	9.5 ± 0.42	—
Ciprofloxacin	8.8 ± 0.58	4.8 ± 0.21	8.2 ± 1.09	5.2 ± 0.03	—

[a] Values are expressed as mean ± SD ($n = 3$).

Table 4 Lipid peroxidation level (LPO) of SLs toward the bacterial pathogens[a]

SL concentration (μg mL^{-1})	Lipid peroxidation efficiency (malondialdehyde, nmol mL^{-1})			
	Staphylococcus aureus (MDR)	*Bacillus cereus* (MDR)	*Pseudomonas aeruginosa* (XDR)	*Klebsiella pneumonia* (XDR)
40	1.111 ± 0.06	2.821 ± 0.23	0.883 ± 0.02	2.055 ± 0.15
80	3.282 ± 0.25	3.383 ± 0.41	1.395 ± 0.11	3.232 ± 0.27
160	4.721 ± 0.51	5.110 ± 0.22	3.119 ± 0.34	4.007 ± 0.21
Ciprofloxacin	7.833 ± 0.09	5.942 ± 0.14	7.218 ± 0.09	6.926 ± 0.04

[a] Values are expressed as mean ± SD ($n = 3$).

involves perturbation of membrane lipid order, compromising microbial viability. Specifically, SLs can disrupt bacterial and fungal membranes *via* reactive oxygen species (ROS) production, leading to oxidative stress and cell damage.[44]

Quantification of reactive oxygen species (ROS) induced by SLs

Investigation of the ROS releases by the targeted compounds could confirm a possible pathway mechanism for the inhibition process. In this way, the increases in killing sensitivity towards bacterial pathogens may be related to the excitation process of the SLs surrogates *via* Reactive Oxygen Species (ROS). Therefore, the quantification of ROS inside the inhibited bacterial cells was determined to investigate the inhibition pathway. Rapid oxidation of the DCFH when reacted with the resulting ROS was spontaneously conducted and the highly fluorescent compound DCF appeared. In brief, the treated bacterial cells were immediately subjected to a fluorescent assay to accurately detect the ROS generated from the produced SLs. As shown in Table 5, the induction of different ROS was indicated by each concentration, and the maximum ROS generated inside bacterial cells was carried out in the case of *Klebsiella pneumonia*. A lower ROS amount was induced inside the bacterial cells at 80 μg mL^{-1}. Furthermore, ROS quantity was found to be increased after increasing the SL concentration, especially in the case of Gram-positive ones. Meanwhile, the ROS induced by SLs at both concentrations was found to be identically in case of Gram-negative strains. In addition, the ROS releases inside *Candida albicans* was also indicated in high concentration, particularly when treated at 160 μg mL^{-1}.

Sophorolipids (SLs), a class of biosurfactants produced by certain non-pathogenic yeast species, have demonstrated notable antimicrobial properties. Their amphiphilic structure enables them to interact with and disrupt microbial cell membranes, leading to increased permeability and cell death. Recent studies have investigated the role of ROS in the antimicrobial activity of SLs. For instance, research on sophorolipid-capped gold nanoparticles (AuNPs-SL) revealed that treatment induced ROS production in *Vibrio cholerae*, leading to DNA damage, membrane potential alterations, ATP depletion, and ultimately apoptotic cell death. The study utilized the DCFH-DA assay to measure ROS levels, confirming a dose-dependent increase in ROS upon treatment with AuNPs-SL.[45] Another study explored the antibacterial effects of lactonic sophorolipids (LSL) on pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The findings indicated that LSL exhibited inhibitory effects on these bacteria, with mechanisms involving disruption of cell membrane integrity and induction of oxidative stress.[46]

Material and methods

Substrates

Lepidium sativum (LS) seeds were sourced from a local market in Cairo, Egypt, while the cake of castor oil was provided by the Pressing and Extracting Natural Oils Unit at the National Research Center, Cairo, Egypt. The crude oil of soybean was collected from the Institute of Food Technology Research, Soy Processing Centre, Agriculture Research Centre, Giza, Egypt. The yeast strain used (*Saccharomyces cerevisiae*) had been previously isolated from castor oil waste, identified, molecularly

Table 5 ROS determination of SL toward the microbial pathogens[a]

SL concentration (μg mL^{-1})	ROS determination (DCF level, counts) using spectrofluorometric (excitation and emission wavelength at 485 nm and 530 nm, respectively)				
	Staphylococcus aureus (MDR)	*Bacillus cereus* (MDR)	*Pseudomonas aeruginosa* (XDR)	*Klebsiella pneumonia* (XDR)	*Candida albicans* (MDR)
80	177 ± 2.22	162 ± 1.35	169 ± 0.87	249 ± 2.44	177.4 ± 0.72
160	238 ± 1.51	295 ± 1.88	183 ± 1.75	301 ± 3.11	367.1 ± 2.94
Ciprofloxacin	296 ± 0.92	362 ± 3.18	251 ± 0.72	313 ± 1.09	—
Amphotericin B	—				338 ± 1.05

[a] Values are expressed as mean ± SD ($n = 3$).

characterized, and registered in the GenBank database under accession number ON644539.1.[15]

Preparation of LS oil cake

The LS seeds were subjected to compression using a laboratory-grade Carver hydraulic press at a pressure of 10 000 lb in^{-2} for one hour at a temperature of 25 °C. After pressing, the LS oil cake was left to air dry at room temperature for 24 hours before being ground using a commercial blender. Finally, the powdered oil cake was stored in polyethylene bags and preserved at −20 °C.[47]

Fermentation process

A loopful of *S. cerevisiae* from a 7-day-old stock culture was transferred into a sterile 50 mL inoculum medium, following the method described by ref. 48. The mixture was then incubated in an Orbital Shaker (Thermo SCIENTIFIC, USA) at 28 °C and 180 rpm for 24 hours. Subsequently, 2 mL of the overnight culture (1×10^8 cells per mL) was introduced into the sterilized solid-state fermentation (SSF) medium containing: 4 g of LS oil cake; 1.5 g of soybean oil, and 4 mL of nutrients solution consisting of (g L^{-1}) NH_4NO_3, 1.0; K_2HPO_4, 2.55; NaH_2PO_4, 0.15; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; $MnSO_4 \cdot H_2O$, 0.02; peptone, 1.0 and the medium was adapted to pH 8. The incubation was carried out in static conditions at 28–30 °C for 8 days.[49]

Extraction of SLs

Based on a modified approach from ref. 17, SLs extract was obtained by adding 100 mL of methanol to the culture and shaking the mixture at 160 rpm for 60 minutes at 40 °C using an Orbital Shaker. The methanol extract was then collected, and the remaining fermented substrate underwent a second extraction using 100 mL of ethyl acetate under the same shaking conditions. Both extracts were filtered using Whatman filter paper no. 40 to separate the methanol and ethyl acetate SLs extracts. The filtrates were individually concentrated using a rotary evaporator (Heidolph Cooling Analog Vacuum Controller G1, Germany) and further dried at 40 °C to yield the crude methanol and ethyl acetate SLs extracts.

Surface tension (ST) and critical micelle concentration (CMC) evaluation

The ring method was conducted to estimate ST and CMC utilizing KrÜss tensiometer-K100 (Germany). Serial concentrations of the produced SLs were prepared, and the surface tension value constant point against a specific concentration was determined, then reported.[30]

Fourier transform infrared spectroscopy (FTIR) of the produced SLs

Bruker VERTEX 80 (Germany) was utilized for attenuated total reflectance (ATR)-FTIR assessment, a combined Platinum Diamond ATR consisting of a diamond disc as an internal reflector (4000–400 cm^{-1}) with a refractive index of 2.4 and 4 cm^{-1} resolution.

^{1}H NMR spectra analysis

NMR spectra were estimated by a Varian Mercury VX-300 NMR spectrometer. In deuterated chloroform ($CDCl_3$), ^{1}H spectra were conducted at 300 MHz. Chemical shifts are quoted in and are related to solvent shifts for CA-SLs.

LC-MS/MS of the produced SLs

The structural characterization of the generated SLs was performed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The analysis was carried out on an ExionLC AC system, and separation was accomplished with a SCIEX Triple Quad 5500+ MS/MS system equipped with an electrospray ionization (ESI). An Ascentis® C18 column with dimensions of 4.6 × 150 mm, 3 μm, was employed for compound separation. The mobile phase consisted of two eluents: eluent A (0.1% formic acid dissolved in water) and eluent B (0.1% formic acid dissolved in acetonitrile, LC grade). The gradient elution program was as follows: 10% B from 0–2 min, rising to 90% B from 2–30 min, maintained at 90% B from 30–36 min, then returning to 10% B at 36.1 min and maintained at 10% B until 40 min. For MS/MS analysis, the flow rate was set at 0.7 mL min^{-1}, with 10 μL injection volume. The positive ionization mode was used with an EMS-IDA-EPI scan range of 100–1000 Da for MS1 under certain conditions: curtain gas at 25 psi, ion spray voltage of 5500 V, source temperature of 500 °C, and ion source gases 1 & 2 at 45 psi. For MS2, the scan range was 50–1000 Da with a decluttering potential of 80, collision energy of 35, and collision energy spread of 20. Compound identification was carried out using MS-DIAL software (version 4.70) with the FiehnHILIC library.

Fatty acid composition of the produced SLs

The composition of fatty acids was analyzed by GC/MS technique (MassHunter GC/MS, SW version: Acquisition B.07.03.2129 18-May-2015, Agilent Technologies, Inc.) at the Central Laboratory, National Research Centre, Egypt.

Antimicrobial activity

The effectiveness of sophorolipids in eliminating clinically significant pathogens was evaluated against a range of bacterial strains, including multidrug-resistant (MDR) species. These included Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus cereus* (Gram-positive), as well as extensively drug-resistant (XDR) bacteria such as Carbapenem-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (CRKP) (Gram-negative). Additionally, the unicellular fungal pathogen *Candida albicans* (MDR) was tested. The microbial pathogens were clinically isolated from urinary tract infections and fully characterized by the Microbiology and Immunology Department, Faculty of Medicine (Boys), Al-Azhar University. Prior to testing, each pathogen was pre-activated in Mueller Hinton Broth (MHB) medium (Condalab, Spain) at 30 °C for 24 hours under shaking conditions. The inoculum size was standardized using the McFarland scale and accurately measured in colony-forming units per milliliter (CFU mL^{-1}) to maintain

a consistent concentration for all experimental assays. The antimicrobial activity of sophorolipids was initially assessed through the agar well diffusion method, following the Clinical and Laboratory Standards Institute (CLSI) protocol.[50,51] To determine the antimicrobial activity of SL, a constant concentration was used (80 μg mL^{-1}) compared to the standard antibiotic agents. After the incubation period, the obtained results of SLs and standard antimicrobial agents were measured based on the inhibition zone diameter (mm) around each targeted molecule.[52]

Assessment of the minimum inhibitory concentration (MIC)

To determine the Minimum Inhibition Concentration (MIC), varying concentrations of SL were prepared following the microdilution method, as described by ref. 53 dilutions of SLs were implemented from the previously prepared stock solution to obtain targeted concentrations ranging from 10–250 μg mL^{-1}. The inhibition activity of treated pathogens was measured according to the turbidometry method and then confirmed by the CFU method. The MIC value of SLs was identified as the minimum concentration of each sample that resulted in a significant reduction in colony-forming units (CFU) compared to the untreated samples.[25]

Effect of SLs on the bacterial lipid peroxidation (LPO)

The oxidation of fatty acids within the bacterial cell membrane is a key mechanism that determines the effectiveness of a targeted molecule in bacterial destruction. This oxidative process compromises membrane integrity, ultimately inhibiting cell proliferation. To evaluate the ability of SLs to induce lipid peroxidation (LPO) in bacteria, an LPO colorimetric assay kit was employed.[54] The presence of malondialdehyde (MDA), a byproduct of fatty acid peroxidation, was detected using thiobarbituric acid (TBA), which forms a pink MDA-TBA complex upon reaction. To assess LPO levels, SLs were tested at concentrations near their minimum inhibitory concentration (MIC) values. Each bacterial sample (1 mL) was thoroughly mixed with 300 μL of MDA lysis buffer at 4 °C. To prevent interference from pigment decomposition, 3 μL of butylated hydroxytoluene (BHT) was added. Following this, samples were centrifuged at 8000 rpm for 10 minutes to remove insoluble substances. A 200 μL portion of the resulting clear supernatant was then mixed with 600 μL of TBA solution and incubated at 95 °C for 60 minutes. After allowing the samples to cool to room temperature, the developed pink color was measured at 532 nm using a spectrophotometer (Agilent Cary 100, USA). Treatment of both bacterial pathogens using 5% hydrogen peroxide for 20 min as a positive control was also involved. The indicated increase in the lipid peroxidation efficiency was calculated from the following equation.

$$\text{Malondialdehyde (nmol mL}^{-1}) = A_{\text{sample}}/A_{\text{standard}} \times 10$$

where A sample is the lipid peroxidation absorbance in the treated bacterial cells, and A standard is the absorbance of the standard lipid peroxidation sample.

Measurement of intracellular reactive oxygen species (ROS) inside the treated microbial cells

Likewise, the induction of ROS from SLs inside the microbial cells was also a suggested mechanism that may contribute to the cell inhibition process. ROS are known as a group of highly reactive molecules like molecular oxygen (O_2), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$), having the ability to induce oxidative stress onto bacterial cells. Therefore, the susceptibility of SLs to release high levels of ROS toward the microbial pathogens was assessed intracellularly. Therefore, ROS could be quantified using a fluorescent prop 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, the treated microbial samples were subjected to a permeabilization process by ultrasonication of cells, and the resultant supernatant was harvested and reacted with 10 μm DCFH-DA for 30 minutes at 37 °C.[52] At the same time, the microbial cells were treated with hydrogen peroxide H_2O_2 at 155 μm as a positive control. Immediately, the DCF fluorescence intensity was measured by a spectrofluorometric apparatus (JASCO FP-6500, light source xenon arc lamp, Japan). The fluorescence intensity for each sample was measured with an excitation and emission wavelength at 485 nm and 530 nm, respectively.

Analysis of statistical data

The presented data were documented as the mean along with the standard deviation (±SD) based on three replicates ($n = 3$).

Conclusion

Lepidium sativum oil cake, a byproduct of oilseed processing, is a sustainable resource due to its rich content. In this study, it was confirmed to be a promising substrate for the production of low-cost sophorolipids from locally isolated *Saccharomyces cerevisiae*, which was previously isolated from another agro-industrial waste (castor oil cake). The production of sophorolipids by growing *Saccharomyces cerevisiae* on *Lepidium sativum* oil cake has not been reported before. Therefore, a considerable yield of SLs (35.8 g/100 g substrate) was produced from two steps of solvent extraction (methanol and ethyl acetate). The findings demonstrated that SLs effectively reduced the surface tension of water from 72 to 48 mN m^{-1}, with a critical micelle concentration of 30 mg/100 mL. Structural analysis using FTIR, LC-MS/MS, ^{1}H NMR spectroscopy, and GC/MS chromatography verified the presence of a novel lactonic form of sophorolipids. The positive response of the produced SLs towards many drug-resistant pathogens clearly appeared. Interestingly, the eradication pathway in the tested pathogens was indicated through lipid peroxidation activity and ROS release, which showed a gradual activity based on SLs doses.

Impact statement

This study demonstrates the sustainable production of antimicrobial sophorolipids from *Lepidium sativum* oil cake, an

agro-industrial waste, using *Saccharomyces cerevisiae*. The biosurfactants exhibit strong activity against drug-resistant pathogens, offering an eco-friendly approach to waste valorization and the development of novel antimicrobial agents.

Author contributions

Amr S. Al-kashef (conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing – original draft, writing – review & editing), Khyreyah J. Alfifi (conceptualization, formal analysis, investigation, methodology, writing – original draft, writing – review & editing), Mohamed U. Nooman (conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing – original draft, writing – review & editing), Abd El-Nasser A. Khattab (conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing – original draft, writing – review & editing), Mohamed M. I. Helal (conceptualization, supervision, writing – original draft, writing – review & editing), Madeha O. I. Ghobashy (conceptualization, formal analysis, investigation, methodology, writing – original draft, writing – review & editing), Farhan Qalib Alruwaili (conceptualization, formal analysis, investigation, methodology, writing – original draft, writing – review & editing), and Mohamed Abdelraof (conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing – original draft, writing – review & editing).

Conflicts of interest

None declared.

Data availability

All data mentioned are available from the original source papers cited in this manuscript. Unpublished data are available on request.

Supplementary information is available. See DOI: **https://doi.org/10.1039/d5ra06594a**.

Acknowledgements

This research was funded by the Research, Development, and Innovation Authority (RDIA) - Kingdom of Saudi Arabia with grant number (13445-Tabuk-2023-UT-R-3-1-SE) and the Deanship of Research and Graduate Studies at University of Tabuk through Research no. (0011-1442-S). The authors would like to acknowledge the facilities available at the National Research Centre, Cairo, Egypt, and also to a research grant funded by the Research, Development, and Innovation Authority (RDIA) - Kingdom of Saudi Arabia with grant number (13445-Tabuk-2023-UT-R-3-1-SE). The authors extend their appreciation to the Deputyship ship for Research & Innovation, Ministry of Education in Saudi Arabia, for funding this research work through project number (0011-1442-S).

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