Preparation Process of Sophorolipids by Vacuum Freeze-Drying and Evaluation of Their Antimicrobial Activity

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Abstract

A simpler and more efficient method for preparing sophorolipids (SLs) facilitates the application of SLs. In this study, a new preparation method for SLs named SLs-WpH was designed firstly: the SLs in fermentation broth were separated by sedimentation in a separatory funnel, then dissolved in distilled water by adjusting the pH to 6-7 and centrifuged to remove the yeast brought in the sedimentation process, and the supernatant was vacuum freeze-dried. Secondly, the purity, composition, and antimicrobial activity of SLs-WpH were compared with the other two types SLs purified by ethanol and ethyl acetate (SLs-EtOH, SLs-EAC) in order to evaluate the feasibility of this preparation process. The purity of SLs-WpH was between that of SLs-EtOH and SLs-EAC and there was no difference in the composition, mainly lactonic SLs by TLC and HPLC analysis. SLs-WpH showed broad-spectrum antimicrobial activity against bacteria, fungi and actinomyces. In addition, both the diameter of the inhibition zone (15 to 18 mm) against Staphylococcus aureus and the mycelium inhibition rate (70 to 78%) against Phytophthora infestans were not significantly different among these three types SLs; SLs-WpH had better inhibitory effect against Streptomyces scab and S. bottropensis with MICs of 32 μg mL-1 than the other two types with MICs [?]64 μg mL-1, which is the first reported that SLs had inhibitory activity against actinomyces. The results of this study indicated that the new preparation process of SLs is feasible and SLs have great application potential in the prevention of potato common scabs caused by pathogenic Streptomyces.

Research article

Preparation Process of Sophorolipids by Vacuum Freeze-Drying and Evaluation of Their Antimicrobial Activity

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Running Title: Preparation and Antimicrobial Activity of SLs by Vacuum Freeze-Drying

Abstract: A simpler and more efficient method for preparing sophorolipids (SLs) facilitates the application of SLs. In this study, a new preparation method for SLs named SLs-WpH was designed firstly: the SLs in fermentation broth were separated by sedimentation in a separatory funnel, then dissolved in distilled water by adjusting the pH to 6-7 and centrifuged to remove the yeast brought in the sedimentation process, and

the supernatant was vacuum freeze-dried. Secondly, the purity, composition, and antimicrobial activity of SLs-WpH were compared with the other two types SLs purified by ethanol and ethyl acetate (SLs-EtOH, SLs-EAC) in order to evaluate the feasibility of this preparation process. The purity of SLs-WpH was between that of SLs-EtOH and SLs-EAC and there was no difference in the composition, mainly lactonic SLs by TLC and HPLC analysis. SLs-WpH showed broad-spectrum antimicrobial activity against bacteria, fungi and actinomyces. In addition, both the diameter of the inhibition zone (15 to 18 mm) against *Staphylococcus aureus* and the mycelium inhibition rate (70 to 78%) against *Phytophthora infestans* were not significantly different among these three types SLs; SLs-WpH had better inhibitory effect against *Streptomyces scab* and *S. bottropensis* with MICs of 32 μg mL^{-1} than the other two types with MICs [?]64 μg mL^{-1}, which is the first reported that SLs had inhibitory activity against actinomyces. The results of this study indicated that the new preparation process of SLs is feasible and SLs have great application potential in the prevention of potato common scabs caused by pathogenic *Streptomyces* .

Practical applications: SLs-WpH could be used as a kind of agricultural antibiotic in the prevention of potato common scabs caused by pathogenic *Streptomyces* .

Keywords: biosurfactant, sophorolipids, preparation process, antimicrobial activity, potato common scab

Abbreviations: SLs, sophorolipids; L-SLs, lactonic sophorolipids; A-SLs, acidic sophorolipids; SLs-WpH, sophorolipids prepared by Vacuum Freeze-Drying; SLs-EtOH, sophorolipids prepared by ethanol; SLs-EAC, sophorolipids prepared by ethyl acetate; PCS, potato common scab; LB, Luria–Bertani; TLC, thin—layer chromatography; MIC, Minimum inhibitory concentration

1 Introduction

Sophorolipids (SLs), one of the most interesting biosurfactants, are secondary metabolites produced by non-pathogenic yeast [1-3]. Their molecular structures can be roughly divided into a hydrophilic part and a hydrophobic part: the hydrophilic part is sophorose composed of two glucose molecules linked by a β-1",2' glycosidic bond, and the hydrophobic part is a saturated or unsaturated long-chain ω-(ω-1) hydroxy fatty acid [4]. These two parts are linked by another glycosidic bond located on the 1' carbon atom position to generate SLs [5]. Compared with traditional surfactants, SLs have the advantages of biocompatibility, biodegradability, and low cytotoxicity [3,6]. Due to these advantages, SLs are widely used in many fields such as soil remediation, petroleum recovery, laundry detergents, food preservation, and medical treatment, especially antimicrobial application [7-11].

SLs usually exist in fermentation broth as a mixture containing two major forms: lactonic sophorolipids (L-SLs) and acidic sophorolipids (A-SLs). When glucose and oleic acid are used as substrates, the product is mainly L-SLs, which are easy to settle down in the form of brown oil [12]. Moreover, a large number of literatures have proved that SLs have pharmaceutical activities, such as antibacterial activity [11]. Therefore, it is necessary to separate and prepare L-SLs for further applications, which is also a key process from fermentation to products.

Currently, the preparation methods of SLs mainly include froth flotation, chromatography technology, crystallization at low temperature, membrane filtration, and extraction [13-16]. Among these methods, extraction technology is the most commonly used and the extractant is mainly ethyl acetate or ethanol (SLs-EAC, SLs-EtOH). The crude SLs was extracted from the broth by an equal volume of extractant, then centrifuged to remove the yeast, and the organic supernatant containing SLs was collected and vacuum spin-dried [13,17-20]. However, there are many drawbacks of extraction technology. Although ethyl acetate can preferentially extract L-SLs whether ethanol or ethyl acetate is as the extractant, the drying efficiency is low and a large amount of organic solvent will be consumed, resulting in high preparation costs. In addition, when ethanol was used as the extractant, both A-SLs and L-SLs could be extracted from the fermentation broth [18,21]. Our previous studies have demonstrated that L-SLs can dissolve in distilled water by adjusting the pH to 6-7 [22]. So, one of the aims of this study is to develop a simpler and more efficient method for preparing L-SLs extracted by distilled water adjusting pH and dried by vacuum freeze (SLs-WpH).

L-SLs have pharmaceutical activities, especial antimicrobial activity, which has been broadly reported [11]. SLs can effectively inhibit pathogenic bacteria, including Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* , *Bacillus subtilis* ,*Listeria* spp., *Escherichia coli* , etc [23-26]. Besides, SLs also show inhibitory effect against pathogenic fungi including plant, human, and food pathogenic fungi [10,27,28]. However, there is no literature reported that the inhibitory effect of SLs on actinomycetes. Pathogenic actinomycetes such as Streptomyces scab and S. bottropensis can cause potato common scab (PCS), which is one of the most common diseases of potatoes [29,30]. Therefore, the other aim of this study is to evaluate the feasibility of this new preparation process by analyzing and comparing the purity, composition, and antimicrobial activity (antibacterial, antifungal and anti-actinomycetes) of SLs extracted by different extractants. This research will also provide new insights into the prevention and treatment of PCS caused by pathogenic actinomycetes.

2 Materials and methods

2.1 Microorganisms and culture conditions

Wickerhamiella domercqiae Y_{2A} currently reclassified as *Starmerella bombicola* by Li et al. [31]) isolated by Chen et al. [32] was grown in the 50 mL yeast extract medium(glucose 2%, yeast extract 1%, peptone 2%)in 300 mL flasks at 30 °C on a rotary shaker (200 rpm) for 24 h. A volume of 2.5 mL was transferred to 50 mL fermentation medium (glucose 8%, yeast extract 0.3%, KH_2PO_4 0.1%, Na_2HPO_4[?]12H_2O 0.1%, $MgSO_4$[?]7H_2O 0.05%, oleic acid 6%, pH 6.5) in 300 mL flasks and incubated at 30 degC and 160 rpm for 144 h.

S. aureus (ATCC 6538) *and P. infestans* were obtained from Shandong Province Culture Collection of Industrial Microorganisms. S. aureus was cultured on Luria–Bertani (LB) agar plate (peptone 10g, yeast extract 5g, NaCl 10g, agar 15g per L) at 37 for 24 h. A single colony of *S. aureus* was scraped and suspended in 15-20 mL sterile isotonic solution (0.9% NaCl), and the bacterial density was determined by McFarland turbidimetry. Mycelia plug (6-mm diameter) of *P. infestans* was cultured on V8 agar plate at 18degC for 5-6 days. *S. scabies* ACCC 40330 and *S. bottropensis* AMCC400023 were obtained from Agricultural Culture Collection of China and Shandong Agricultural University respectively. Mycelia of *S. scabies* and *S. bottropensis* were cultured on Gauze's Synthetic Agar Medium (Soluble starch 20.0 g, KNO_3 1.0 g, K_2HPO_4 0.5 g, $MgSO_4$*7H_2O 0.5 g, NaCl 0.5 g, $FeSO_4$ 0.01 g, agar 15.0 g per L) at 26 for 48 h; then*S. scabies* and *S. bottropensis* were transferred on Oatmeal Agar plate at 26 for 3-4 d to produce spores. The surface of the actinomycetes was rinsed with sterile water, and the spores were rinsed with sterile water and counted by spreading plate method.

2.2 Preparation of SLs

2.2.1 Preparation of SLs by Ethyl Acetate and Ethanol

A total of 300 mL of fermentation broth was transferred to a separatory funnel, and after standing for 30 min, SLs in the form of brown oil at the bottom were collected. The SLs was extracted with an equal volume of extractant, then centrifuged at 8000 r min^{-1} for 8 min to collect the supernatant and discard the yeast. Then the supernatant was concentrated by a rotary evaporator, washed with n-hexane to remove excess oleic acid, dried at room temperature; and L-SLs solids (SLs-EtOH, SLs-EAC) were obtained.

2.2.2 Process for Preparing L-SLs by Vacuum Freeze drying adjusting pH

In the same steps mentioned above, the crude SLs in the form of brown oil at the bottom of the separatory funnel were collected; added distilled water and adjusted pH to 6-7 using 0.01 mol mL^{-1} NaOH to completely dissolve the SLs; then centrifuged to collect supernatant discarding the residual yeasts. The supernatant was first pre-cooled at -40 for 24 h and dried by vacuum freezer with different conditions.

2.3 Characterization of SLs Prepared by Three Methods

2.3.1 Relative Purity of SLs by anthrone method [33,34]

The SLs powder was dissolved in distilled water at the concentration of 0.05 mg mL^{-1}. Anthrone reagent was prepared by the following: 0.1 anthrone was dissolved in 100 mL of concentrated sulfuric acid in the dark. 1 mL of 0.05 mg mL^{-1} SLs solution and 4 mL of anthrone reagent were added into the stoppered test tube, heated in boiling water for 10 min, cooled in ice water, and measured optical density (OD) at 600 nm. at 600 nm. SLs are composed of disaccharide sophorose and a long fatty acid chain. According to the ratio between the molecular weights of SLs and glucose (1g of glucose is equivalent to 1.91g of SLs), the actual concentrations of the three SLs solutions were calculated, and the relative purity of SLs was defined as: relative purity (%) = (equivalent glucose concentration) x 1.9 / (Prepared concentration) x 100 %.

2.3.2 Composition Analysis of SLs by TLC and HPLC

Five-microliter samples of 20 mg mL^{-1} SLs solution prepared by different methods were applied to thin—layer chromatography (TLC) for analysis of SLs composition. Stationary phase: silica gel GF254; developing system: $CHCl_3/CH_3OH/H_2O$ (65:15:2, v/v/v); visualizing reagents: a-naphthol/sulfuric acid [4].

SLs composition was analyzed by HPLC (SHIMADZU, Japan) with a Venusil MP-C18 column (250 mmx4.6 mm). The mobile phase was acetonitrile/water starting from 20% acetonitrile, then gradually increased from 20 to 30% within 10 min, from 30 to 60% within another 10 min, from 60 to 70% within 15 min, from 70 to 90% within 20 min, then reduced from 90 to 20% as stop condition within 5 min at a flow rate of 1.0 mL min^{-1}. 5 μL of 50 mg mL^{-1} SLs methanol solution prepared by ethyl acetate, ethanol and distilled water at pH 6.8 was injected and the eluent was monitored with UV detector at 207 nm [35].

2.4 Comparation of Antimicrobial Activities of Three Types of SLs

2.4.1 Inhibition of mycelial growth of *P. infestans*

Mycelia plugs (6 mm diameter) of*P. infestans* were transferred to V8 agar plates (V8 vegetable juice 200g, $CaCO_3$ 2g, agar 15g per L). These plates were divided into 4 groups: control, SLs-EtOH, SLs-EAC, SLs-WpH. All plates were incubated at 18 for 6 days and the diameter of each fungal colony was recorded at 4, 5, 6 days, respectively. The inhibition rate of mycelial growth was calculated according to the following formulation: mycelial growth (%) = (colony diameter of control - colony diameter of SLs treatment) / (colony diameter of control-6 mm of plug diameter) × 100 %.

2.4.2 Measurement of Inhibition Zone Diameter by Agar Diffusion

Inhibition zone diameter was measured by drilling method with slight modification [36]. In brief, 100 μL of spore suspension of tested*Streptomyces* (*S. scabies* ACCC 40330 and *S. bottropensis* AMCC400023) and 100 μL of bacterial suspension of *S. aureus* was spread on Gauze's synthetic agar plates and LB plates, respectively; then every agar plate was drilled evenly five holes of 5 mm diameter by the puncher, and 50 μL of SLs solutions at the concentrations of 2.5, 5, 7.5 and 10 mg mL^{-1} was added, respectively. All the plates were placed at 25 at least 2 h for spreading adequately. The Gauze's synthetic agar plates and the LB agar plates were incubated at 30 and 37, respectively.

2.4.3 Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was determined by test tube double dilution method with minor modifications. Briefly, 36 tubes (13 mm×100 mm) were divided into three groups for SLs-EtOH, SLs-EAC and SLs-WpH, and marked from 1 to 12, respectively. Test tubes from 1 to 10 were treatments of SLs, 11 and 12 were blank and negative control, respectively. For *S. aureus* , LB liquid medium (2 mL) was transferred into all test tubes except 1, and 2 mL of SLs (1024 μg/mL) diluted by LB liquid medium was transferred to test tube 1 of each group. Double dilution series was carried out and the concentration of the tube 10 was 2 μg mL^{-1}. A volume of 100 μL bacterial suspension (about 10^7 CFU mL^{-1}) was added to all tubes expect 11 to reach a final concentration of 5×10^5 CFU mL^{-1}, and 100 μL of LB medium was transferred to tubes 11. For *S. scabies* and *S. bottropensis* , 2 mL Gauze's synthetic medium was transferred to 2 to 12 tubes. 2mL of SLs solution (1024 μg mL^{-1}) diluted by Gauze's synthetic medium was added to the No.1 tube. Double dilution series was as same as mentioned above. Volume of 100 μL spore suspension about the

tested actinomycetes was 10^5 CFU mL^{-1} to reach the final concentration about 10^3 CFU mL^{-1} and 100 μL of Gauze's synthetic medium was transferred to tubes 11.

The Gauze's synthetic medium tubes were incubated at 30 for 48 h and the LB medium tubes were incubated at 37 for 24 h, then the tested bacteria growth was observed. MIC was defined as the lowest concentration of SLs that completely inhibited growth of the bacterium as detected with the naked eye, corresponding to the clear tube.

2.5 Statistical Analyses

All experiments were conducted twice under the same conditions and the data were expressed as the mean ± standard error. Statistical analyses were performed and analysis of comparing means was used to determine the effect of the treatments using SPSS ver. 26.0 (SPSS, Inc., Chicago, IL). Means were separated by the least significant difference tests at$p$ =0.05.

3 Results

3.1 Process for Preparing L-SLs by Vacuum Freeze Drying Adjusting pH

Comparing the solubility of SLs in distilled water at different pH ranging from 6 to 7, it was found that SLs had the highest solubility at pH 6.8 with the volume ratio of SLs to water being 1.5. After centrifugation, the collected supernatant was first pre-cooled at -40 for 48 h, and then dried by vacuum freeze at different temperatures of -40, -50 and -70, respectively, and the drying time was controlled for 12, 24 and 48 h, respectively. The drying parameters were -70 and 12 h, the drying efficiency was the highest. It was reflected in the fact that the texture of the SLs obtained by the three drying temperatures was all white crispy powder without obvious difference, but the drying time at -70degC was much less. According to the data, the process for preparing L-SLs by vacuum freeze drying by adjusting pH was determined (Figure 1).

3.2Characterization of SLs-WpH, SLs-EAC, SLs-EtOH

The purity of SLs was 98.13%(SLs-EtOH),81.80%(SLs-EAC), and 97.27%(SLs-WpH), respectively. The purity of SLs-EtOH was similar to SLs-WpH, and there were no significant differences between them. SLs-EAC had the lowest purity that was significantly lower than the other two SLs.

Due to different polarity, L-SLs distributed faster than A-SLs, thus the L-SLs showed a much higher R_f value than A-SLs. R_f values of TLC bands of SLs were 0.93 (SLs-WpH), 0.89 (SLs-EAC), and 0.92 (SLs-EtOH), respectively, which showing these bands corresponded to L-SLs forms mainly and there were no significant differences in R_f among different preparation methods (Figure 2B).

The composition of SLs-WpH, SLs-EAC and SLs-EtOH was further analyzed by HPLC (Figure 2C). There were more than six peaks in each type of SLs and the retention time of each peak was basically the same, at 22.97, 23.99, 26.34, 27.10, 28.66 and 34.21min. The relative area of these peaks was slightly different, especially peak NO.4, 5, and 6, which are L-SLs according to the analysis of relevant literatures,[37] peak areas were accounted for 34.28, 15.50 and 10.93% (SLs-WpH); 31.61,17.05 and 11.37% (SLs-EAC); 31.76, 17.04 and 11.57% (SLs-EtOH) of the total area, showing that the three SLs were mainly lactonic. All the data showed that preparation method can affect the proportion of L-SLs to some extent without affecting the composition.

3.3 Comparison of Antimicrobial Activity amongSLs-WpH, SLs-EAC and SLs-EtOH

3.3.1 Antimicrobial Activity of SLs against Bacteria and Fungi

S. aureus cells were treated with three types of SLs at 7.5 mg mL^{-1}. The inhibition zone diameters were 18.00 mm (SLs-WpH), 15.48 mm (SLs-EAC) and 16.42 mm (SLs-EtOH), respectively. It showed no significant differences in inhibition zone diameters among the SLs. The MICs of SLs-EAC and SLs-EtOH were 128 μg mL-1, which were four times higher than that of SLs-WpH (32 μg mL-1) (Table 1 and Table 3).

Three types SLs inhibited the mycelial growth of *P. infestans* in a time-dependent manner. The mycelial inhibition rates were recorded from the fourth day to the sixth day, ranged from 74.26-78.89% (SLs-WpH), 70.90-75.06% (SLs-EAC), and 71.71-78.17% (SLs-EtOH) (Figure 4). SLs-WpH showed an inhibitory effect of mycelium growth similar to SLs-EAC and SLs-EtOH without significant difference.

3.3.2 Antimicrobial Activity of SLs against Actinomycetes

Growth of *S. scabies* ACCC 40330 and *S. bottropensis*AMCC400023 was inhibited effectively by three types SLs showing a dose-dependent manner. When *S. scabies* cells were treated with SLs at 2.5-10 mg mL^{-1}, inhibition zone diameters ranged from 17.83-26.25 mm (SLs-WpH), 13.88–24.17 mm (SLs-EAC), and 13.08–28.17 mm (SLs-EtOH), and inhibition zone appeared only when the concentrations of SLs-EAC and SLs-EtOH were not less than 5 mg mL^{-1}; in addition, in the concentration range of 2.5 to 5 mg mL^{-1}, the inhibition zone diameters of SLs-WpH were higher than those of SLs-EAC and SLs-EtOH, and the difference was significant. Likewise, for *S. bottropensis* cells, inhibition zone diameters ranged from 16.83-23.67 mm (SLs-WpH), 16.75-22.02 mm (SLs-EAC), and 17.70-20.06 mm (SLs-EtOH), and inhibition zone appeared only when the concentrations were not less than 5 (SLs-WpH) and 7.5 mg mL^{-1} (SLs-EAC, SLs-EtOH), respectively; in the concentration range of 5 to 7.5 mg mL^{-1}, the inhibition zone diameters of SLs-WpH were higher than those of SLs-EAC and SLs-EtOH, showing different significantly. When the concentration was greater than 7.5 mg mL^{-1}, whether *S. scabies* or *S. bottropensis* , the inhibition zone of the three types of SLs did not show significant differences anymore (Table 2, Figure 3b and 3c).

Test tube double broth dilution method was used to determine MICs of SLs. For *S. scabies* , MICs of SLs-WpH, SLs-EAC, and SLs-EtOH were 32,128, and 64 μg mL^{-1}, respectively; for *S. bottropensis* , MICs of SLs-WpH, SLs-EAC, and SLs-EtOH were 32,128, and 128 μg mL^{-1}, respectively. The data showed whether for*S. scabies* or *S. bottropensis* , the MIC of SLs-WpH was lower and significantly different compared to SLs-EAC and SLs-EtOH (Table 3).

4. Discussion

SLs are biosurfactants produced by non-pathogenic yeasts and present in fermentation broth as a mixture. Traditionally, the preparation methods of SLs on the laboratory scale mainly rely on single gravity separation and extraction, which results in high preparation costs [15,38]. At pH 5 or lower value, SLs were dispersed in water. Beyond 5.6-5.8, the solubility is improved, and at pH 6, SLs are totally soluble. They are unstable at pH values higher than 7.0–7.5; beyond this point, irreversible hydrolysis of the acetyl groups and ester bonds is observed [3,39]. Based on this, it is feasible that SLs in fermentation broth settle in a separatory funnel and then dissolve in distilled water adjusting pH. In this study, SLs had the highest solubility at pH 6.8 with the volume ratio of SLs to water being 1.5. This result was also consistent with our previous conclusion [22].

Pure SLs are colorless and form a white powder when completely dry [40]. In the lab-scale extraction, the organic phase containing SLs is usually concentrated by rotary evaporation and the excess water is removed by oven drying or air drying. Because of the low volatility of water, a more efficient drying method is needed. Vacuum freeze-drying is an efficient drying method used in the food and biology industry. Since the process occurs at low temperatures and under vacuum conditions, vacuum freeze drying generates products have significantly high quality and good biological activity [41-44]. Therefore, vacuum freeze-drying was appropriately used in the process for preparing L-SLs in this study. The freeze-drying temperature was lower, the freeze-drying period was shorter. The drying rate was affected by some factors such as freezing temperature, ice crystal shape, frozen layer thickness and so on [38,45-47], which may explain why the freeze-drying period was the shortest at -70. The dried SLs-WpH after grinding were yellowish white powder.

The purity and composition of SLs are important for evaluating the feasibility of new preparation methods. In this work, we found that the purity of SLs-WpH was about 10% higher than SLs-EAC and equal to SLs-EtOH. Besides, SLs are mixture with similar fundamental structures, the type of SLs obtained by the new preparation method was investigated whether lactone or acid forms. The results of TLC showed that the R_f value of the three bands was around 0.90, without other spots with low $R_f$value, which preliminarily indicated that the three SLs were mainly lactonic SLs. The conclusion was further confirmed by analytical

HPLC. Generally, the retention time of lactonic SLs is longer than that of acid SLs, around 30 minutes or later, which varies from the type of yeast and the fermentation substrate [34,48]. Figure 2 showed that the retention peaks of three SLs mainly appeared in about 27, 28 and 34 minutes, and these peaks areas were accounted for about 60% of the total area, which proved that SLs-WpH, SLs-EAC and SLs-EtOH were mainly lactonic.

Drug activity, particularly antimicrobial activity, is an important parameter to evaluate the biological activity of SLs. *S. aureus*is a typical pathogen which can cause a series of diseases, such as food poisoning, skin infection, and postoperative infection [49]. In this study, treated at the concentration of 7.5 mg mL^{-1}, the diameter of inhibition zones of three SLs were similar without significant difference (Table 1). MIC refers to the lowest concentration of compounds that inhibit the visible growth of microorganisms, which can more accurately evaluate the bacteriostatic effect of the durgs [50]. SLs-WpH showed the lowest MIC of 32 μg mL^{-1}, which was one-fourth of the MIC of SLs-EAC and SLs-EtOH. The same MIC of SLs was reported by Silveira et al. and by our previous research [23,51]. The consistency with other research results showed that SLs had good antibacterial activity.

The Antifungal activity of SLs has also been widely verified, especially on plant pathogenic fungi. Caretta found that SLs were more effective against *P. ultimum* with 95% inhibition of mycelial growth, followed by *B. cinerea* with 75.7%, *Rhizoctonia solani*with 64.3% and *S. rolfsii* with 28.5%.[52] The mycelium is a filamentous or tubular fungal structure that grows from a germinal tube and extends continuously by growing at the top [53]. The growth of filamentous fungi mainly depends on the spread and extension of mycelia, and the repeated branches form a network of mycelia [54]. Inhibition of mycelium formation is the most direct reflection of inhibition of fungal growth. Compared with the control group (without SLs), SLs-WpH, SLs-EAC and SLs-EtOH could significantly inhibit the growth of mycelia in a time-dependent manner (Table 1). Moreover, the inhibitory effect showed by SLs-WpH was roughly equal to the other two SLs without significant difference, and the antifungal activity was consistent with our previous study (treated under the same concentration, the mycelial inhibition rate was 73.98%) [22].

Actinomycetes, more than a special strain between bacteria and fungi, are a group of gram-positive bacteria, but their cell morphology and reproduction mode are quite different from those of cocci and bacilli [55]. Potato common scab is one of the five major pathogens of potato, which is mainly caused by multiple pathogenic actinomycetes such as *S. scabies* and *S. bottropensis* [56]. The surface of tubers infected with PCS often has flat spots, raised spots, or concave scabs, resulting in a decline in tuber quality and severe economic losses [57]. A safe and effective antimicrobial method is urgently needed to prevent and control PCS. In this work, SLs were proven to significantly inhibit the growth of actinomycete, and there was no obvious inhibition zone around the negative control group. In addition, SLs-WpH could inhibit the growth of two actinomycetes at a lower concentration. When treated at 2.5 mg mL^{-1} and 5 mg mL^{-1} of SLs-WpH, the inhibition zone of *S. scabies* and *S. bottropensis* was appeared respectively. When the concentration was greater than 7.5 mg mL^{-1}, the inhibition zone diameter of the SLs-WpH was more than 20 mm and did not show significant differences compared the other two SLs (Table 2). The inhibitory effect was further supported by the MIC results. For *S. scabies* , the MIC of SLs-EAC was twice and three times higher than that of SLs-EtOH and SLs-WpH respectively. Similar to *S. bottropensis* , SLs-EAC and SLs-EtOH had the highest MIC, which was twice higher than SLs-WpH (Table 3). To verify the reason why SLs inhibit actinomycetes, it is necessary to further explore the antibacterial mechanism. The destruction of SLs on actinomycete mycelium morphology can be seen intuitively by observing the microscopic morphology [28]. As actinomycete is a special bacteria, it is helpful to analyze whether SLs damage the cell structure or metabolic pathway of actinomycetes by verifying the effects of SLs on the activities of several key enzymes of bacteria [58-60]. In addition, to testify the feasibility of applying SLs to protect PCS, a large number of in vitro field experiments will be the focus of future research.

The inhibitory effects showed by SLs-WpH was not lower than SLs-EAC and SLs-EtOH towards bacteria, fungus and actinomycetes. This may be related to the higher proportion of lactonic SLs in SLs-WpH. Researchers have paid a lot of attention to improving the antimicrobial activity of SLs by changing the

fermentation substrate or directly modifying SLs [61-64]. However, few reports have been made to improve the antimicrobial effect of SLs by optimizing the method of preparation. Solaiman et al. described a low-temperature protocol that preferentially separated 6'-monoacetylated 22:0-SLs from the mixture. Since the purification after the crystallization of SLs at a specific temperature, the SLs had high purity and wonderful antibacterial activity [15]. However, this preparation method still depended on the use of organic solvent and was not suitable for mass production. The new preparation method of SLs-WpH seems to solve the problem. In addition, previous studies discussed too much about the inhibition of SLs to bacteria and fungi, while there have been no reports of SLs inhibiting actinomycetes until now. Our study proved that SLs were able to inhibit the growth of actinomycetes. This may be achieved by inhibiting the mycelial growth of actinomycetes or reducing the activity of certain enzymes.

In this study we proposed a more efficient and cheaper method for the preparation of SLs, and verified the composition and antimicrobial activity of the SLs-WpH. More interestingly, the inhibitory effect of SLs on actinomycetes was firstly demonstrated, which will be expected to be an active substance for the prevention of potato common scab.

Conflict of Interest

The authors declare no conflict of interest.

Reference

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