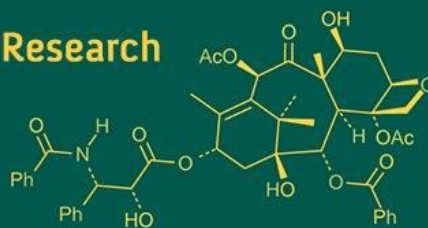
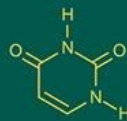
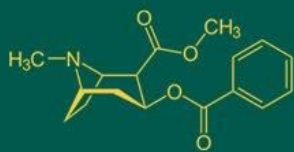


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Structure-activity relationships of lipopeptides produced by *Bacillus methylotrophicus* DCS1 over time

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Abstract

The present study aims to assess the functional properties and biological activities of lipopeptide mixtures produced by *Bacillus methylotrophicus* DCS1 strain and recovered from Landy medium during fermentation, after 24, 48 and 72 h. These lipopeptide mixtures are composed of different homologues and various isoforms, belonging to surfactin, iturin and fengycin families, identified in our previous study. The purpose of this research was to investigate some physicochemical properties and to test antibacterial, antifungal and antioxidant activities. Lipopeptide DCS1 solutions exert interesting functional properties such as their ability to reduce the surface tension of water down to low values with weak critical micelle concentration (CMC). The best surface tension reduction from 59.53 mN m⁻¹ (initial surface tension of water) to 30.1 mN m⁻¹, with a CMC value of 100 mg l⁻¹, was obtained with LP (48 h). All the LP mixtures are able to emulsify hydrocarbons but with different emulsification indexes, LP (72 h) exhibit the highest emulsifying activity with vegetable oil with an emulsification index of 47.5%. In addition, they are endowed with antibacterial and antifungal activities against several pathogenic microorganisms. A significant antagonistic activity was observed with LP (48 h) against *Salmonella typhimurium* and *Fusarium solani*. The different LP mixtures exhibit interesting antioxidant activities using DPPH radical-scavenging and β -carotene bleaching inhibition methods. The highest activity was observed with LP (72 h), the scavenging effect on DPPH radicals at a concentration of 2 mg ml⁻¹ was 86.27%. Moreover, LP (72 h) exhibited a strong inhibition of β -carotene bleaching by linoleic acid assay with almost 86% at 4 mg ml⁻¹ concentration. Therefore, these bioactive lipopeptide families mixtures, having a synergistic effect, could be potential for pharmaceutical purposes, cosmetic and food industries applications.

Keywords: *Bacillus methylotrophicus*, DCS1, Lipopeptide mixtures, Functional properties, Antimicrobial, Antioxidant activities

1. Introduction

Lipopeptides are produced by a variety of microorganisms. Microbial lipopeptides have gained the interest of microbiologists, chemists and biochemists for their high structural diversity as well as efficient action, low toxicity and good biodegradability in comparison with synthetic counterparts.

Lipopeptides from *Bacillus* are the most widely studied, and can be divided into three families according to the structure of the cyclic peptide: surfactin, iturin and fengycin-plipastatin and kurstakin [1]. *Bacillus* lipopeptides possess amphiphilic features that facilitate numerous functional properties and multiple biological activities including antibacterial, antifungal, antiviral and antitumor activities. Therefore, they can be used in various fields of biotechnology applications such as medical, pharmaceutical, cosmetic, food and agriculture [2].

The long-term use of antibiotics as feed additives can lead to drug resistance, drug residuals in animals, and environmental pollution. In *Bacillus* species, the genetically and biochemically elucidated biosynthesis of antimicrobial metabolites constitutes a principal mechanism underlying their biological control activity [3]. The *Bacillus* lipopeptides have the potential to be developed as new feed additives due to their antimicrobial and antioxidant properties and their good thermal and chemical stability. Lipopeptides are typically small in molecular weight, and they can maintain high antibacterial activity. In the intestines, lipopeptides can effectively inhibit the growth and reproduction of pathogenic

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microorganisms and regulate the balance of intestinal flora without development of drug resistance or causing side effects of drug residue. These materials are expected to be new green antibacterial feed additives.

The different lipopeptide families produced by *Bacillus* species have been considered as key factors due to their biological control activity [4, 5, 6, 7, 8]. Fengycin and iturin homologues exhibit antifungal activity and microorganisms producing these lipopeptides are sought after as first generation biocontrol agents in the agricultural sector [9]. Khan *et al.* [10] reported that species of *Bacillus* represent the most promising biocontrol agents. However, the advanced medical and agricultural applications of lipopeptides demand production under strictly regulated conditions to enhance the selective synthesis of these compounds [11].

2. Materials and methods

2.1. Bacterial strain and lipopeptides production

Lipopeptides-producing strain used in this study is *Bacillus methylotrophicus* DCS1 [12]. The lipopeptides DCS1 production in Landy medium and their recovery has been achieved at different times of fermentation, 24, 48 and 72 h, by acid precipitation method as described in our previous study [13]. The freeze dried crude lipopeptides were used to perform the physicochemical properties and biological activities.

2.2. Critical Micelle Concentration (CMC) determination

Lipopeptides are characterized by their CMC value, it's the lipopeptide concentration at which the surface tension reaches its minimum and no further decrease is detected because there is formation of lipopeptide micelles. The CMC was determined by monitoring surface tension across different concentrations of diluted lipopeptides in distilled water, until the surface tension reached a constant value. Surface tension measurements were performed using the Wilhelmy plate method with a manual digital surface tensiometer (GIBERTINI Elettronica™ TSD Digital Tensiometer). The surface tension measurement was output digitally in $\text{mN}\cdot\text{m}^{-1}$. CMC value was determined by graphically plotting the curve representing the surface tension as a function of the lipopeptides concentration.

2.3. Emulsification activity

Emulsification activity of lipopeptides recovered after each 24 hours of incubation was estimated by measuring the emulsification index (E_{24}) using the method of Padmavathi and Pandian [14]. 2 ml of each LP solution of 1 g l^{-1} concentration was mixed with 2 ml of hydrocarbon substances, diesel and vegetable oil, the mixture was vortexed vigorously for 2 min. After incubating the tubes at room temperature for 24 h, the emulsification index (E_{24}) was calculated. The emulsion height (He) obtained, compared to the total height (Ht) was measured. The emulsification index (E_{24}) was determined using the following formula:

$$E_{24} (\%) = (He / Ht) \times 100$$

2.4. Hemolytic activity

Hemolytic activity of different LP solutions was tested on blood agar plate containing 5% of human blood (v/v). This method consists of making wells with 3 mm depth and 8

mm diameter, using sterile cones, then placing 60 μl of crude lipopeptide solutions, LP (24 h), LP (48 h) and LP (72 h), at a concentration of 0.5 g l^{-1} in each well of the Petri dish. The plates were incubated at 4°C for 2 h to allow diffusion of LP solutions into the agar, then, they were incubated at 37°C for 72 h. The evolution of the lysis diameter of the red blood cells was monitored every 24 hours by measuring the diameter of the lysis zones (in mm). This test was performed in triplicate.

2.5. Antimicrobial activities

2.5.1. Microbial strains

The antimicrobial activities of the crude LP (24 h), LP (48 h) and LP (72 h), were investigated against four species of pathogenic bacteria: *Micrococcus luteus* (ATCC 4698), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 19430), and against two fungal strains: *Fusarium solani* and *Rhizoctonia solani*.

2.5.2. Agar diffusion method

The agar diffusion method, described by Nanda and Saravanan [15], was applied for the determination of antimicrobial activities of the different recovered LPs. Prior to analysis, the lyophilized lipopeptide mixtures were dissolved in sterile distilled water to get a final concentration of 1 mg ml^{-1} . The tested bacteria were cultured overnight in liquid Luria-Bertani (LB) medium, each prepared culture was used with a concentration of 10^6 CFU ml^{-1} (monitored by the absorbance at 600 nm). For fungal strains, cultures of 10^8 spores ml^{-1} were prepared in the malt extract medium (monitored by counting on a Malassez cell). Then, 200 μl of each suspension was spread on agar plates.

After boring wells (3 mm depth, 4 mm diameter) in the inoculated agar, each one was loaded with 60 μl of each LP sample. The Petri dishes were kept at 4°C for 1 h, to permit diffusion of the lipopeptide solution through the agar medium; then, they were incubated for 24 h at 37°C for bacteria and for 72 h at 30°C for fungi. The antimicrobial activity was evaluated by measuring the growth inhibition zone diameter in millimetres (including well diameter of 4 mm).

2.6. DPPH radical-scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity of DCS1 lipopeptide solutions was evaluated at concentrations ranging from 0.2 to 2 mg ml^{-1} , following the method described by Bersuder *et al.* [16]. The percentage of DPPH radical scavenging was calculated using the standard formula:

$$\text{DPPH radical-scavenging activity } (\%) = \frac{A_{\text{control}} + A_{\text{blank}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

A_{control} represents the absorbance of the control reaction, which contains all reagents except the lipopeptides, A_{blank} denotes the absorbance of the lipopeptide sample in the absence of DPPH solution, while A_{sample} corresponds to the absorbance of the lipopeptide sample in the presence of DPPH. A decrease in the absorbance of the reaction mixture indicates an increase in DPPH radical-scavenging activity. Butylated hydroxyanisole (BHA, 2.0 mM) was used as the positive control. All assays were performed in triplicate, and the results are presented as mean values.

2.7. β -carotene bleaching assay

The ability of DCS1 lipopeptide solutions to inhibit β -carotene bleaching was assessed at concentrations ranging from 0.2 to 4 mg ml⁻¹, following the method described by Koleva *et al.* [17]. Antioxidant activity was expressed as the percentage of β -carotene bleaching inhibition, calculated using the following equation:

$$\beta\text{-carotene bleaching inhibition} = \left[1 - \left(\frac{A_0 - A_t}{A'_0 - A'_t}\right)\right] \times 100$$

A_0 and A' represent the initial absorbance values of the test sample and the control, respectively, while A_t and A'_t correspond to their absorbance after incubation. BHA was used as the positive reference standard. All assays were conducted in triplicate, and the results are reported as the mean of the three independent measurements.

3. Results and discussion

3.1. Lipopeptides mixtures produced over time

Identification of lipopeptides by mass spectrometry (LC/MSD-TOF) showed that lipopeptide isoforms produced by *B. methylotrophicus* DCS1 during the first 24 h and 48 h of fermentation are the same, belonging to bacillomycin D and fengycins A and B homologues. Whereas lipopeptides produced after 72 h comprise different homologues and isoforms belonging to the surfactin, iturin and fengycin families [13].

3.2. CMC determination

The CMC value is the concentration of lipopeptide biosurfactants required to reduce surface tension to its minimum value. Above the CMC, surface tension remains relatively constant because the interface of the culture medium becomes saturated by lipopeptides, and micelles are formed. The CMC is an important characteristic in several applications and the minimum value of surface tension merely shows the effectiveness of lipopeptides [18].

Figure 1 demonstrates that the CMC value of lipopeptides produced by *B. methylotrophicus* DCS1 after 24 and 48 h is approximately 0.1 g l⁻¹; this is explained by the presence of the same lipopeptide isoforms. The lipopeptide isoforms produced from the first 24 hours of fermentation are composed of fatty acids considered to have long hydrocarbon chains, ranging from C₁₄ to C₁₉ [13]. Indeed, CMC value decreases as the number of carbons in the fatty acid chain increases. The CMC is also around 0.1 g l⁻¹ for lipopeptides obtained after 72 h of fermentation. LP (48 h) and LP (72 h) exhibit a nearly identical profile of the surface tension reduction curve at low concentrations. LP (48 h) exhibited the highest surface tension reduction, lowering it from 59.53 mN·m⁻¹ (initial surface tension of water) to 30.1 mN·m⁻¹ with a CMC value of 0.1 g l⁻¹.

Carolyn *et al.* [19] reported that CMC values of lipopeptides span a broad range, from approximately 22 to 507 mg l⁻¹. CMC value of lipopeptides varies widely depending on their specific chemical structure such as amino acid sequence, fatty-acid chain length, and net charge of the molecule.

3.3. Emulsification activity

Biosurfactants play a very important role in the emulsification of oils by trapping hydrophobic molecules in the pseudo-hydrophobic phase formed by micelles [20]. The E₂₄ index is a parameter based on the stability of the

emulsion formed with diesel and vegetable oil after 24 hours.

Figure 2 showed that all the LP solutions are able to emulsify hydrocarbons but with different emulsification indexes. LP (24 h) and LP (72 h) emulsify vegetable oil more efficiently than diesel oil, the emulsification indexes (E₂₄) are 25% and 47.5%, respectively, with vegetable oil. Whereas, for LP (48 h), the emulsification index of diesel (45%) is higher than that of vegetable oil (30%). LP (24 h) have the lowest emulsifying activity, while LP (72 h) are the most effective at emulsifying diesel and vegetable oil with an emulsification indexes of 47% and 47.5%, respectively. This may be explained by the wealth of LP (72 h) with surfactin and pumilacidin isoforms endowed with interesting functional properties.

These results are comparable with previous studies that have reported the emulsifying activity of lipopeptides. Pathak *et al.* [21] isolated *B. subtilis* K1 strain from aerial roots of banyan tree, producer of surfactins, iturins and fengycins mixture exhibiting a very good emulsification activity as well as excellent emulsion stability. Lee *et al.* [22] reported that lipopeptide produced by *B. amyloliquefaciens* LP03 exhibit an important crude-oil emulsifying activity.

3.4. Hemolytic activity

Based on the results presented in Figure 3, it can be seen that during the incubation period, the lysis diameter of the red blood cells is in the following order: LP (72 h) > LP (48 h) > LP (24 h). After 72 h of incubation, the largest lysis diameter is around 21 mm, observed with LP (72 h). This result may be due to the fact that LP (72 h) comprises homologues and isoforms belonging to surfactin family known for their potent hemolytic activity, as well as isoforms of iturin A/mycosubtilin known for their strong hemolytic activity. The lowest lysis diameters were obtained with LP (24 h) over time, which are very close to those obtained with LP (48 h). This can be mainly due to the presence of three fengycin A isoforms and three fengycin B isoforms in LP (24 h) and LP (48 h), known for their low hemolytic activity, and the absence of surfactins and iturins A/mycosubtilins. Chen *et al.* [23] reported that iturin exhibits strong hemolytic activity, whereas fengycin shows comparatively weaker hemolytic effects. In contrast to both iturin and fengycin, surfactin is primarily recognized for its pronounced hemolytic properties. The results obtained are in accordance with those described by Walter *et al.* [24], who showed that the amphiphilic properties of surfactins allow them to interact with biological membranes and disrupt them.

3.5. Antibacterial and antifungal activities

The antibacterial activity of lipopeptides DCS1 against various Gram-positive and Gram-negative bacteria was determined by measuring the diameter of the inhibition zone (Fig.4). Lipopeptides DCS1 are able to spread on the agar and they are responsible for the antibacterial effect against all the pathogenic bacteria tested, with different degrees of growth inhibition. The strongest antibacterial effect was observed against *S. typhimurium*, with a maximum inhibition diameter of 24 mm with LP (48 h) and growth inhibition diameters of 19 and 14.5 mm, were observed with LP (72 h) and LP (24 h), respectively. The antibacterial activity is less important against *P. aeruginosa* with a diameter of 10.5 mm for LP (48 h) and a diameter of 9 mm for LP (24 h) and (72 h). For *B. cereus* strain, the results are

very close to those of *P. aeruginosa*. While the lowest antibacterial activity has been observed with LP (48 h) and LP (72 h) against *M. luteus* with an inhibition diameter of 8 mm.

Antifungal activity of the LP solutions, LP (24 h), LP (48 h), and LP (72 h) has been tested against the two phytopathogenic fungi, *F. solani* and *R. solani* (Fig. 5A). LPs present antagonistic activities against the tested fungi with different degrees of growth inhibition. All LP solutions showed stronger antifungal activity against *F. solani* than against *R. solani*, with LP (48 h) producing the largest inhibition zone around the well, measuring 31 mm. Average inhibition diameters of 23 and 19 mm were obtained with LP (24 h) and LP (72 h), respectively, against *F. solani*. In contrast, *R. solani* shows smaller average growth inhibition diameters, between 13 and 14.5 mm, compared with *F. solani* (Fig. 5B).

These findings indicate that the lipopeptide mixture obtained after 48 h of fermentation, comprising bacillomycin D and fengycin A and B isoforms, exhibits noteworthy antibacterial and antifungal activities, higher than those of LP (72 h). Therefore, it can be inferred that LP (72 h), containing surfactins, pumilacidins, and iturins A, in addition to bacillomycins D and fengycins A and B, does not enhance antimicrobial activities, on the contrary, the inhibitory activities decreased. This may be due to an antagonistic interaction between the various lipopeptide families, in some cases, competition for the microbial membrane can slightly reduce the efficiency of one of the lipopeptide families.

The antimicrobial activity of lipopeptides is attributed particularly to their amphiphilic nature, which allows them to interact with the membrane composed of a lipid bilayer. Our results align with the observations of Ortiz *et al.* [25], who reported that lipopeptides are biological antimicrobials with a distinctive strategy, they have the ability to disrupt biological phospholipid membranes causing cell lysis by increasing membrane permeability and therefore the escape of metabolites.

3.6. Antioxidant activities

3.6.1. DPPH radical-scavenging assay

DPPH is a free radical with purple color and a maximum absorbance at 517 nm. The purple color of DPPH generally disappears when an antioxidant is present in the reaction medium. Antioxidants can neutralize DPPH free radicals

and turn them pale yellow in color, through the transfer of an electron or hydrogen atom [26].

Figure 6A showed that at concentrations ranging from 0.2 to 2 g l⁻¹, LP (24 h), LP (48 h) and LP (72 h) are capable of neutralizing the DPPH radical and they exerted a dose dependent anti-radical activity. Our results are in accordance with those reported by Yalçın and Çavusoglu [27], indicating that DPPH scavenging activity increased with increasing the concentration of biosurfactants synthesized by *B. subtilis* RW-1.

BHA has the highest scavenging activity at all the concentrations tested. For lipopeptide solutions, an anti-radical activity of 86.27%, the highest recorded, was observed for LP (72 h), followed by 70% for LP (48 h) and 49.82% for LP (24 h) at a concentration of 2 g l⁻¹. This result can be explained by the abundance of several homologues and isoforms in LP (72 h) belonging to the surfactin, iturin and fengycin families. When comparing the results obtained by LP (48 h) and LP (24 h), it can be seen that the anti-radical power of LP (48 h) is greater than that of LP (24 h), this can be explained by the presence of larger quantity of bacillomycin D, fengycin A and fengycin B isoforms in LP (48 h).

Our results are of significant importance when compared with those of Dussert *et al.* [28], who reported that the maximum inhibition of DPPH reached 22.88% at the mixture surfactins/plipastatins concentration of 0.5 g l⁻¹. The free radical scavenging effect can be attributed to the presence of the fatty acid carbon chain and some active residues in the peptide chain, which act as electron or hydrogen donors and can react with DPPH free radicals and convert them into more stable products.

3.6.2. β -carotene bleaching test

β -carotene bleaching by linoleic acid assay is widely used to measure the antioxidant activity of bioactive compounds. The β -carotene bleaching inhibition is tested at different concentrations of lipopeptide DCS1 solutions (0.2 to 4 g l⁻¹) and compared against BHA (Figure 6B). All the lipopeptide DCS1 solutions inhibit β -carotene bleaching and the activity is dose-dependant. The highest activity was obtained with LP (72 h) with a maximum inhibition percentage of 85.96% at 4 g l⁻¹ concentration, while the lowest β -carotene bleaching inhibition percentage was observed with LP (24 h) with a value of 35.9%. BHA had the best inhibition of β -carotene bleaching at all concentrations tested.

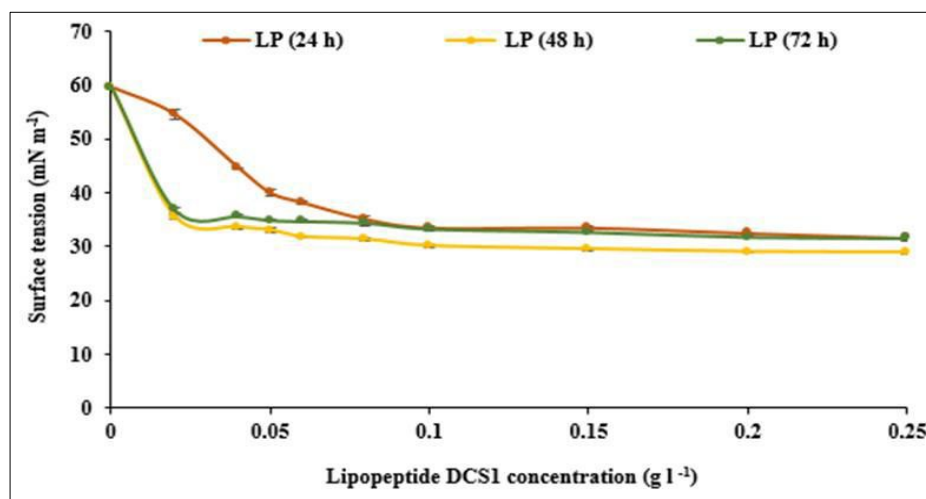


Fig 1: Critical micelle concentration determination of lipopeptides DCS1 recovered at different times of incubation.

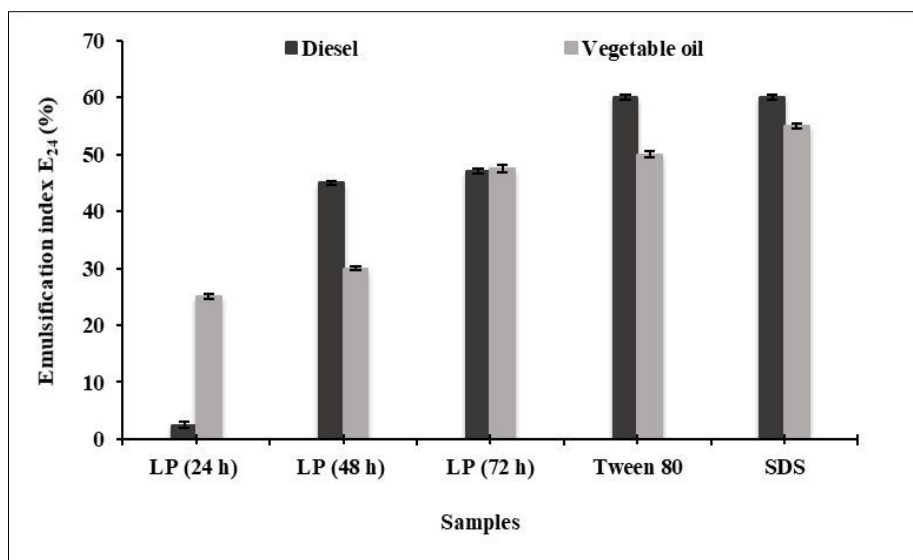


Fig 2: Emulsification activity (E₂₄) of lipopeptide DCS1 solutions towards diesel and vegetable oil compared with SDS and Tween 80.

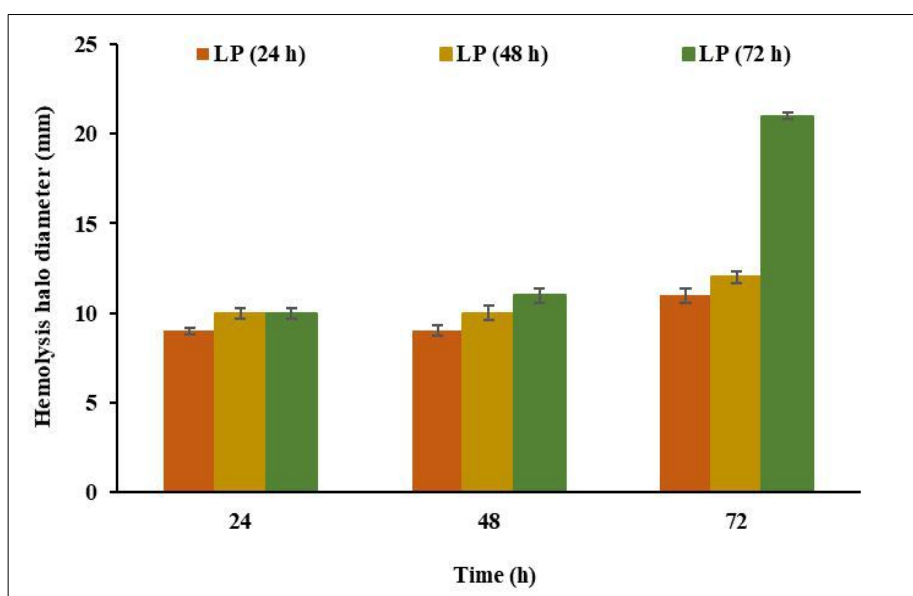


Fig 3: Hemolytic activity of lipopeptide DCS1 solutions.

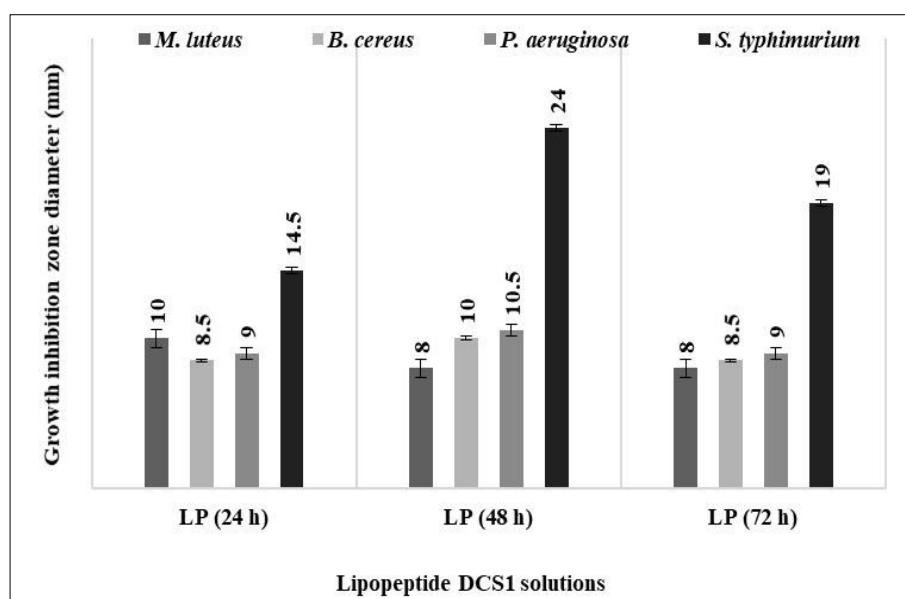


Fig 4: Antibacterial activity spectrum (inhibition zone diameter, mm) of lipopeptides DCS1 produced at different fermentation times (1 mg ml⁻¹).

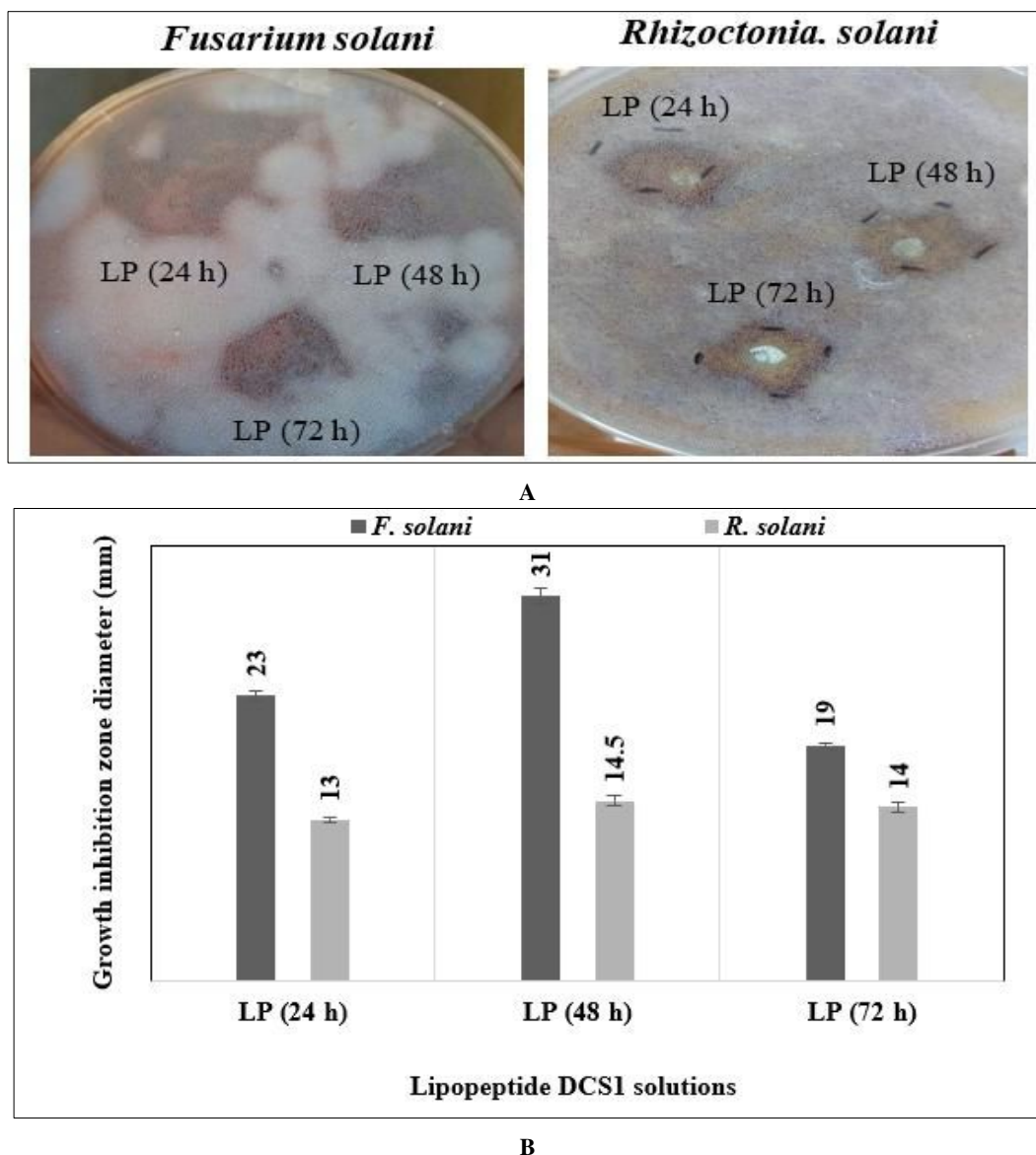
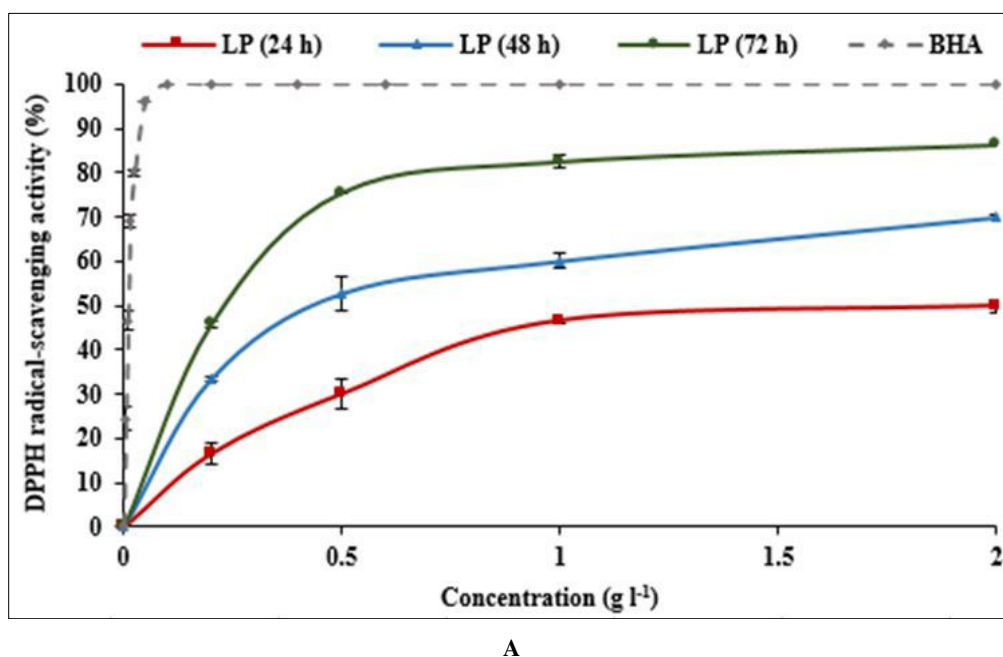


Fig 5: Antifungal activity of lipopeptide DCS1 solutions against *F. solani* and *R. solani* (A), growth inhibition zone diameter (mm) (B).



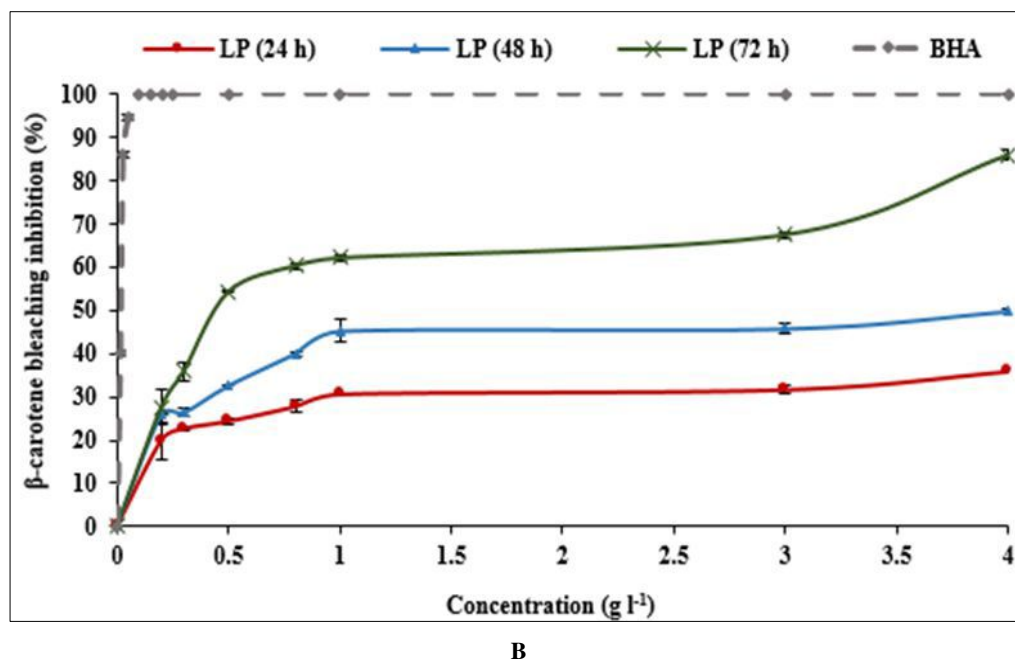


Fig 6: Scavenging effect on DPPH free radical (A) and β -carotene bleaching assay (B) of DCS1 lipopeptide solutions at different concentrations. BHA (2.0 mM) was used as positive control. Values presented are the mean of triplicate analysis.

4. Conclusion

This work focused on the evaluation of certain functional properties of the different lipopeptide mixtures produced during fermentation, such as CMC determination and emulsification activity, and biological activities by testing the antimicrobial activities against Gram-positive and Gram-negative bacteria and two fungi strains, as well as the antioxidant activities. Results imply that LP (48 h) could be applied as tensioactives as well as an antimicrobial agent to reduce infection with pathogenic microbes, instead of LP (72 h). For food and pharmaceutical applications, LP (72 h) is the most effective lipopeptide mixture due to its notable antioxidant capacity.

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