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Efficient conversion of black cumin cake from industrial waste into lipopeptide biosurfactant by *Pseudomonas fluorescens*

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ABSTRACT

Most biosurfactants are obtained using costly culture media, which limits their wider industrial use. In the present study, a low-cost culture medium, containing the agro-industrial residue black cumin cake, was developed for amphisin production by *Pseudomonas fluorescens* DSS73. By using black cumin cake as the substrate, not only was the production cost reduced but also a higher production yield was achieved. A Box-Behnken experimental design was applied to maximize lipopeptide biosurfactant production. The optimal conditions for amphisin production, such as black cumin cake (6.6%) and NaCl (8.0 mM) concentration, and cultivation time (6.5 days), were determined. Yield of amphisin production, performed in optimal conditions, reached 16.51 ± 0.49 g/L. Such high production has not been evidenced previously for *Pseudomonas* lipopeptide biosurfactants. Moreover, active utilization of the substrate, observed with the aid of scanning electron microscopy (SEM), documented by numerous holes and pitting on the black cumin cake surface, was confirmed. Finally, antifungal activity of amphisin against *Aspergillus carbonarius* was demonstrated. Hence, amphisin production by *P. fluorescens* was achieved with statistical optimization using an inexpensive agro-industrial by-product for the first time.

1. Introduction

Biosurfactants, classified as secondary metabolites, are assigned to amphiphilic compounds due to the presence of a hydrophilic as well as a hydrophobic moiety. Synthesized outside the microbial cell or attached to the cell surface, the biosurfactant possesses surface active properties, that is the ability to reduce surface or interfacial tension between two immiscible solutions [1]. In addition, biosurfactants are known for their emulsifier activities, supporting dissolution of hydrocarbons in an aqueous environment and vice versa. Due to their unique properties, biosurfactants are used in industry for detergent production, emulsification, lubrication, foam formation, dispersion and solubilization of different phases [2]. Moreover, features such as low toxicity, high biodegradability, stability in various environmental conditions, biocompatibility and low critical micellization concentration (CMC), which correspond to significant power, prioritize biosurfactants over chemical surfactants [1,2]. Biosurfactants exhibit an exceptionally wide spectrum of activities, which makes them appropriate compounds for medical applications [3]. The antibacterial, antifungal, antiviral and anti-mycoplasma effects represent only a part of the wide range of their properties [4,5]. Furthermore, several studies have shown that selected biosurfactants can improve the effectiveness of anticancer drugs [6–8]. Biosurfactants are classified by their origin, molecular weight or mode of action [9]. Nevertheless, the most significant criterion for biosurfactant classification is the chemical structure; therefore, glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids and polymeric surfactants have been distinguished. While glycolipids are the most studied biosurfactants, lipopeptides definitely deserve far more attention [10]. The lipopeptide molecule contains two main regions: an acyl tail(s) and a short oligopeptide sequence. They are synthesized during the stationary and exponential phase of microbial growth with the aid of water-soluble or water-immiscible substrates. Among lipopeptides, cyclic forms are described as highly active and therefore applied in industry, environmental protection and medicinal fields [11]. The genus

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Bacillus is the prime producer of various lipopeptides. However, the diversity of compounds produced in cultures of *Pseudomonas* spp. is noteworthy [12]. According to Raaijmakers and co-workers [13], several plant-associated *Pseudomonas* species, such as *Pseudomonas syringae*, *Pseudomonas tolaasii*, *Pseudomonas fuscovaginae*, *Pseudomonas corrugata*, *Pseudomonas fluorescens* and *Pseudomonas putida*, are known for lipopeptide production. *Pseudomonas* species produce a diverse spectrum of cyclic lipopeptides, assigned to four major groups – viscosin, amphisin, tolaasin, and syringomycin-like compounds – each of which includes lipopeptides of significant structural similarities. Amphisin is produced by *P. fluorescens* DSS73. The two-component regulatory system GacA/GacS (GacA is a response regulator and GacS is a sensor kinase) controls the amphisin synthetase gene (*amsY*) [14]. The National Center for Biotechnology Information (NCBI) accession numbers for the partial DNA sequence of the *gacS* and *amsY* genes are AJ416155 and AJ416154, respectively. Cyclic lipopeptides included in the amphisin family consist of a peptide chain, made of 11 amino acids, linked to 3-hydroxydecanoic acid (3-HDA). The peptide chain, with the sequence Leu-Asp-Thr-Leu-Leu-Ser-Leu-Gln-Leu-Ile-Asp, creates a lactone ring between C-terminal amino acids and the Thr residue. Tensin, pholipeptin A, lokisin, arthrofactin and hodersin are the remaining, so far poorly characterized members of the amphisin family. In addition, among members of the amphisin group, several amino acid substitutions in the structure of the peptide chain have been observed [15,16]. The molecular weight of compounds included in this family varies from approximately 1350 to 1430 Da. Production of amphisin-like compounds is affiliated with *P. fluorescens* strains, while *P. fluorescens* DSS73 is the only producer of amphisin known so far [14]. Moreover, according to the studies performed by Nielsen et al. (2002), all amphisin-like compound-producing strains are characterized by their ability to utilize putrescine, which distinguish them from the other studied bacteria [17].

In order to incorporate lipopeptides into industrial application, one of the main limitations, i.e. the low yield and consequently high cost of production, needs to be overcome. Therefore, one of the greatest challenges for scientists is to improve the production process, which represents a fundamental issue in the global production. Although several strategies have been implemented so far to scale up industrial production, optimization studies seem to be of key importance [18]. Certain factors such as the type of substrate, divalent cations, temperature of incubation, initial pH and the speed of agitation can affect lipopeptide production. Bearing in mind that the amount and type of a raw material contribute considerably to the production cost, accounting for 10–30% of the total production cost [19], the use of cheap, waste substrates is of great importance. According to Carolin et al. (2021), numerous waste substrates such as those originating from olive, sunflower, canola and coconut oil production, as well as some agro-waste substrates such as wheat or rice straw, cassava flour, sugarcane or beet molasses, bagasse of sugarcane, bran and corn have been examined so far for lipopeptide production [11].

In this study, black cumin cake, a waste from oil manufacturing, was used as the substrate for amphisin production. Black cumin (*Nigella sativa* L.) is a common herb belonging to the *Ranunculaceae* family. Although it is native to Mediterranean countries, it is widely cultivated in India, Iran, Syria, etc. [20]. Due to its unique properties, black cumin seeds and oil are applied on a global scale as food supplements, natural flavoring agents and traditional folk medicines [21,22]. According to the Centre for the Promotion of Imports from developing countries (CBI), the total world black cumin seed production is between 580 and 600 thousand tonnes, and black cumin seed oil is a product that attracts significant interest (https://www.cbi.eu/market-information/spices-herbs/cumin/market-potential). However, the process of oil production is accompanied by the generation of a large amount of waste biomass, called black cumin cake. Although the amount of oilcake generated worldwide is unknown, according to Rajpoot et al. (2022), about 250–350 g of oil is extracted through mechanical pressing of 1 kg of oilseeds, meaning that about 65% of the oilseeds end up as residue (de-oiled cake), which is a significant quantity [23]. Black cumin cake is mainly utilized as a food and animal feed ingredient due to its high amino acid and protein content, with significant presence of essential amino acids such as lysine, methionine and threonine [24]. Black cumin cake protein was proposed for production of bioactive peptides and novel biomaterials [25,26]. Due to the high content of hydrocarbons, proteins and fatty acids, black cumin cake is a promising substrate for microbiological processes [27].

Black cumin cake was evaluated in this study as a substrate for production of the lipopeptide amphisin. Amphisin was synthesized in a culture of *P. fluorescens* DSS73 optimized in terms of substrate concentration, time of production and NaCl supplementation to enhance its production. Finally, due to the fact that lipopeptides often exhibit a wide spectrum of biological activities, the antifungal activity of amphisin against *Aspergillus carbonarius* MUM 05.18, *Aspergillus flavus* MUM 17.14 and *Aspergillus niger* MUM 92.13 was determined.

2. Materials and methods

2.1. Black cumin cake composition analysis

Chemical composition analysis of black cumin cake was performed, in order to confirm its suitability as a substrate for amphisin production. Black cumin cake used in this study was obtained as a generous gift from a local company dealing in the production of healthy oil-based products, AleOlej.pl (Wrocław, Poland). It was generated as a waste by-product from black cumin originating from India. Dry matter (DM) (%), crude protein (% of DM), neutral detergent fiber (NDF) (% of DM), acid detergent fiber (ADF) (% of DM), acid detergent lignin (ADL) (% of DM) and crude ash (% of DM) were determined in the National Laboratory for Feedingstuffs of the National Research Institute of Animal Production (Lublin, Poland). Dry weight, fiber fractions and ash were analyzed using the gravimetric method, while crude protein concentration was determined using the Kjeldahl titration method. In addition, the composition of fatty acids (FAs) (% of DM), including palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids, was established using gas chromatography mass spectrometry (GC-MS), described in detail in our previous work [28]. The procedure was based on the generation of fatty acid methyl esters (FAMEs), using a methanol solution of 2.5% (v/v) sulfuric acid. Obtained esters were extracted, examined on a GC-MS-QP2010 Plus system (Shimadzu, Kyoto, Japan) equipped with a Zebron ZB-FAME capillary column (30 m × 0.25 mm × 0.20 μm; Phenomenex, Torrance, CA, USA) and compared to the commercial standard (Supelco 37 Component FAME mix, Sigma-Aldrich, St. Louis, MO, USA) for identification and quantification.

2.2. Optimization of amphisin production in the culture of P. fluorescens DSS73 performed in the black cumin cake based medium

P. fluorescens DSS73 [17] used in this study for amphisin production was graciously provided by Dr. Ole Nybroe (University of Copenhagen, Denmark) and stored at the Department of Biotechnology and Food Microbiology, Wrocław University of Environmental and Life Sciences (Wrocław, Poland) as a glycerol stock (20% v/v) at − 80 °C. In order to perform optimization studies, pre-cultures were prepared by inoculating 50 mL of Luria-Bertani medium (LB; 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl, pH 7.0) with 100 μL from a frozen stock, and were incubated at 28 °C and 160 rpm for 24 h. Amphisin biosynthesis was carried out in 300 mL Erlenmeyer flasks containing 50 mL of culture medium supplemented with NaCl and black cumin cake as the only source of carbon and nitrogen. Each flask was inoculated using 1% of *P. fluorescens* DSS73 pre-culture and incubated at 28 °C with rotary agitation at 180 rpm. The biosynthesis process was optimized using a Box-Behnken experimental design, in order to establish the influence of three selected variables on the final lipopeptide concentration. Substrate

(black cumin cake) concentration (X_1), concentration of NaCl (X_2) and cultivation time (X_3) served as independent variables. Three experimental levels (−1; 0; 1) were designated for each variable, according to Table 1. The experiment comprised 15 runs, each performed in duplicate (Table 2). Flasks were incubated as long as indicated by the model and centrifuged in the next stage at 9500g for 20 min to separate biomass and determine amphisin concentration.

In addition, the relationship between the independent variables and the response (amphisin concentration) was modeled in the form of the polynomial Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{33} X_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is the predicted response; β_0 the intercept; β_1, β_2, β_3 linear regression coefficients; β_{11}, β_{22}, β_{33} quadratic regression coefficients; and β_{12}, β_{13}, β_{23} interaction effects. Prior to the evaluation of the model, insignificant terms were reduced. The analysis of the model, response surface plots and ANOVA statistics were performed in Statistica 13 (TIBCO Software Inc.).

2.3. Quantification of amphisin production

Determination of amphisin concentration in the samples obtained as a result of optimization studies was performed using high-performance liquid chromatography (HPLC (Shimadzu, Kyoto, Japan)) with a Hypersil GOLD column (5 μm; 4.6 ×150 mm) according to the methodology described by Ciurko et al. (2022) [28]. Therefore, culture supernatants in the amount of 100 μL were dissolved in 900 μL of methanol (Chempur, Piekary Śląskie, Poland) and analyzed. As the mobile phase, solvents A (0.1% trifluoroacetic acid) and B (0.1% trifluoroacetic acid in acetonitrile) were applied according to the following scheme (% A:B v/v): 0 min (50:50), 5 min (20:80), 9 min (10:90), 15 min (0:100), 21 min (0:100), 24 min (50:50) and 25 min (50:50). Trifluoroacetic acid (99%) and acetonitrile (99%) used in this study were provided by Sigma-Aldrich Corporation (St. Louis, MO, USA). Tested samples injected onto a column in the amount of 10 μL were eluted for 25 min with a flow rate of 0.5 mL/min and detected at the wavelength of 210 nm. A calibration curve performed for the amphisin standard (purified by HPLC; ≥99.0%) was used to estimate the biosurfactant concentration and to select conditions ensuring the highest production. All determinations were performed in triplicate.

2.4. Production of amphisin in optimal conditions

Optimization studies allowed us to establish the best conditions for amphisin production. Hence, large-scale production in a 300 mL Erlenmeyer flask containing 50 mL of the medium, performed in the optimal conditions, i.e. black cumin cake concentration 6.6% and NaCl concentration 8.0 mM, was applied. The bacterial culture was inoculated with 1% of a pre-culture from *P. fluorescens* DSS73 grown for 24 h, and incubated at 28 °C and 180 rpm for 6.5 days. Finally, post-culture medium was centrifuged at 9500g for 20 min to separate the precipitate.

2.5. Black cumin cake surface analysis using scanning electron microscopy (SEM)

Precipitate containing a mixture of bacterial biomass and black cumin cake residues, obtained as a by-product of amphisin production, was analyzed to confirm substrate utilization. The precipitate was dried at 40 °C for 2 days and examined using a HITACHI S–3400 N scanning electron microscope. Samples were coated with a gold alloy thin film using the thermal evaporation physical vapor deposition (PVD) method to ensure electric conductivity.

Table 1
Experimental values of independent variables.

	Unit	-1	0	+ 1
X_1: substrate	%	2.5	5.0	7.5
X_2: NaCl	mM	1	51	101
X_3: cultivation time	day	3	5	7

Table 2
Experimental layout of the Box-Behnken design, with the obtained response outcomes and predicted responses.

	Independent variables			Amphisin (g/L)	
Run	X_1: Substrate (%)	X_2: NaCl (mM)	X_3: Time (day)	Actual response	Predicted response
1	-1	-1	0	6.71 ± 0.27	6.88
2	1	-1	0	15.09 ± 0.31	15.00
3	-1	1	0	4.14 ± 0.17	4.23
4	1	1	0	7.46 ± 0.18	7.29
5	-1	0	-1	4.02 ± 0.40	4.06
6	1	0	-1	8.83 ± 0.02	9.12
7	-1	0	+ 1	8.43 ± 0.77	8.14
8	1	0	+ 1	14.30 ± 0.26	14.27
9	0	-1	0	8.57 ± 0.42	8.37
10	0	1	0	3.55 ± 0.02	3.43
11	0	-1	1	13.10 ± 0.16	13.22
12	0	1	1	7.61 ± 0.56	7.81
13	0	0	0	10.53 ± 0.01	10.96
14	0	0	0	10.86 ± 0.17	10.96
15	0	0	0	11.48 ± 0.35	10.96

2.6. Purification of amphisin using solid-phase extraction (SPE)

Supernatant containing amphisin was lyophilized, dissolved in distilled water and loaded onto cartridges of the Chromabond C18 SPE system (Macherey-Nagel, Düren, Germany). Then, crude biosurfactant was eluted using an acetonitrile gradient (40%, 60%, 80% and 100% acetonitrile–water (v/v)), and the 80% acetonitrile–water (v/v) solution (containing the lipopeptide) was concentrated through nitrogen drying as described by Ciurko et al. (2022) [28]. These fractions were subsequently subjected to HPLC on a Hypersil GOLD column using a gradient according to the scheme described in Section 2.3. The peak with a retention time of 10.93 min corresponded to pure amphisin (Fig. S1). Mass spectrometry of the purified amphisin revealed over 99% purity (Fig. S2). The purified amphisin fraction was dried and stored at − 20 °C for further studies.

2.7. Biosurfactant characterization

The purified amphisin was characterized by Fourier transform infrared spectroscopy (FTIR) in the wavenumber range of 4000–400 cm^{-1}. The IR spectra were recorded using the IRSpirit FTIR spectrophotometer (Shimadzu, Japan) at 25 °C. In addition, surface tension (γ) measurements were performed at 25 °C according to du Noüy's ring method [29] using a KRÜSS K20 Tensiometer (KRÜSS GmbH, Hamburg, Germany). Each determination was performed in triplicate. The surface tension data were analyzed using the Origin software provided with the equipment to obtain the CMC value.

2.8. Research on the antifungal activity against Aspergillus species

A. carbonarius MUM 05.18, *A. flavus* MUM 17.14 and *A. niger* MUM 92.13 were obtained from the culture collection of Micoteca da Universidade do Minho (MUM). Fungal strains were stored in the form of glycerol stocks (20% v/v) at − 80 °C. The antifungal activity was

investigated in Petri dishes (55 mm diameter) containing malt extract agar (MEA) medium previously adjusted to pH 5.5. Amphisin was applied as one of the culture components and used at the concentration of 1500 mg/L or 3000 mg/L. Investigation of the antifungal activity required in addition preparation of fungal spore suspensions. Therefore, filamentous fungi were cultured on MEA plates at 25 °C for 7 days and washed afterwards with 1 mL of sterile distilled water in order to recover the spores. Spore suspensions were transferred to sterile tubes and diluted to the concentration of 10^5 spores/mL with the aid of a Neubauer improved cell counter (Marienfeld GmbH, Germany). Then, spore suspensions were spotted, each in the volume of 10 μL, in the center of MEA plates (with and without amphisin) and incubated at 25 °C for 5 days. To maintain accuracy, spore suspensions were stored at 4 °C and used within 3 weeks. Finally, the antifungal activity was determined by measuring the diameter of the fungal mycelium in the presence of amphisin and on the corresponding control plate and calculated as follows:

$$Growth\ inhibition\ (\%) = \left(1 - \frac{diameter\ x}{diameter\ c}\right) \times 100 \quad (2)$$

where *diameter x* (cm) represents the diameter of the fungal growth in the medium containing amphisin and *diameter c* represents the diameter of the fungal growth on the corresponding control plate. All experiments were performed in triplicate.

3. Results and discussion

3.1. Black cumin cake composition

Black cumin seed composition is strongly affected by the geographic distribution, time of harvest and agronomic practices. However, the dominant fractions are oil, hydrocarbons, and proteins [27]. Black cumin cake used in this work contained 94.6 ± 5.0% DM, 23.84 ± 0.9% crude protein (% of DM) and 4.84 ± 0.3% ash (% of DM) (Table 3). For comparison of our results with those obtained by Thilakarathna et al. (2018), the concentration of crude protein (% of DM) of Indian and Ethiopian cake was 18.44% and 19.29%, respectively, while ash content (% of DM) totaled 5.31% and 4.98% [30]. In another study, a higher concentration of crude protein (40.60% (% of DM)), and similar content of ash (6.46% (% of DM)), in Siberian black cumin cake were established [31]. Finally, a cake of Turkish origin contained 23.95% crude protein (% of DM), and 9.45% ash (% of DM) [32]. Therefore, the contents of protein and ash of black cumin cake analyzed in our work were similar to those of Indian, Ethiopian [30] and Turkish origin [32]. The higher concentration of protein present in Siberian cake could be explained by different environmental conditions related to the climate of Siberia. Research has confirmed a strong relationship between protein concentration and cultivation conditions of black cumin. Acar et al. (2016) identified genetics, oil processing variables and accuracy of detection and lipid extraction as sources of variability in fatty acid composition [32]. Dominant fatty acids (%) of black cumin cake studied in this research were palmitic (12.64), oleic (24.31) and linoleic acid (57.48) (Table 3), the same as the Turkish cake, containing palmitic (12.25%), oleic (24.74%) and linolenic acids (58.62%). Despite their different origin, the fatty acid composition of black cumin cake herein studied and the Turkish one was very similar. It indicates that the effect of the growth and processing variables and detection precision on the fatty acid content is not as strong as in the case of protein or ash.

Table 3
Composition of black cumin cake.

Component	Black cumin cake
Dry Matter (DM), (%)	94.6 ± 5.0
Crude Protein, (% of DM)	23.84 ± 0.9
Fiber	
NDF, % of DM	29.7 ± 1.5
ADF, % of DM	15.61 ± 0.9
ADL, (% of DM)	5.68 ± 0.6
FA, (% of DM)	18.4 ± 0.5
FA composition, (%)	
Palmitic (C16:0)	12.64 ± 0.3
Oleic (C18:1)	24.31 ± 0.1
Linoleic (C18:2)	57.48 ± 0.3
Others	5.57 ± 0.2
Ash, (% of DM)	4.84 ± 0.3

According to Cameotra and Makkar (1998), the type of substrate and growth conditions affect the type and yield of the biosurfactant produced in microbial cultures [33]. The carbon source may induce or repress biosurfactant synthesis. Moreover, addition of water-immiscible substrates in some cases results in the induction of biosurfactant production. The key role of nitrogen concentration in the regulation of biosurfactant synthesis was underlined. Therefore, black cumin cake, containing high amounts of protein, fiber and fatty acids, seems to be a suitable substrate for amphisin synthesis. High content of water-insoluble fatty acids may lead to maximization of biosurfactant production. Finally, black cumin cake, as a substrate, ensures the appropriate amount of carbon and nitrogen for metabolic synthesis.

3.2. Effects of black cumin cake concentration, salinity and cultivation time on amphisin production

A Box-Behnken experimental design was applied to establish the combined effect of three selected parameters on the production of amphisin in a culture of *P. fluorescens* DSS73. According to the Pareto chart, nearly all independent variables exerted a significant impact on amphisin production (Fig. 1, Table 4). Linear effects were dominant in the model, of which the substrate content (black cumin cake) and cultivation time were directly proportional to the product concentration. Quadratic effects in the model were less pronounced, but statistically significant, except for the quadratic term of substrate content. Additionally, a single significant interaction between substrate and salt concentration was observed.

Analysis of variance (ANOVA) was performed in order to verify the significance of the main and interacting effects of parameters in the created model. The regression model was highly significant, according to the F-value of 123.48. The suitability of the model was confirmed by an exceptionally high coefficient of determination, $R^2 = 0.9955$, which implied that over 99% of the obtained response values were explained by the model. The "lack of fit" test of insignificant rank further confirmed its correctness (Table 5).

The obtained results made it possible to define a polynomial equation to illustrate the regression model (significant terms underlined) (2):

$$Y = \underline{-11{\cdot}76} + \underline{2{\cdot}91X_1} + \underline{0{\cdot}07X_2} + \underline{3{\cdot}70X_3} - \underline{0{\cdot}15X_1X_1} - 0{\cdot}00X_2X_2 - \underline{0{\cdot}28X_3X_3} - \underline{0{\cdot}01X_1X_2} + 0{\cdot}05X_1X_3 - 0{\cdot}00X_2X_3 \quad (2)$$

Analysis of response surface plots allows us to illustrate the combined effect of pairs of independent variables on lipopeptide production. Fig. 2A depicts the simultaneous influence of substrate and NaCl concentration on the production of amphisin.

Increasing concentration of substrate exhibited an increasing trend with experimental boundaries, and the curvature of this relationship was confirmed to be statistically insignificant. Hence, the highest amphisin concentration was achieved in the presence of the highest analyzed substrate content. The concentration of NaCl exerted a variable impact, dependent on the substrate, which illustrated the significant interaction between these two variables. Nevertheless, the highest concentration of amphisin occurred in the proximity of the lowest concentration of NaCl. In Fig. 2**B**, the impact of the third independent variable, cultivation time, is plotted against the substrate content. Taking into consideration the curvature of the response surface, it was confirmed that further extension of cultivation time should not result in

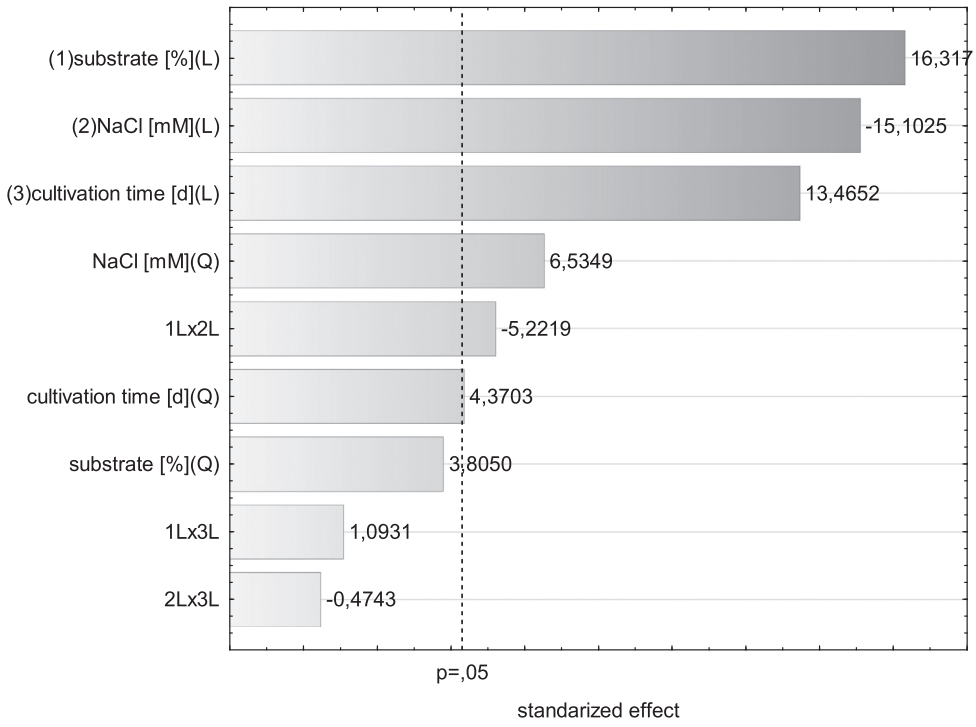

Fig. 1. Pareto chart of standardized effects.

Table 4
Summary of effects from the regression model.

Variable	Coefficient	Standard error	*t*-value	*p*-value
Intercept	8.4848	0.1399	60.6329	0.0003
X_1	5.5930	0.3428	16.3170	0.0037
X_2	-5.1767	0.3428	-15.1025	0.0044
X_3	4.6155	0.3428	13.4652	0.0055
X_1X_1	0.9599	0.2523	3.8050	0.0627
X_2X_2	1.6486	0.2523	6.5349	0.0226
X_3X_3	1.1025	0.2523	4.3703	0.0486
X_1X_2	-2.5314	0.4848	-5.2219	0.0348
X_1X_3	0.5299	0.4848	1.0931	0.3884
X_2X_3	-0.2299	0.4848	-0.4743	0.6820

Table 5
Analysis of variance (ANOVA) for the regression model.

Error source	Sum of squares (SS)	Degrees of freedom (DF)	Mean square (MS)	F-value	p-value
Regression model	181.2556	9	20.1395	123.48	< 0.001
Residual error	0.8153	5	0.1631	-	-
Lack of fit	0.3453	3	0.1151	0.49	0.7243
Pure error	0.4700	2	0.2350	-	-
Cor. total	182.0709	14	-	-	-

$R^2 = 0.9955$, R^2adj.$= 0.9875$

higher amphisin production. The response surface plot of cultivation time against NaCl concentration shown in Fig. 2**C** produced a plateau at the edge of the experimental model, in the area of maximum cultivation time and minimum NaCl content. This implies that optimal values for these variables could be determined to maximize amphisin production.

The positive effect of the increasing substrate concentration on lipopeptide production observed in our study corroborates the research of Nielsen et al. (2000), in which optimization studies of tensin production were performed [34]. Tensin is another cyclic lipopeptide produced by *P. fluorescens*, included in the group of amphisin-like compounds. Tensin production was conducted using different carbon sources and applying different initial concentrations of carbon and nitrogen. Availability of both carbon and nitrogen was necessary for efficient tensin production; as in our study, black cumin cake, being a source of carbon and nitrogen, influenced amphisin production. Moreover, a positive effect of addition of sugar beet seeds on tensin production was observed. The use of natural substrates of agricultural origin seems to have an advantage over synthetic reagents as previously reported by Nielsen et al. (2000) [34] and confirmed in our study. Materials such as sugar beet or black cumin cake provide increased yield of lipopeptide production.

Likewise, Ray et al. (2022) observed maximum lipopeptide production by *Pseudomonas* sp. IITISM 19 in the culture medium supplemented with the highest tested substrate concentration (10 g/L of glucose) [35]. Therefore, determination of the optimal conditions seems to be a crucial step for efficient lipopeptide production in *P. fluorescens* cultures. Optimization studies, like the one presented here, are highly required. According to the results obtained, addition of NaCl was of key importance for the production of amphisin. The lowest concentration of NaCl in the culture medium resulted in the most efficient production. Similarly, Lotfabad et al. (2009) reported an inverse relation between NaCl concentration and biosurfactant production by *P. aeruginosa* MR01 [36]. The gradual addition of NaCl to the glucose-containing MS medium led to a decrease in biosurfactant production by this strain. However, in the study performed by Dubern and Bloemberg (2006), putisolvin, another cyclic lipopeptide produced by *Pseudomonas* ssp., was produced in a medium supplemented with 1 M NaCl [37]. Surprisingly, contrary to our study, the addition of NaCl had a positive effect on the production of this lipopeptide, and putisolvin concentration increased twofold in comparison with a medium without salt addition. Therefore, the results obtained in this study, together with those previously performed [37], indicate strong differences regarding NaCl tolerance between different *Pseudomonas* strains. In some cases, NaCl works as an inductor, but in most cases it seems to inhibit biosurfactant biosynthesis. Additional research on the mechanism of action of NaCl on lipopeptide production should be performed in the future to explain it.

According to the regression model obtained, optimal conditions for

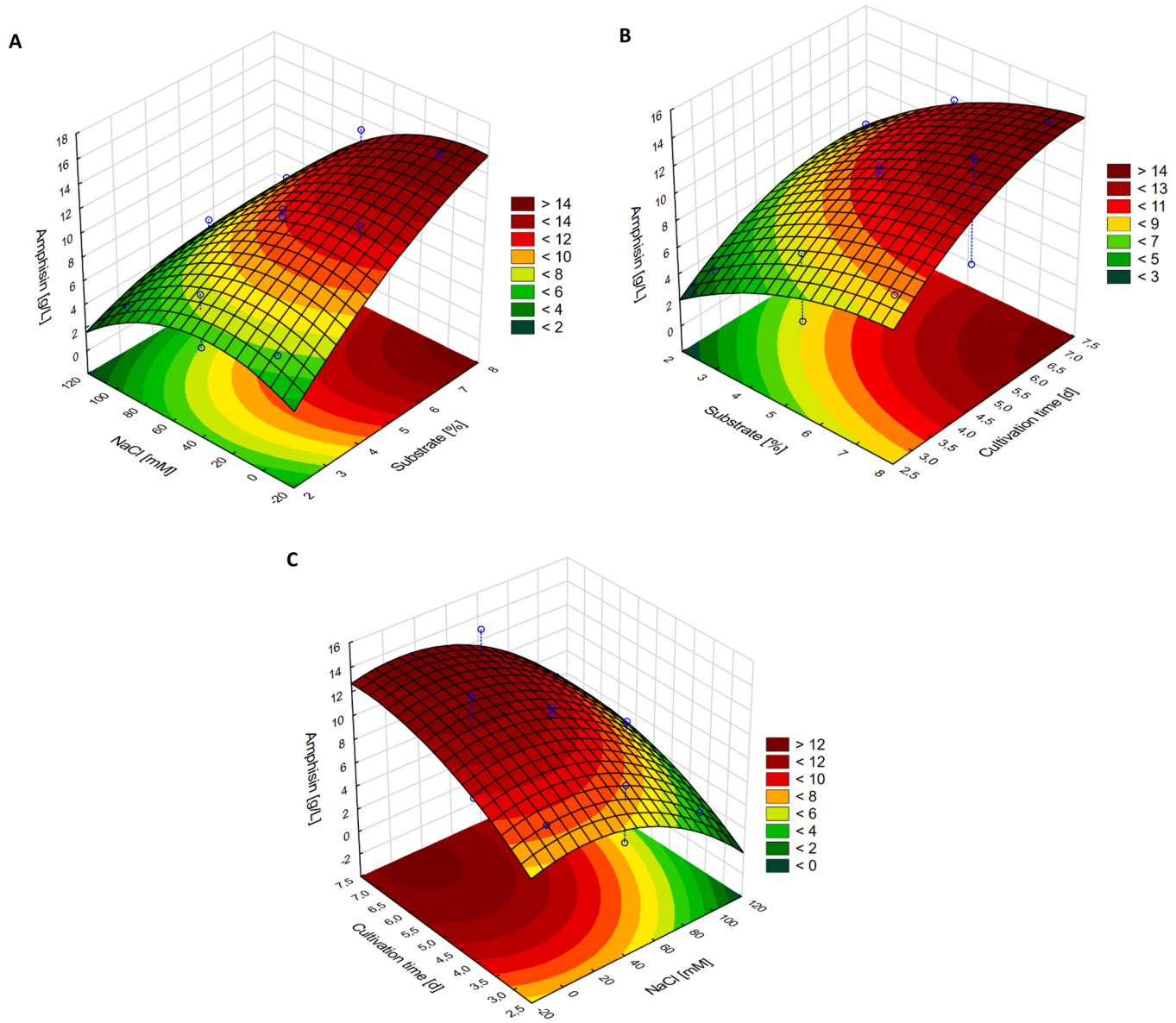

Fig. 2. The simultaneous influence of: **(A)** substrate and NaCl concentration, **(B)** cultivation time and substrate content, **(C)** cultivation time and NaCl concentration on the production of amphisin by *P. fluorescens* DSS73.

amphisin production, such as substrate (6.6%) and NaCl (8.0 mM) concentration, and cultivation time (6.5 days), were determined. These conditions correspond to the predicted response value of 15.37 g amphisin/L with a confidence interval of 13.86–16.77 g/L. Moreover, when the process was conducted at the optimal conditions, the amount of amphisin produced reached 16.51 ± 0.49 g/L, which validated the model predictions.

In the available literature, there are not many studies related to the quantification of lipopeptide production in the culture of *Pseudomonas* species. Researchers tend to focus on the search for new compounds, on structural studies and on the analysis of molecular mechanisms regulating the production of cyclic lipopeptides [13,38,39]. However, quantification of lipopeptide production in *Pseudomonas* ssp. cultures is crucial for their future application. Quantitative analysis was performed in research on milkisin production. Milkisin is a relatively new cyclic lipopeptide with a structure similar to amphisin. The peptide chain contains 11 amino acids, among which 9 are conserved in the amphisin family. In a glucose-containing medium, the average production of milkisin by *Pseudomonas* sp. UCMA 17988 was 47.6 ± 1.4 mg/L [40]. The research of Biniarz et al. (2018) included complex optimization studies of pseudofactin production and was completed with a quantitative analysis [41]. Critical factors promoting lipopeptide production, such as high glycerol concentration, supplementation with amino acids (leucine or valine) and complex additives (e.g. tryptone), as well as high aeration, were determined. Pseudofactin concentration after successful high-throughput optimization in the culture of *P. fluorescens* BD5 reached 1.19 g/L [41], which is much lower than the result obtained in our study.

Compared to the data reported by other authors, the yield of amphisin production was substantially high, which indicates a good selection of substrate and culture conditions. The yield of amphisin secretion was similar to the one obtained in surfactin studies. Surfactin is one of the most efficient lipopeptide biosurfactants known so far, generated by the Gram-positive, endospore-producing bacterium *Bacillus subtilis* [42]. Nowadays, due to the numerous studies performed on optimization of surfactin production, it can be synthesized with high efficiency. One example is the research of Haddad et al. [43], where the yield of surfactin production reached 47.58 g/L. In another study,

surfactin production was influenced by carbon source concentration, pH, temperature and salinity of the culture medium. At the optimum conditions, surfactin concentration was found to be 7.46 ± 0.39 g/L [44]. Finally, productivity levels reaching 3.65 g/L and 3.16 g/L were detected when cashew apple juice and grape juice, respectively, were applied as substrates for surfactin production [45]. Therefore, lipopeptide production is strongly affected by the culture conditions, especially by the type of substrate used.

Nowadays, when the high cost of production is a bottleneck for the industrial application of biosurfactants, our research showed, due to the high yield of amphisin production and thanks to the use of low-cost waste substrates, that it is possible to overcome those limitations.

3.3. Structural changes observed on the surface of black cumin cake as a consequence of bacterial colonization

Black cumin cake applied as a substrate for amphisin production was actively utilized in *P. fluorescens* DSS73 culture. It was confirmed through SEM, where the structural differences between control and bacterial-treated samples were detected. The surface of black cumin cake treated by bacteria was ragged compared to the control (Fig. 3).

Numerous holes, formed as a result of bacterial activity, were observed. Moreover, large biomass accumulation on the substrate surface was evidenced. The greatest damage to the black cumin cake structure was observed in the SEM micrographs taken at 10 kV with magnification x 20 000, where multiple cracks were visible.

Numerous researchers have used SEM in order to observe changes

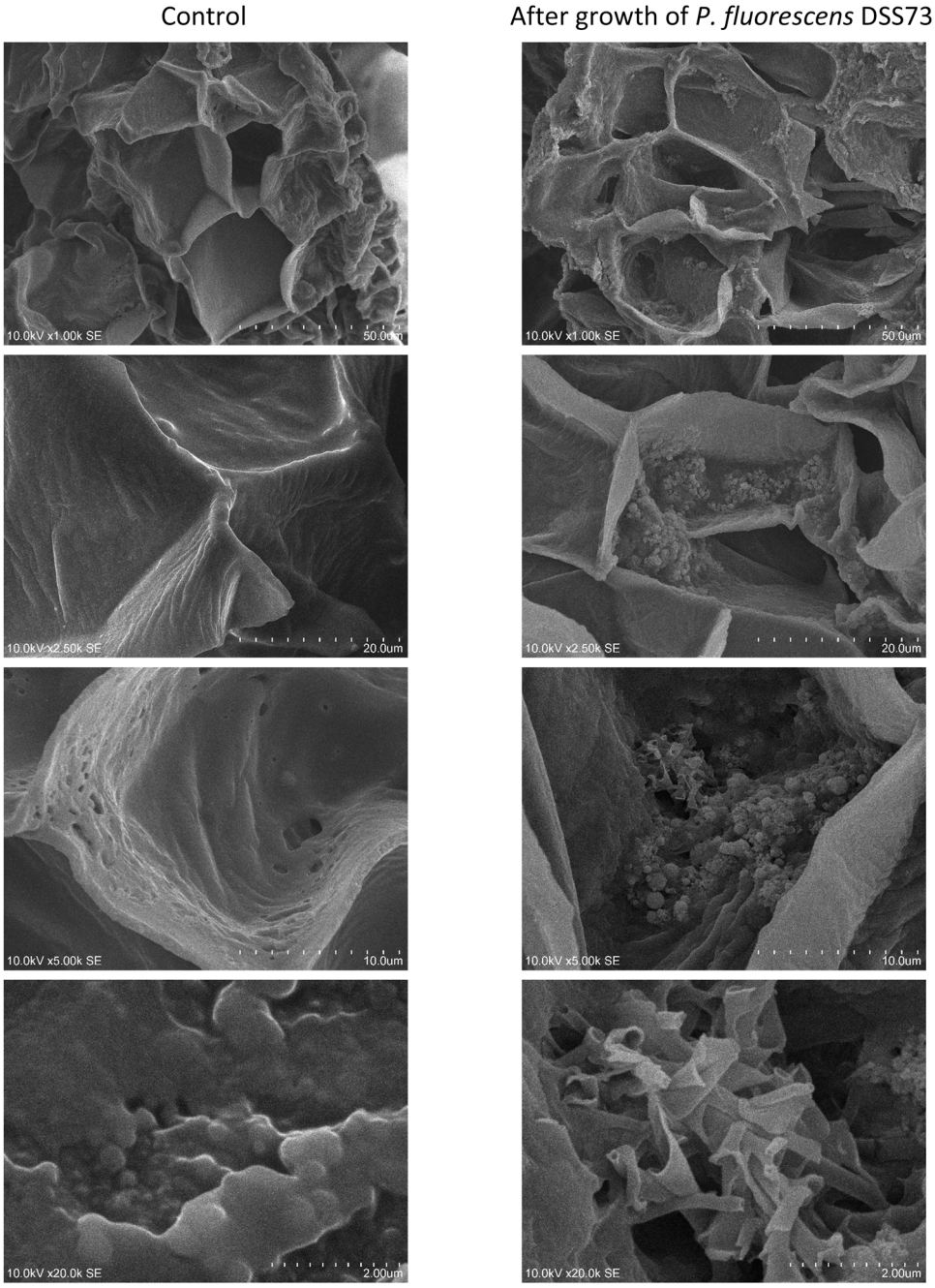

Fig. 3. The surface of black cumin cake after the growth of *P. fluorescens* DSS73 (right column) compared to the control (left column) observed under increasing magnification.

caused by bacteria during substrate colonization. Sharma et al. [46] noted bacterial colonization of the polyhydroxyalkanoates' surface using SEM. In the polymer surfaces pitting was observed, being the result of *Pseudomonas chlororaphis* and *Acinetobacter lwoffii* biodegradation [46]. Xiudong et al. (2016) observed a large number of *Lactobacillus plantarum* 70810 cells attached to the okara matrices used as a natural immobilization carrier in the soymilk fermentation process [47]. In accordance with our study, SEM enables observation of bacterial adhesion. Finally, traces of polyethylene microbial degradation, similar to the one observed on the black cumin cake surface, were detected by Montazer et al. (2019) [48]. Active adhesion of the tested bacterial strain to the substrate was direct evidence for plastic biodegradation. Our research and the above-mentioned studies confirm SEM as an excellent tool providing direct evidence for substrate utilization in microbial cultures.

3.4. Characterization of amphisin

The chemical nature of amphisin was determined based on the presence of different types of functional groups reported in the literature [49–51]. Characteristic absorption peaks at 3297 cm^{-1} and 1640 cm^{-1} representing stretching of N-H and the stretching mode of CO–N, respectively, were detected. In addition, the deformation mode of the N–H bond combined with C–N stretching was represented by the presence of a peak at 1548 cm^{-1}. Several peaks at 1400 cm^{-1}, 1446 cm^{-1}, 2874 cm^{-1}, and 2926 cm^{-1} corresponding to the aliphatic long hydrocarbon chain were observed. Finally, the presence of a hydroxyl group of amino acids was detected by the peak at 932 cm^{-1}. Numerous researchers have observed similar peaks while studying lipopeptides secreted by *Pseudomonas* ssp. [49–51]. Therefore, with the aid of FTIR analysis, we confirmed that the compound produced in *P. fluorescens* DSS73 culture belongs to the lipopeptide family. Furthermore, in order to establish the effect of amphisin to reduce surface tension, the CMC point was determined. CMC was designated as 75 mg/L, decreasing surface tension to 28 mN/m (Fig. 4).

In the research of Portet-Koltalo et al. (2013), the CMC of amphisin was estimated as 300 mg/L [52], while in the study of Janek et al. (2018) [53] it was reported to be 0.075 mM, which corresponds to a value of ~100 mg/L. Such differences may be due to the differences in the purity of analyzed compounds. However, the results obtained in this study as well as previously published data indicate that amphisin is a powerful lipopeptide from the point of view of industrial application.

3.5. Antifungal activity of amphisin against Aspergillus species

The fungal species selected for this study are common contaminants of agricultural products. Furthermore, they produce toxic compounds (mycotoxins), representing a substantial problem for food and agriculture industries. Amphisin produced by *P. fluorescens* DSS73 showed antifungal activity against *A. carbonarius* MUM 05.18. Fungal growth was inhibited to 51 ± 9% of the control level after 72 h of incubation at an amphisin concentration of 3000 mg/L (Table 6). Further colonization of the substrate surface by the fungus was inhibited until the end of the experiment (120 h). However, at the concentration of 1500 mg/L, the growth of *A. carbonarius* MUM 05.18 was almost uninhibited and was 88 ± 9% compared to the control. Likewise, antifungal activity against *A. flavus* MUM 17.14 and *A. niger* MUM 92.13 was not observed.

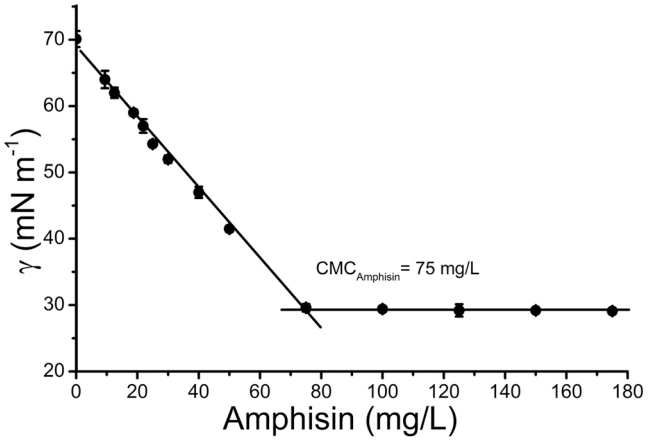

Fig. 4. CMC analysis of amphisin produced by *P. fluorescens* DSS73.

The antifungal activity of amphisin was previously reported by Nielsen et al. (2002) [17]. Purified amphisin as well as other amphisin-like compounds, that is lokisin, hodersin and tensin, showed antagonistic effects against *Rhizoctonia solani* and *Pythium ultimum*. However, the antifungal activity of the lipopeptide-producing *Pseudomonas* ssp., cultured together with the fungal strains, was higher than that of the purified lipopeptide. That can be explained in reference to observations reported by Andersen et al. (2003) [54]. Co-cultures of amphisin-producing *Pseudomonas* sp. DSS73 and the relevant fungal strains *R. solani*/*P. ultimum* were performed in order to identify the factor responsible for the antifungal activity. Whereas the wild type strain and its amsY$^-$ mutant (amphisin-deficient) showed significant antifungal activity, demonstrated by a mycelium-deficient zone around the bacterial colony, the gacS$^-$ mutant (unable to produce amphisin, chitinases, proteases and hydrogen cyanide) was not active [54]. Therefore, the antifungal activity of *Pseudomonas* spp. is determined by a whole set of metabolites, including proteases, cellulases, chitinases and hydrogen cyanide, with amphisin being one of them, as demonstrated by the results obtained in the present work. According to Andersen et al. (2003), amphisin is a crucial factor for bacterial motility, and therefore its production is essential to obtain efficient inhibition of fungal growth [54].

4. Conclusions

In this study, successful optimization of amphisin production by *P. fluorescens* DSS73 was performed. A significant impact of black cumin cake content, NaCl supplementation and time of production on the final amphisin concentration was found. Due to the use of waste substrate and because of optimization, a high yield of amphisin production (about 16 g/L) was obtained, while maintaining cost at a low level. Amphisin produced in this research was a powerful biosurfactant, with a CMC value of 75 mg/L and minimum surface tension of 28 mN/m, enabling commercial application. Finally, activity of amphisin as an antifungal agent against *Aspergillus* species was determined. Although antifungal activity against *A. flavus* MUM 17.14 and *A. niger* MUM 92.13 was not observed, amphisin (3000 mg/L) inhibited the growth of the mycotoxigenic *A. carbonarius* MUM 05.18 by 50%. With high probability, antifungal activity would be greater if, instead of purified amphisin, *P. fluorescens* DSS73 cells were applied. The results obtained suggest that amphisin can be a useful weapon against the mycotoxigenic fungus *A. carbonarius*, and find potential applications in food and agriculture sectors. Despite the promising results, the scalability of the process should be further investigated with a view to the development of a sustainable bioprocess.

Ethics approval

This article does not concern any studies with human participants or animals performed by any of the authors.

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Table 6
Effect of amphisin concentration on the growth of *A. carbonarius* MUM 05.18, *A. flavus* MUM 17.14 and *A. niger* MUM 92.13.

Amphisin mg/L	*A. carbonarius* MUM 05.18			*A. flavus* MUM 17.14			*A. niger* MUM 92.13		
	Control	1500	3000	Control	1500	3000	Control	1500	3000
	Diameter (mm)								
48 h	21 ± 4	18 ± 2	14 ± 1	21 ± 1	24 ± 1	25 ± 1	22 ± 1	25 ± 1	22 ± 1
72 h	39 ± 5	34 ± 3	20 ± 3	39 ± 2	35 ± 1	37 ± 3	41 ± 2	35 ± 1	35 ± 1
96 h	45 ± 4	40 ± 3	24 ± 7	45 ± 1	39 ± 2	42 ± 2	48 ± 2	43 ± 2	45 ± 1
120 h	50 ± 0	44 ± 2	26 ± 10	49 ± 1	45 ± 2	47 ± 2	50 ± 0	49 ± 1	49 ± 1
	Growth (%)								
48 h	100	85 ± 10	68 ± 4	100	113 ± 5	118 ± 7	100	115 ± 3	102 ± 6
72 h	100	88 ± 9	51 ± 9	100	92 ± 2	97 ± 7	100	86 ± 2	86 ± 3
96 h	100	90 ± 8	53 ± 17	100	88 ± 5	94 ± 5	100	89 ± 3	94 ± 1
120 h	100	89 ± 5	51 ± 20	100	92 ± 4	95 ± 4	100	99 ± 2	99 ± 1

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CRediT authorship contribution statement

WŁ, AK, and TJ conceived and designed research. DC, AK, ŁJ, EJG and TJ conducted the experiments. DC wrote the manuscript. EJG, ZL, and TJ reviewed the manuscript. TJ supervised the study. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2023.108981.

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