Research Article

Improved biosurfactant production from *Aspergillus niger* through chemical mutagenesis: characterization and RSM optimization

Muhammad Asgher[1] · Sadaf Arshad[1] · Sarmad Ahmad Qamar[1] · Nimrah Khalid[1]

Received: 25 February 2020 / Accepted: 15 April 2020 / Published online: 25 April 2020

Abstract
Biosurfactants are surface-active natural polymers produced within several microorganisms and are secreted outside the cellular environment. They are the focus of modern researches due to their eco-friendly nature and high production capability using low-cost agro-industrial wastes. In this research, we have evaluated *Aspergillus niger* (*A. niger*) for its biosurfactant production potential during solid state fermentation of banana stalks powder. The native strain of *A. niger* produced 2.3 g/L of biosurfactant with 49.74 cm^2 oil displacement, 57% emulsification index and an emulsification activity of 1.024 (OD_{540}). Sequential mutagenesis was induced in the native strain of *A. niger* by exposing the strain with different concentrations of ethidium bromide (EtBr), for different time periods. Significantly higher amount of biosurfactant (3.3 g/L) was obtained from the mutant strain *A. niger* M2 exposed to 50 µg/10 mL of EtBr for 60 min. The screening tests revealed the improvement in oil displacement (59.81 cm^2), emulsification index (62.3%) and emulsification activity (OD_{540}, 1.262) of biosurfactant. FTIR analysis confirmed the presence of amine, amide, fatty acids and triglycerides functional groups. The maximum biosurfactant synthesizing mutant (*A. niger* M2) was further optimized using RSM under CCD. After optimization, the highest biosurfactant (5.50 g/L) was obtained at 35 °C temperature, 7 pH, 5.75 g substrate concentration and 168 h of overall incubation period. In conclusion, the cost-effective production of biosurfactant, along with novel structural and multifunctional characteristics, this study may be useful for different industrial and biotechnological applications.

Keywords Biosurfactant · *Aspergillus niger* · Mutagenesis · Structural properties · Statistical optimization · Characterization

1 Introduction

Surfactants are surface-active chemical substances which have gained high importance in fluid mechanics because of their ability to reduce surface tension of liquids. Most of these surface-active compounds are synthesized chemically, which are causing serious toxicological and environmental problems. Biosurfactants are biopolymers, produced by several microorganisms (bacteria and fungi), which possess hydrophobic and hydrophilic moieties. They provide the best alternative to synthetic surfactants in term of low toxicity, high biodegradability, environmentally friendly nature, and the production capability using low-cost agro-industrial raw materials "green technology" [1]. Biosurfactants comprise a large group of chemical compounds e.g. glycolipids, lipopeptides, phospholipids, lipoproteins and lipid-polysaccharide complexes [2]. They present various applications as emulsifiers, conditioners, cosmetics and food industries [3, 4].

Biosurfactants have been reported to possess excellent biomedical and therapeutic properties [5–8] and also found to be effective to tackle environmental pollution

✉ Sarmad Ahmad Qamar, sarmad_qamar@uaf.edu.pk | [1]Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

through bioremediation [4, 9]. They are important in food digestion and respiratory action, plant pathogenicity, paints, beverages, cosmetics and cement industries. Moreover, biosurfactants can be produced from renewable resources and commonly effective in extreme environmental conditions. Biosurfactants also have applications in laundry formulations, pesticides and herbicides, agriculture, textile, household cleaning products, detergents, petroleum and paper industries, food processing, and pharmaceutical industries. They are also important in enzyme biotechnology because of their regulatory effects and bio-stimulatory effects [10].

Surfactant display properties like foaming, emulsification, detergency and dispersion [11–13]. Microorganisms have ability to produce different kinds of surfactants, from these, high molecular weight polymers are highly efficient emulsifiers [14, 15], while low molecular weight polymers efficiently reduce interfacial and surface tension of liquids [16]. Biosurfactants can bear extreme environmental conditions e.g., wide range of pH, high salinity and temperature [17]. In previous decade, much attention has been devoted towards biosurfactants because of their exceptional functional properties. These properties allow a possible replacement of chemically synthesized surfactants for a number of industrial and biotechnological operations [18].

The identification and optimization of fermentation parameters that influence the surfactant yield represent the major points for the development of cost-effective procedures [19]. There are several different factors controlling the microbial biosurfactant production during fermentation which are needed to be maintained within a specific range. It will allow maximum microbial multiplication, which ultimately leads to higher biosurfactant production. In this regard, several physical and nutritional parameters are needed to be optimized for enhance biosurfactant yield. The yield of biosurfactant highly depends upon the availability of carbon and nitrogen sources and other limiting nutrients [20]. Factors influencing biosurfactant production have extensively been investigated particularly for *Pseudomonas* spp. [21, 22], *Candida* spp. [23–25], *Rhodococcus* spp. [26, 27] There is a dirt of information related to biosurfactant production from bacterial spp. and the optimization of several nutritional and physical parameters influencing the overall yield [28–30].

Microbial biosurfactant production can only be maximized if fermentation parameters are maintained at the optimal growth conditions of microbe. For this purpose, one of the most appropriate approach could be response surface optimization. RSM is a combination of statistical and mathematical tool that is frequently being used in fermentation biotechnology for optimization of fermentation parameters [31–33]. RSM is a statistical design experimental process in which several factors are varied simultaneously. In fact, the relationship between the independent variables and the response variable is usually unknown in an experiment. Therefore, the initial step is to approximate the response variable through analyzing the independent variables.

This study was designed to investigate *A. niger* for its ability to synthesize biosurfactant. The native fungal strain was mutated using different concentrations of EtBr for different time periods. The lyophilized biosurfactant from both native and mutant strain was structurally characterized using FT-IR spectroscopy. The maximum biosurfactant producer mutant strain (*A. niger* M2) was further optimized using RSM under CCD. An insight of primary characteristics of fungal biosurfactant have also been provided in this article.

2 Materials and methods

2.1 Materials

Banana stalks were obtained from Botanical Garden of University of Agriculture, Faisalabad (UAF), Pakistan. After pretreatment, stalks were chopped and placed in the incubator (LabTech LDO-150 N) at 50 °C for 48 h. The dried substrate was ground into fine powder and stored in an airtight jar at 25 °C temperature. All other chemicals/materials used in this study were obtained from distributors of international companies (Merck KGaA, USA and Sigma Aldrich, Germany).

2.2 Fungal strain

Purified culture of a black rot fungus (*A. niger*) was collected from Industrial Biotechnology Laboratory of UAF, Pakistan. Culture was streaked on a freshly prepared PDA slants of pH 6.5 and incubated at 35 °C temperature for 6–8 days. Fungal spore suspension was prepared by transferring the culture to Vogel's minimal media [34]. Media was added with glucose (2%) as carbon source and placed in shaking incubator at 160 *rev*/min and 37 °C temperature.

2.3 Chemical mutagenesis

Ethidium bromide (EtBr) was used for the induction of mutation in *A. niger*. For this purpose, mutagen stock solution was prepared by the addition of EtBr 500 µg/10 mL of distilled water. Solutions of different concentrations i.e. 50, 100, 150 and 200 µg/10 mL were prepared from EtBr stock. Serial dilutions of vegetative cells of fungal inoculum were formed and pellets were suspended in EtBr solution for different time periods of 30, 60 and 90 min. The

fungal suspension was centrifuged, and the pellets were washed thrice with saline solution [composition (g/L) of NaCl (8.9) and yeast extract (1.0) in ddH_2O]. Agar media containing triton X-100 was used for the development of genetically modified colonies [35]. The final screening of mutant colonies was achieved by using 2-deoxy-D-glucose as metabolic inhibitor that was used for the isolation of mutant strains [36]. The inocula were prepared from native as well as selected mutant strain and preserved at refrigerator temperature (4 °C).

2.4 Development and recovery of biosurfactant

The solid-state fermentation was carried out using banana stalk powder (5 g) as substrate moistened with 5 mL of fungal growth suspension prepared by mixing g/L of $NaNO_3$ (3.0), $MgSO_4.7H_2O$ (0.5), KH_2PO_4 (1.0), yeast extract (1.0) and peptone (3.0) with a pH 7.0. The media was sterilized by autoclaving for 20 min at 121 °C temperature. Fermentation medium was added with 2 mL of *A. niger* inoculum containing 10^7 spores/mL and incubated at 37 °C for 6–8 days. All the experiments were conducted in triplicate. Media was centrifuged for 20 min at 5600 *rev*/min to get cell-free supernatant. Cell-free supernatant was acidified with 6 N HCl to get a final pH of 2.0 and was kept in refrigerator temperature (4 °C) for the precipitation of lipids and proteins [1]. The pallets were harvested by centrifugation for 15 min at 10,000 *rev*/min and pH was adjusted to normal by the addition of sufficient amount of distilled water. The extracted pallets were lyophilized for the quantification of biosurfactant.

2.5 Screening of biosurfactant

2.5.1 Oil displacement

Oil displacement was performed using the method adopted from Morikawa et al. [37] with little modifications. Briefly, distilled water (40 mL) was poured in a glass petri plate of 90 mm diameter. Cottonseed oil (8.0 mL) was poured on the water surface to form a thin layer. Cell-free supernatant containing biosurfactant (3 mL) was gently poured at the center of petri plate. The area (cm^2) of displaced oil layer was measured for determination of biosurfactant.

2.5.2 Emulsification index

Emulsification index (E_{24}) was determined by the method of Alvarez et al. [38]. Briefly, the cell free supernatant was added in the cottonseed oil with 3:2 (biosurfactant: oil) ratio. The mixture was gently vortexed, and the height (cm) of emulsion layer and total height of solution was calculated after 24 h. The emulsification index was calculated by using the equation.

$$\text{Emulsification index}\left(E_{24}\right) = \frac{\textit{Hight of emulsion layer}\,(\text{cm})}{\textit{Total hight of solution}\,(\text{cm})} \times 100$$

2.5.3 Emulsification activity assay

The determination of emulsification assay was performed by the method described by Patel and Desai, [39]. Briefly, a solution of Tris-Mg was prepared by the addition of Tris–HCl (20 mM) and $MgSO_4$ (10 mM) with a final pH of 8.0. Tris-Mg solution (7.5 mL) was added in a test tube and added with 0.5 mL of cell-free supernatant (biosurfactant) and 0.1 mL of kerosene oil. The tubes were gently vortexed and allowed to stand for 60 min. Absorbance was recorded at 540 nm using UV/visible spectrophotometer (Dynamica Scientific, HALO DB-20). Patel and Desai (1997) described the emulsification activity as the measured optical density of a sample. The activity was calculated by using the following relationship:

$$\text{Emulsification activity}\left(OD_{540}\right) = {}^{A}/_{L}$$

here 'A' is absorbance and 'L' is optical pass length.

2.6 Structural characterization of biosurfactant

2.6.1 Fourier transform infrared spectroscopy (FT-IR)

Biosurfactant obtained from native as well as mutant strains of *A. niger* was analyzed using FT-IR (Bruker Alpha FTIR, Germany) for the detection of major functional groups. The lyophilized biosurfactant samples (0.5 mg) were placed directly under the infrared beam and spectrum was recorded at 500 – 4000 nm wavelength for detailed structural analysis.

2.7 Optimization of biosurfactant production

Optimization is a technique used to get the best possible results with maximum achievable performance and cost-effectiveness under certain limitations. The biosurfactant production from mutant *A. niger* was optimized using response surface methodology (RSM) under central composite design (CCD). RSM is a combination of statistical and mathematical techniques used to analyze the effect of dependent variables on one or more independent variables (response). Four parameters i.e., temperature (X1), pH (X2), incubation time (X3) and substrate concentration (X4) were optimized using four-factors, six-level CCD requiring 30 runs with six central points and α 0.5. The biosurfactant was harvested by acid-precipitation followed by

centrifugation at 10,000 *rev*/min for 15 min. The extracted biosurfactant was freeze-dried and weighted for quantification. The RSM model was designed using Design Expert software v11.1.0.1.

3 Results

3.1 Screening for best biosurfactant producer strain

Biosurfactant produced by native strain of *A. niger* (2.3 g/L) showed 49.74 cm^2 oil displacement area, 57% emulsification index and 1.024 emulsification activity. The wild fungal strain was mutated with EtBr to determine the consequence of mutagenesis on total biosurfactant yield. Table 1 summarizes the data obtained after sequential mutagenesis in *A. niger* using different concentrations and exposure time to mutagen (EtBr). Maximum biosurfactant yield (3.3 g/L) was attained from *A. niger* M2 specie exposed to 50 µg/10 mL EtBr for 60 min. In addition, screening tests revealed an emulsification index of 62.30% and emulsification activity of 1.262 (OD_{540}) with oil displacement area of 59.81 cm^2. All the mutants produced significant amount of biosurfactant ranging from 1.5 – 3.3 g/L depending upon mutagen concentration and exposure time (Fig. 1a). Figure 1b provides a representation of emulsification index (E_{24}) from different mutant strains of *A. niger*. The emulsification and oil displacements conformed the biosurfactant production, and the dry weight estimation after lyophilization made the selection easier for maximum producing mutant colonies (*A. niger* M2) which was subjected to RSM optimization.

Table 1 Screening of *A. niger* mutants for their biosurfactant production potential depending upon total biomass, oil displacement area, emulsification index and emulsification activity

EtBr conc. (µg/10 mL)	Mutant strain ID	Exposure time (min.)	Displacement area (cm^2)	Emulsification index % (E_{24})	Emulsification activity (OD_{540})	Biosurfactant yield (g/L)
50	M1	30	47.76 ± 3.44	57.63 ± 2.30	1.024 ± 0.01	2.9 ± 0.20
	M2	60	59.81 ± 4.20	62.30 ± 2.41	1.262 ± 0.00	3.3 ± 0.21
	M3	90	52.78 ± 4.01	59.95 ± 1.80	1.175 ± 0.01	3.0 ± 0.18
100	M4	30	30.18 ± 3.32	42.45 ± 2.81	0.820 ± 0.03	2.1 ± 0.28
	M5	60	37.37 ± 3.41	47.50 ± 2.73	0.963 ± 0.02	2.6 ± 0.30
	M6	90	35.24 ± 3.00	47.23 ± 2.89	0.894 ± 0.01	2.7 ± 0.27
150	M7	30	25.50 ± 2.91	39.60 ± 2.31	0.679 ± 0.00	1.6 ± 0.10
	M8	60	24.62 ± 3.31	38.24 ± 2.40	0.652 ± 0.01	1.5 ± 0.13
	M9	90	32.96 ± 4.29	43.04 ± 2.44	0.740 ± 0.00	1.7 ± 0.11
200	M10	30	40.69 ± 4.10	49.90 ± 7.20	0.826 ± 0.00	1.9 ± 0.51
	M11	60	44.39 ± 6.21	63.45 ± 7.61	1.260 ± 0.02	3.0 ± 0.39
	M12	90	42.99 ± 4.02	61.20 ± 6.91	1.187 ± 0.00	2.4 ± 0.42

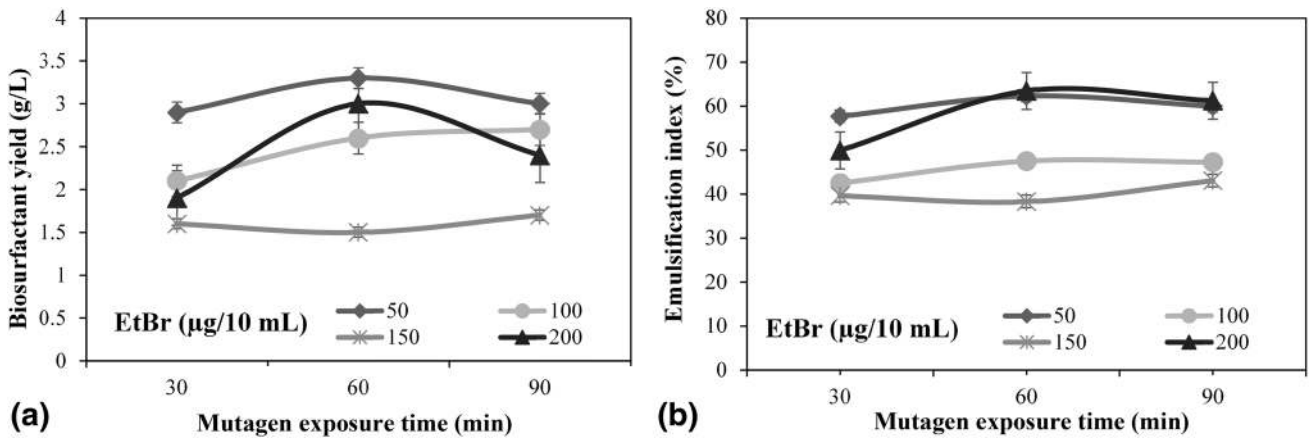

Fig. 1 Representation of **a** Biosurfactant yield **b** Emulsification index (E_{24}) of biosurfactant produced from *A. niger* after exposure to different concentrations of EtBr for different time periods

3.2 Fermentation for biosurfactant production

Biosurfactant production was monitored after 168 h of fermentation. The maximum biosurfactant production (3.3 g/L) was attained from *A. niger* M2 spp. with highest emulsification index of 62.30%. The extraction of biosurfactant was performed using acid-precipitation. Biosurfactant is generally soluble at basic or neutral pH (i.e. 7). By decreasing the pH, biosurfactant start to precipitate and increase the turbidity of the solution. This effect was determined by gradually decreasing the pH using 6 N HCl and taking the absorbance at 600 nm at UV/vis spectrophotometer (Dynamica Scientific, HALO DB-20). The maximum biosurfactant precipitation was achieved at pH 2. Further decrease in pH did not resulted any significant change (Fig. 2).

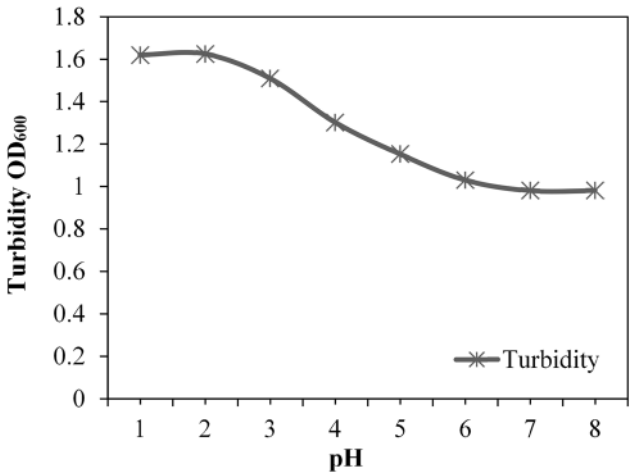

Fig. 2 Representation of biosurfactant precipitation (increase in turbidity of solution) at acidic range of pH

3.3 Major functional groups: FTIR

Spectrum of biosurfactant produced from native *A. niger* (Fig. 3, orange line) showed a broad peak at 3366 cm^{-1} which shows asymmetric stretching of N–H bonds of secondry amides of proteins. A peak at 2890 cm^{-1} shows strong stretching of C–H bonds of alkane alkene and alkynes. A intense peak at 1655 cm^{-1}indicate C=O, C–N stretching and N–H bending of protein amides. A short peak at 1710 cm^{-1} shows the presnece of C=O stretching of carboxylic acid, aldehyde and keton functional groups. A peak at 1500 cm^{-1} indicate the presence of C=C bond of aromatic skeloton and peak at 1571 cm^{-1} shows N–H bonds of amines group. Peek at 1220 cm^{-1} indicate the presence of P=O bond of phosphodiester group of nuclic acid and phospholipids.

Biosurfactant from mutant strain *A. niger* M2 has been presented in Fig. 3 (blue line), which shows a broad peak at 3349 cm^{-1} which shows weak N–H stretching of primary and secondary amines and amides. Successive vibrations between 2000 and 2500 cm^{-1} indicates the presence of N–H, C–H bonds of alkane alkene and alkynes. A peak at 1710 cm^{-1} show plane stretching of C=O bond which indicate the presence of ester, lipid and triglycerides functional group. A peak at 1551 cm^{-1} indicates the presence of N–H bending which is coupled with C–N bond stretching of amide functional group. Peaks at 1456, 1435 and 1400 cm^{-1} indicate the presence of CH_3 asymmetric vibration, C–H bending and CH_3 symmetric vibrations of protein respectively. A broad peak at 1051 cm^{-1} shows C-O stretch of COH tyrosine protein. Peek at 702 cm^{-1} show N–H out of plane deformation of protein.

3.4 Optimization of biosurfactant

The biosurfactant yield from *A. niger* M2 was further optimized in SSF using statistical tools (RSM). 4-level, 6-factors CCD was used which required 30 experiments and

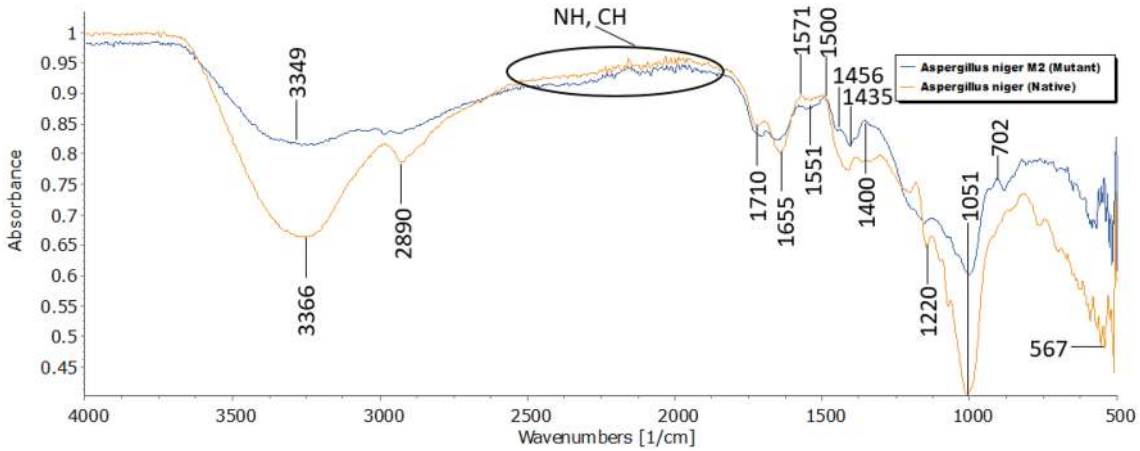

Fig. 3 FT-IR spectra of lyophilized biosurfactant produced by native and mutant strains of *A. niger*

the response (g/L of biosurfactant yield) was determined. Total biosurfactant yield was in the range of 2.8–5.5 g/L depending upon fermentation conditions provided. The lowest yield was obtained at 35 °C temperature, 5.5 pH, 4.5 g substrate concentration and incubation of 168 h. The maximum biosurfactant yield (5.5 g/L) was obtained at 35 °C temperature, 7 pH, 5.57 g substrate concentration and an overall incubation period of 168 h. The model was analyzed using Design-Expert software and the 3D response surface graphs were constructed.

The response surface model significance and fitness were analyzed by the determination of ANOVA variant for quadratic model and each individual factor was tested using F-values and p-values. ANOVA for RSM quadratic model for biosurfactant production under optimized conditions has been represented in Table 2. The high model F value (2.743) showed that the model is significant. The lack of fit value was insignificant indicating the model appropriateness. These results showed the quality and the fitness of the RSM and its capability to predict the response within the range of variables used. RSM model suggested the quadratic equation for the total biosurfactant yield which can be expressed as follows:

where X1, X2, X3 and X4 are the codded values of the relating factors temperature, pH, incubation time and substrate concentration.

Temperature and pH are the great influencer of biosurfactant production during fermentation. The optimum production occurs in the specific range where the microorganism is most active. In case of *Aspergillus* strains, the optimum growth temperature lies between 30 and 40 °C and a pH ranging from 6 to 7. This indicates the high temperature and pH dependency of biosurfactant production process. In this study, maximum biosurfactant production was obtained at pH 7 and 35 °C temperature. Figure 4a presents the effect of temperature and pH on biosurfactant yield at fixed value of substrate concentration and fermentation time. Since the optimum pH for *Aspergillus* strains is 6.5–7, the production was drastically reduced at end points of pH variable. Temperature was also the critical parameter that have been controlled in bioprocess. Strain *A. niger* M2 showed maximum activity at 30–40 °C temperature with maximum biosurfactant production at 35 °C (Fig. 4a).

Incubation time was found to be one of the most influencer of biosurfactant production. Biosurfactant

$$\begin{aligned}\text{Biosurfactant yield (g/L)} = {} & 2.05 + 0.0784(X1) + 0.0166(X2) + 0.0157(X3) \\ & + 0.0353(X4) + 0.0441(X1 \times X2) + 0.0206(X1 \times X3) \\ & + 0.0212(X1 \times X4) - 0.0027(X2 \times X3) + 0.0234(X2 \times X4) \\ & - 0.0134(X3 \times X4) + 0.3274\left(X1^2\right) - 0.5901\left(X2^2\right) \\ & - 0.9827\left(X3^2\right) + 1.09\left(X4^2\right)\end{aligned}$$

Table 2 Analysis of variance (ANOVA) for response surface model for the development of biosurfactant by *A. niger* M2

Source	Sum of squares	*df*	Mean square	F value	*p* value	
Model	22.15	14	1.58	36.29	0.000	Significant
A-temperature	0.116	1	0.117	2.68	0.002	
B-pH	0.136	1	0.136	3.13	0.007	
C-incubation time	0.434	1	0.434	9.96	0.006	
D-substrate concentration	0.012	1	0.013	0.294	0.005	
AB	1.95	1	1.95	44.63	< 0.000	
AC	0.230	1	0.231	5.29	0.036	
AD	0.024	1	0.024	0.551	0.009	
BC	0.176	1	0.176	4.05	0.002	
BD	0.000	1	0.001	0.009	0.925	
CD	0.144	1	0.144	3.31	0.088	
A^2	0.176	1	0.176	4.04	0.062	
B^2	0.469	1	0.470	10.77	0.005	
C^2	0.030	1	0.031	0.702	0.415	
D^2	0.073	1	0.074	1.69	0.213	
Residual	0.654	15	0.044			
Lack of fit	0.588	10	0.059	4.46	0.0562	Not significant
Pure error	0.065	5	0.014			
Cor total	22.80	29				

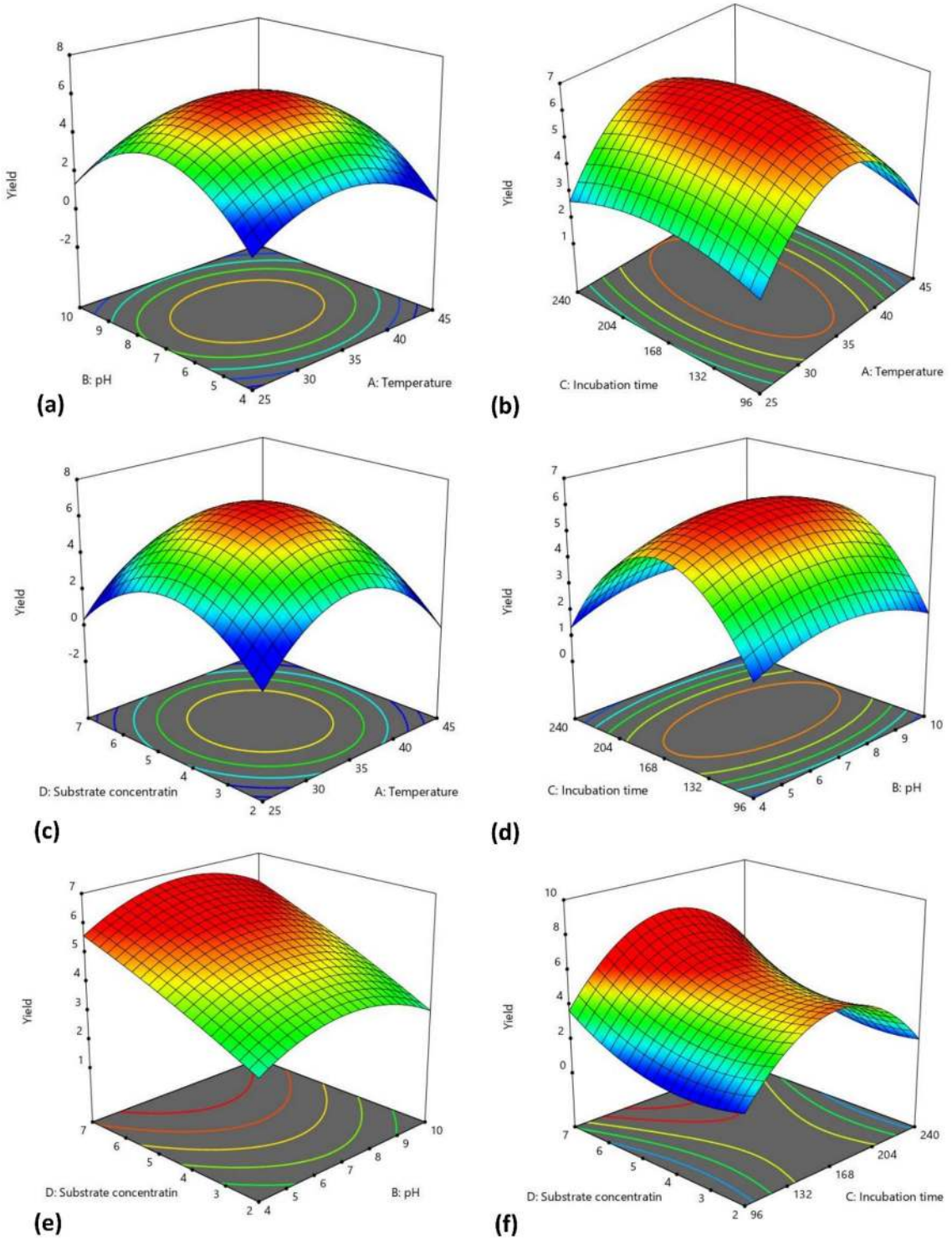

Fig. 4 Response surface three-dimensional (3D) plots showing interaction between **a** temperature versus pH **b** temperature versus incubation time **c** temperature versus substrate concentration **d** pH versus incubation time **e** pH versus substrate concentration **f** incubation time versus substrate concentration

production was maximum after 168 h of incubation in SSF of banana stalks powder. The production was equally decreased at higher or lower values of incubation time than 168 h. Figure 4b shows the interaction between temperature and incubation time by keeping other factors constant. Any deviation from 168 h of incubation leads to

the decline in biosurfactant production from *A. niger* M2. The strains showed maximum production after 168 h in the exponential growth phase of the specie.

The joint effect of temperature and substrate concentration has been represented in Fig. 4c by keeping pH and incubation time constant. The biosurfactant yield was maximum at 4–5 g of substrate and the yield was equally decreased at upper and lower level of factor. The maximum biosurfactant production was achieved at 5.75 g of substrate concentration where the factor was at + 1 level of the design. Figure 4d shows the joint effect of pH and incubation time on biosurfactant production by keeping other factors constant. Maximum biosurfactant production was obtained at pH 6 to 7 and 168 h of incubation in SSF. The production was equally declined at higher and lower level of these factors, but the pH was less influencing the production as compared with incubation time.

The effect of pH versus substrate concentration has been displayed in Fig. 4e by keeping constant values of temperature and incubation time. In the figure, substrate concentration positively influenced the biosurfactant production, means the yield was increased by increase in substrate concentration, whereas, at pH 6 to 7, yield was optimum which gradually decreased by moving towards extreme points, but the reduction was generally lower. Figure 4f shows the interaction between incubation period and substrate concentration by keeping temperature and pH constant. Highest yield was obtained at 6 to 7 g substrate concentration and incubation period of 168 h.

4 Discussion

The production of microbial surfactants has gained high importance from previous decade, especially due to their potential applications in biomedical and industrial biotechnological sectors. Oil displacement, Emulsification activity and emulsification index has been used for the estimation of biosurfactant [32, 38–40]. Therefore, using such strategy, biosurfactant production from *A. niger* was screened after random mutagenesis for the selection of highest biosurfactant producer mutant strain. Furthermore, quantification using dry weight estimation made the selection more convenient. Biosurfactant production from fungal species have extensively been described from *Aspergillus* spp. [41–44] using various synthetic and biobased substrates, but herein, mutant *A. niger* (M2) produce significantly higher amount of biosurfactant (3.3 g/L) as compared with previous studies using native *A. niger* strain. The mutant (*A. niger* M2) exhibited high emulsification index and oil displacement as compared to native strain (Table 1).

Despite having several commercially attractive characteristics compared with synthetic surfactants, the production of commercial scale biosurfactants from microbial origin has not been realized because of their high production cost and significantly low yield. There are two basic strategies that are being adopted by the biotechnologists for economical biosurfactant production, which corresponds, the use of low-cost waste materials to reduce the overall substrate cost and the efficient bioprocesses, including optimized fermentation parameters involving multiple physical and nutritional factors [45]. The selection of appropriate substrate is a crucial step in the production of biosurfactant, as the substrate account for overall 10–30% cost of the experiment [45, 46]. Therefore, in this context, herein, a cost-effective process was developed for the production of biosurfactant using agro-industrial raw-material (banana stalks powder) with waste-to-value approach. The production of significant amount of biosurfactant using banana stalks waste revealed the compatibility of the medium. Hence, the reported medium composition was selected for the next experiments.

The biosurfactant production during logarithmic growth phase and stationary phase of microorganisms have already been studied from various fungal species e.g., *A. ustus* [42], *A. niger* [43, 44], *A. flavus* [43, 46], *A. versicolor* [47], and *Piper hispidum* [48], and various factors influencing the total biosurfactant yield during fermentation have broadly been studied in previous few decades [42, 49–54]. From these, several studies have reported classical methods for optimization purpose by placing one factor as constant and modifying another one. In this research, we have applied a proper tool (RSM) for the optimization of different factors that influence biosurfactant production from *A. niger* M2 during fermentation. One of the advantages of this mutant (*A. niger* M2) is the highest amount of biosurfactant production with maximum emulsification activity, which make it capable for several industrial applications such as microbially enhanced oil recovery [30, 38].

After optimization using RSM-CCD, maximum biosurfactant production (5.5 g/L) was attained which was 2.5-folds higher than the biomass obtained from native strain and about 1.7-folds higher than the mutant strain (*A. niger* M2). These results clearly suggest a significantly higher amount of biosurfactant as compared with previous studies. The central composite design (CCD) during RSM enabled us to investigate different fermentation parameters that support biosurfactant overproduction. A close association was observed between R^2 (0.820) and predicted R^2 (0.794), which reflected the compatibility and suitability of methodology to optimize biosurfactant production. The maximum yield was obtained at 35 °C temperature, 7 pH, 5.75 g substrate concentration and 168 h of overall fermentation period. Any change

(increase of decrease) in one of these parameters may cause decrease in biosurfactant production. The biosurfactant yield (5.5 g/L) from *A. niger* M2 is the highest reported yield from *Aspergillus* species in solid state fermentation.

In addition, the use of banana stalks waste as a substrate in SSF for biosurfactant production by *A. niger* is novel. The production cost would be significantly reduced via experimental design described in this study using optimized fermentation conditions. The insights of fungal surfactants and their significant characteristics have also been studied in this research. The stability of biosurfactant obtained from *A. niger* M2 over a wide temperature range indicates its activity at extreme environmental conditions, which is an amazing feature for its potential use in biomedical and industrial biotechnological sectors including microbial enhanced oil recovery and bioremediation [9, 12]. In conclusion, we have successfully improved the biosurfactant production about 1.5-folds after random chemical mutagenesis of *A. niger* using banana stalks powder as low-cost substrate following waste-to-value idea (from 2.3 to 3.3 g/L).

5 Conclusion

In this study, biosurfactant production was successfully enhanced about 1.5-folds after EtBr mutagenesis of *A. niger* using agro-industrial waste (banana stalks powder) as low-cost substrate. Optimization of fermentation parameters was performed using statistical approach (RSM-CCD) to maximize the yield. After optimization, maximum biosurfactant yield (5.5 g/L) was attained, which was significantly higher from both native as well as mutant strains (before optimization) i.e., 2.5-folds and 1.7-folds respectively. In conclusion, the biosurfactants produced from *A. niger* were a kind of surface-active substances, having potential applications in biomedical and industrial biotechnological sectors including enhanced oil recovery and bioremediation. To the best of our knowledge, this the first study reporting mutagenesis of *A. niger* for improved biosurfactant production and process optimization using cost-effective agro-industrial waste as substrate.

Acknowledgements This study was supported by Higher Education Commission (HEC), Islamabad, Pakistan under grant number NRPU 6417. The analytical facilities provided by Department of Biochemistry and Punjab Bioenergy Institute (PBI), University of Agriculture, Faisalabad, Pakistan are thankfully acknowledged.

Compliance with ethical standards

Conflict of interest The authors declared that they have no conflict of interest.

References

1. Mukherjee S, Das P, Sen R (2006) Towards commercial production of microbial surfactants. Trends Biotechnol 24:509–515. https://doi.org/10.1016/j.tibtech.2006.09.005
2. Van Hamme JD, Singh A, Ward OP (2006) Physiological aspects: part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnol Adv 24:604–620. https://doi.org/10.1016/j.biotechadv.2006.08.001
3. Mulligan CN (2009) Recent advances in the environmental applications of biosurfactants. Curr Opin Colloid Interface Sci 14:372–378. https://doi.org/10.1016/j.cocis.2009.06.005
4. Banat IM, Rengathavasi T (2018) Microbial biosurfactants and their environmental and industrial applications. CRC Press. ISSN: 9781315271767. https://doi.org/10.3390/app8091555
5. Janek T, Czyznikowska Z, Lukaszewicz M, Gałęzowska J (2019) The effect of *Pseudomonas fluorescens* biosurfactant pseudofactin II on the conformational changes of bovine serum albumin: pharmaceutical and biomedical applications. J Mol Liq 288:111001. https://doi.org/10.1016/j.molliq.2019.111001
6. Satpute SK, Mone NS, Das P, Banat IM (2019) Inhibition of pathogenic bacterial biofilms on PDMS based implants by *L. acidophilus* derived biosurfactant. BMC Microbiol. 19:39. https://doi.org/10.1186/s12866-019-1412-z
7. Satpute S, Mone N, Das P, Banpurkar A (2018) *Lactobacillus acidophilus* derived biosurfactant as a biofilm inhibitor: a promising investigation using microfluidic approach. Appl Sci 8:1555. https://doi.org/10.3390/app8091555
8. Mnif I, Ghribi D (2015) Review lipopeptides biosurfactants: mean classes and new insights for industrial, biomedical, and environmental applications. J Pept Sci 104:129–147. https://doi.org/10.1002/bip.22630
9. Silva EJ, Correa PF, Almeida DG, Luna JM (2018) Recovery of contaminated marine environments by biosurfactant-enhanced bioremediation. Colloids Surf B 172:127–135. https://doi.org/10.1016/j.colsurfb.2018.08.034
10. Reis RS, Pacheco GJ, Pereira AG, Freire DM (2013) Biosurfactants: production and applications, biodegradation-life of science, Dr. Rolando Chamy. InTech, London. https://doi.org/10.5772/56144
11. Law SQ, Mettu S, Ashokkumar M, Scales PJ (2018) Emulsifying properties of ruptured microalgae cells: barriers to lipid extraction or promising biosurfactants? Colloids Surf B 170:438–446. https://doi.org/10.1016/j.colsurfb.2018.06.047
12. Bouassida M, Fourati N, Ghazala I, Ellouze-Chaabouni S (2018) Potential application of *Bacillus subtilis* SPB1 biosurfactants in laundry detergent formulations: compatibility study with detergent ingredients and washing performance. Eng Life Sci 18:70–77. https://doi.org/10.1002/elsc.201700152
13. Santos DK, Resende AH, de Almeida DG, Soares da Silva RD (2017) *Candida lipolytica* UCP0988 biosurfactant: potential as a bioremediation agent and in formulating a commercial related product. Front Microbiol. 8:767. https://doi.org/10.3389/fmicb.2017.00767
14. Peele KA, Ch VR, Kodali VP (2016) Emulsifying activity of a biosurfactant produced by a marine bacterium. 3 Biotech 6:177. https://doi.org/10.1007/s13205-016-0494-7
15. Gaur VK, Regar RK, Dhiman N, Gautam K (2019) Biosynthesis and characterization of sophorolipid biosurfactant by *Candida*

spp.: application as food emulsifier and antibacterial agent. Bioresour Technol 285:121314. https://doi.org/10.1016/j.biortech.2019.121314
16. Mohammed IU, Deeni Y, Hapca SM, McLaughlin K (2015) Predicting the minimum liquid surface tension activity of pseudomonads expressing biosurfactants. Lett Appl Microbiol 60:37–43. https://doi.org/10.1111/lam.12331
17. Nitschke M, Pastore GM (2016) Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. Bioresour Technol 97(2):336–341. https://doi.org/10.1016/j.biortech.2005.02.044
18. Lang S, Wagner F (2017) Structure and properties of biosurfactants. Biosurfactants Biotechnol 2017:21–45. https://doi.org/10.1201/9781315138428
19. Banat IM, Franzetti A, Gandolfi I, Bestetti G (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87:427–444. https://doi.org/10.1007/s00253-010-2589-0
20. Abouseoud M, Maachi R, Amrane A, Boudergua S (2008) Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination 223:143–151. https://doi.org/10.1016/j.desal.2007.01.198
21. Kumar AP, Janardhan A, Radha S, Viswanath B (2015) Statistical approach to optimize production of biosurfactant by *Pseudomonas aeruginosa* 2297. 3 Biotech 5:71–79. https://doi.org/10.1007/s13205-014-0203-3
22. Oluwaseun AC, Kola OJ, Mishra P, Singh JR (2017) Characterization and optimization of a rhamnolipid from *Pseudomonas aeruginosa* C1501 with novel biosurfactant activities. Sustain Chem Pharm 6:26–36. https://doi.org/10.1016/j.scp.2017.07.001
23. Brasileiro PP, de Almeidab DG, de Lunaa JM, Rufinoa RD (2015) Optimization of biosurfactant production from *Candida guilliermondii* using a rotate central composed design. Chem Eng. https://doi.org/10.3303/CET1543236
24. Luna JM, Santos Filho A, Rufino RD, Sarubbo L (2016) Production of biosurfactant from *Candida bombicola* URM 3718 for environmental applications. Chem Eng Trans 49:583–588. https://doi.org/10.3303/CET1649098
25. Almeida DG, Soares da Silva RD, Luna JM, Rufino RD (2017) Response surface methodology for optimizing the production of biosurfactant by Candida tropicalis on industrial waste substrates. Front Microbiol 8:157. https://doi.org/10.3389/fmicb.2017.00157
26. Suryanti V, Hastuti S, Andriani D (2016) Optimization of biosurfactant production in soybean oil by *Rhodococcus rhodochrous* and its utilization in remediation of cadmium-contaminated solution. IOP Conf Ser Mater Sci Eng 107:012018. https://doi.org/10.1088/1757-899X/107/1/012018
27. Kim CH, Lee DW, Heo YM, Lee H (2019) Desorption and solubilization of anthracene by a rhamnolipid biosurfactant from *Rhodococcus fascians*. Water Environ Res. https://doi.org/10.1002/wer.1103
28. Fooladi T, Moazami N, Abdeshahian P, Kadier A (2016) Characterization, production and optimization of lipopeptide biosurfactant by new strain *Bacillus pumilus* 2IR isolated from an Iranian oil field. J Petrol Sci Eng 145:510–519. https://doi.org/10.1016/j.petrol.2016.06.015
29. Moshtagh B, Hawboldt K, Zhang B (2018) Optimization of biosurfactant production by *Bacillus Subtilis* N3-1P using the brewery waste as the carbon source. Environ Technol 2018:1. https://doi.org/10.1080/09593330.2018.1473502
30. Ghazala I, Bouallegue A, Haddar A, Ellouz-Chaabouni S (2018) Characterization and production optimization of biosurfactants by *Bacillus mojavensis* I4 with biotechnological potential for microbial enhanced oil recovery. Biodegradation 2018:1. https://doi.org/10.1007/s10532-018-9844-y
31. Deepika KV, Kalam S, Sridhar PR, Podile AR (2016) Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium *Pseudomonas aeruginosa* KVD-HR42 using response surface methodology. Biocatal Agric Biotechnol 5:38–47. https://doi.org/10.1016/j.bcab.2015.11.006
32. Mouafi FE, Elsoud MM, Moharam ME (2016) Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. Biotechnol Rep 9:31–37. https://doi.org/10.1016/j.btre.2015.12.003
33. Maheshwari N, Kumar M, Thakur IS, Srivastava S (2017) Recycling of carbon dioxide by free air CO_2 enriched (FACE) *Bacillus* sp. SS105 for enhanced production and optimization of biosurfactant. Bioresour Technol 242:2–6. https://doi.org/10.1016/j.biortech.2017.03.124
34. Vogel HJ (1964) Distribution of lysine pathways among fungi: evolutionary implications. Am Nat 98:435–446
35. Lee CA, Jones BD, Falkow S (1992) Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci 89:1847–1851. https://doi.org/10.1073/pnas.89.5.1847
36. Fiedurek J, Paszczynski A, Ginalska G, Ilczuk Z (1987) Selection of amylolytically active *Aspergillus niger* mutants to 2-deoxy-D-glucose. Zentralblatt für Mikrobiologie. 142:407–412. https://doi.org/10.1016/S0232-4393(87)80089-6
37. Morikawa M, Hirata Y, Imanaka TA (2000) A study on the structure–function relationship of lipopeptide biosurfactants. BBA-Mol Cell Biol Lipid 1488:211–218. https://doi.org/10.1016/S1388-1981(00)00124-4
38. Alvarez VM, Jurelevicius D, Marques JM, de Souza PM (2015) *Bacillus amyloliquefaciens* TSBSO 3.8, a biosurfactant-producing strain with biotechnological potential for microbial enhanced oil recovery. Colloids Surf B 136:14–21. https://doi.org/10.1016/j.colsurfb.2015.08.046
39. Patel RM, Desai AJ (1997) Surface-active properties of rhamnolipids from Pseudomonas aeruginosa GS3. J Basic Microbiol 37:281–286. https://doi.org/10.1002/jobm.3620370407
40. Ghribi D, Abdelkefi-Mesrati L, Mnif I, Kammoun R (2012) Investigation of antimicrobial activity and statistical optimization of *Bacillus subtilis* SPB1 biosurfactant production in solid-state fermentation. J Biomed Biotechnol. https://doi.org/10.1155/2012/373682
41. Bezerra KG, Rufino RD, Luna JM, Sarubbo LA (2018) Saponins and microbial biosurfactants: potential raw materials for the formulation of cosmetics. Biotechnol Prog 34:1482–1493. https://doi.org/10.1002/btpr.2682
42. Kiran GS, Hema TA, Gandhimathi R, Selvin J (2009) Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. Colloids Surf B 73:250–256. https://doi.org/10.1016/j.colsurfb.2009.05.025
43. Adekunle AT, Ester BB, Olabisi A, Peter OS (2015) Characterization of new glycosophorolipid-surfactant produced by *Aspergillus niger* and *Aspergillus flavus*. Eur J Biotechnol Biosci 3:34–39
44. Superb JG, Costa TM, Bertoli SL, Tavares LB (2018) Simultaneous production of biosurfactants and lipases from *Aspergillus niger* and optimization by response surface methodology and desirability functions. Braz J Chem Eng 35:857–868. https://doi.org/10.1590/0104-6632.20180353s20160400
45. Asgher M, Urooj Y, Qamar SA, Khalid N (2020) Improved exopolysaccharide production from Bacillus licheniformis MS3: optimization and structural/functional characterization. Int J Biol Macromol. https://doi.org/10.1016/j.ijbiomac.2019.11.094
46. Ishaq U, Akram MS, Iqbal Z, Rafiq M (2015) Production and characterization of novel self-assembling biosurfactants from *Aspergillus flavus*. J Appl Microbiol 119:1035–1045. https://doi.org/10.1111/jam.12929

47. Gul UD, Donmez G (2012) Effects of dodecyl trimethyl ammonium bromide surfactant on decolorization of Remazol Blue by a living *Aspergillus versicolor* strain. J Surfactants Deterg 15:797–803. https://doi.org/10.1007/s11743-012-1377-5
48. da Silva ME, Duvoisin S Jr, Oliveira RL, dos Banhos EF (2019) Biosurfactant production of *Piper hispidum* endophytic fungi. J Appl Microbiol. https://doi.org/10.1111/jam.14398
49. Seghal Kiran G, Thajuddin N, Hema TA, Idhayadhulla A (2010) Optimization and characterization of rhamnolipid biosurfactant from sponge associated marine fungi *Aspergillus* sp. MSF1. Desalin Water Treat 24:257–265. https://doi.org/10.5004/dwt.2010.1569
50. Velioglu Z, Urek RO (2015) Optimization of cultural conditions for biosurfactant production by *Pleurotus djamor* in solid state fermentation. J Biosci Bioeng 120:526–531. https://doi.org/10.1016/j.jbiosc.2015.03.007
51. Meneses DP, Gudina EJ, Fernandes F, Goncalves LR (2017) The yeast-like fungus *Aureobasidium thailandense* LB01 produces a new biosurfactant using olive oil mill wastewater as an inducer. Microbiol Res 204:40–47. https://doi.org/10.1016/j.micres.2017.07.004
52. Reis CB, Morandini LM, Bevilacqua CB, Bublitz F (2018) First report of the production of a potent biosurfactant with α, β-trehalose by *Fusarium fujikuroi* under optimized conditions of submerged fermentation. Braz J Microbiol 49:185–192. https://doi.org/10.1016/j.bjm.2018.04.004
53. Nalini S, Parthasarathi R (2018) Optimization of rhamnolipid biosurfactant production from *Serratia rubidaea* SNAU02 under solid-state fermentation and its biocontrol efficacy against Fusarium wilt of eggplant. Ann Agrar Sci 16:108–115. https://doi.org/10.1016/j.aasci.2017.11.002
54. Hema T, Kiran GS, Sajayyan A, Ravendran A (2019) Response surface optimization of a glycolipid biosurfactant produced by a sponge associated marine bacterium *Planococcus* sp. MMD26. Biocatal Agric Biotechnol 18:101071. https://doi.org/10.1016/j.bcab.2019.101071