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Optimization of the Cultivation Conditions of *Candida Catenulata* in Synthesizing Acidic Sophorolipid

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ABSTRACT

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The application of the agro-industrial waste as the feedstock helps to decrease the operational cost of the fermentation process. Soapstock is a by-product of the vegetable oil refinery and enriched with fatty acids including linoleic acid which has a high potential application in the production of biosurfactants. In this study, a dual carbon source system, including glucose and free fatty acids recovered from a sunflower soapstock, was used for the synthesis of sophorolipid (SL) by Candida catenulata. The production of SL showed a major dependence on the initial carbon sources and the concentration of urea as the nitrogen source. The inoculum size was another influential factor in the fermentation process. The optimization of these factors was evaluated by the one-factor-at-a-time and the response surface methodology (RSM). The one-factor-at-a-time approach gained the best SL productivity (Y_1) of about 52.08 mg L^{-1} h^{-1} and SL-to-biomass yield (Y_2) of 712 mg_{SL} g_{cell}^{-1} at the inoculum size of 4% vv^{-1}, 100 g L^{-1} of glucose, 80 g L^{-1} of soapstock, and 7.5 g L^{-1} of urea. While the RSM, due to considering interactional effects of the factors, obtained the best condition at 100 g L^{-1} of glucose, 100 g L^{-1} of the soapstock, 9.3 g L^{-1} of urea, and an inoculum size of 6.3% vv^{-1} with the Y_1 and Y_1 values of about 58.10 mg L^{-1} h^{-1} and 713 mg_{SL} g_{cell}^{-1}, respectively. The characterization of the produced SLs by the GC-MS analysis indicated that a di-acylated C16:1 acidic sophorolipid with an m/z ratio of 679 amu was the main product.

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1. Introduction

Sophorolipid (SL) is among useful surface active glycolipid molecules that consist of two main moieties of sophorose and fatty acid chains [1]. SLs are widely used to form a stable

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emulsion from immiscible liquids in cosmetics, food, pharmaceuticals, agriculture, and detergent industries [2]. The application of biosurfactants have some advantages in comparison to chemical surfactants in terms of lower toxicity and biodegradability. However, a main drawback to developing the applications of SL is its high production cost in comparison with chemical surfactants [3].

The use of waste resources like food industrial wastes as the feedstock of the fermentation process could help to overcome this problem [4]. Previous studies showed the production of SLs modified using hydrophobic substances including the oil refinery wastes, residual oil cake, and oily sludge as the carbon source of the SL fermentation processes [5, 6]. Soapstock is a by-product of the neutralization step of refining vegetable oils. Soapstock is generated at a rate of about 6% of the total mass of crude oils. It is riche in free fatty acids such as linoleic acid [7]. The presence of the high concentration of the mono-unsaturated fatty acid in soapstock progressed the production of SL and it enhanced the performance of biosurfactant-producing cells to gain a higher productivity in comparison with the hydrophilic carbon sources such as glucose or molasses [8-11]. However, the recent findings in the application of soapstock showed that the presence of impurities such as hydratable and non-hydratable phosphates in raw soapstock decreased the performance of the production of SL due to the limitation of the accessibility of cells to free fatty acids in the culture [12].

In this work, the production of SL was studied with the use of a sunflower soapstock as the hydrophobic carbon source in the batch cultivation of *Candida catenulata* after the removal of the phosphate impurities by applying a degumming treatment. To the best of our knowledge, the optimization of the production of SL by using sunflower soapstock as the hydrophobic carbon source is not reported in literatures. It is crucial because the extra usage of soapstock results in the mixing problems in the medium during fermentation and also increases the difficulty of downstream processes for the separation and purification of the product. So, the cultivation conditions including the initial concentration of glucose (X_1), initial concentration of soapstock (X_2), initial concentration of urea (X_3), and size of the inoculum (X_4) were examined to simultaneously achieve a high SL productivity (Y_1) and the SL-to-biomass yield (Y_2). Due to the importance of interactional effects between these parameters in the fermentation process, the one-factor-at-a-time approach and the response surface methodology (RSM) as the optimization strategies were used and compared in the production of SL. The characterization of the produced SL by high-the pressure liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS) analysis were performed.

2. Material and methods

2.1. Raw sunflower soapstock and degumming process

Raw sunflower soapstock was obtained from Nazgol Oil Factory (Kermanshah, Iran). In order to removal of hydratable and non-hydratable gums, a degumming process which is presented in Figure 1, was performed on the raw soapstock [12]: Initially, 1 kg of soapstock was mixed with 600 mL of thermostat distilled water at 60 °C and stirred for 1 h at 150 rpm. Then, 155 mL of the sulfuric acid solution (37 w%) was added to the mixture and vigorously mixed for 30 min. After that, the mixture was transferred to a settling tank with a temperature of 55 °C and maintained for 5 h. The upper phase which contains FFAs is washed with hot water until the complete removal of sulfuric acid. Then an equal volume of n-hexane was

added to FFAs. The solution was vigorously mixed for 5 min. After that, it was centrifuged at 6000 rpm for 15 min. The n-hexane was completely removed from the supernatant using the rotary vacuum evaporation. The free fatty acid composition after the degumming process was as follows (w%): Myristic acid, 0.69; Palmitic acid, 4.96; Stearic acid, 2.52; Oleic acid, 65.00; Linoleic acid, 16.77; and Linolenic acid, 5.68; Arachidonic acid, 0.79; Gadoleic acid, 1.52.

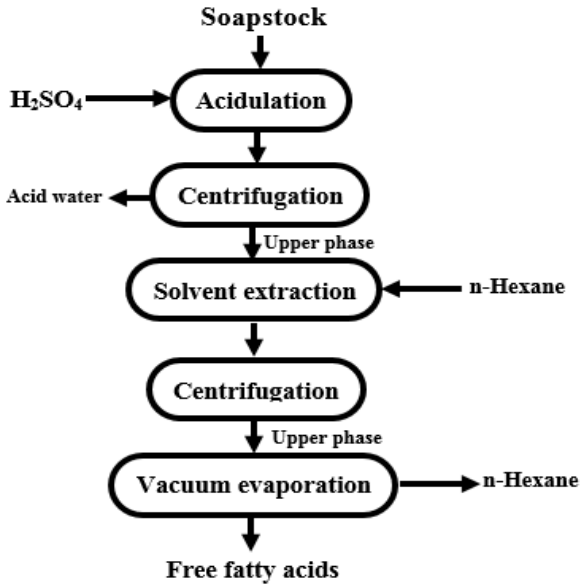

Figure 1. Fluid diagram of the degumming treatment performed on sunflower soapstock.

2.2. Microorganism and cultivation

Candida catenulata (accession number KP324968) was obtained from the Faculty of Agricultural Engineering at Razi University. The strain was cultivated on potato dextrose agar at 30 °C for 24 h. Then, the cell was transferred to a nutrient broth (NB) medium and cultivation was followed for 48 h in a rotary shaker at 150 rpm at 30 °C.

2.3. Production of SL

In each experiment, appropriate amounts of glucose and soapstock were added to 90 mL of a mineral solution with the following ingredients (g L^{-1}): $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 1; and K_2HPO_4, 1. All experiments were performed in a 250 mL Erlenmeyer flask. Prior to sterilization, the pH of the solution was set at 5.5 by 2 N NaOH or HCL solutions [7]. The solution was sterilized in an autoclave at 121℃ for 20 min. Based on previous works, urea was selected as the nitrogen source [8]. To prevent the Maillard reaction, the urea solution was separately autoclaved and added to the medium. Inoculation was performed by the 48-h grown culture of *C. catenulata* cells on the NB medium with a population density of 6.2×10^6 cfu mL^{-1}. The cultivation was continued for 144 h in a rotary shaker at 150 rpm at 30 °C.

2.3.1. Optimization by the one-factor-at-a-time approach

In experiments with a one-factor-at-a-time approach, one of the studied factors was varied at each evaluation, and other variables were kept constant. The effect of the initial concentration of glucose was evaluated at 50, 100, and 150 g L^{-1} when those of soapstock and urea were respectively at 80 g L^{-1} and 7.5 g L^{-1}. At the next step, three initial concentrations of 60, 80, and 100 g L^{-1} of soapstock were examined in the presence of 100 g L^{-1} of glucose and 7.5 g L^{-1} of urea. The initial concentrations of 2.5, 5.0, 7.5, and 10.0 g L^{-1} of urea were studied when those of glucose and soapstock were 100 g L^{-1} and 80 g L^{-1} respectively. In all experiments on the concentrations of glucose, soapstock, and urea, the inoculation was performed by 4% vv^{-1} of the 48-h grown culture with a cell population density of 6.2×10^6 cfu mL^{-1}. To study the effects of the initial concentration of the biomass, the inoculations with 2, 4, 6, and 8% vv^{-1} were performed. In these experiments, the concentrations of glucose, soapstock, and urea were 100, 80, and 7.5 g L^{-1} respectively. All experiments with the one-factor-at-a-time approach were performed in triplicates and the

mean value of observations ± error was reported.

2.3.2. Optimization by the RSM

The RSM was used to statistically determine the interactional effects of the test factors including the initial concentration of glucose (X_1), initial concentration of soapstock (X_2), initial concentration of urea (X_3), and size of the inoculum (X_4) on the SL productivity (Y_1) and the SL-to-biomass yield (Y_2). The design of experiments was performed according to the Box-Behnken design. A total of 30 experiments presented in Table 1 were conducted. Each factor was investigated at three levels which are coded to -1 (low), 0 (medium), and +1 (high). The actual levels of the test factors were selected based on the results obtained in the one-factor-at-a-time approach. To convert the actual factor level to the coded factor level, the following equation was used:

$$X = \frac{2\,x_i\,(x_{high} - x_{low})}{x_{high} + x_{low}} \quad (1)$$

where X and x are coded and actual values respectively.

The central point of the design (0, 0, 0) was performed in triplicates to estimate the pure error. The software of *Design-Expert* (Version 11) was used for the experimental design and data analysis. The following expression used to show the dependency of the responses on variables:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1X_2 + \beta_{13} X_1X_3 + \beta_{14} X_1X_4 + \beta_{23} X_2X_3 + \beta_{24} X_2X_4 \beta_{34} X_3X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 \quad (2)$$

In this model, β_0 is a mean of observations at the center points. The β_1, β_2, β_3, and β_4 are the main effects of parameters. The β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} represent the interactions of the variables, and β_{11}, β_{22}, β_{33}, and β_{44} show the quadratic effects of each factor.

2.4. Analytical methods

At the end of each experiment, the suspended cells were separated from the culture by the centrifugation at 6000 rpm for 10 min. The sedimented cells were washed with the NaCl solution (10 w%) and then dried in an oven overnight at 100 °C. The dry cell weight was measured and divided into the cultivation volume to obtain the concentration of the biomass. To isolate the SL, the supernatant was vigorously mixed with ethyl acetate at an equal volume. Settling the mixture at 4 °C for 24 h and after that the ethyl acetate phase was transferred to a rotary vacuum evaporator. The residue of the vacuum evaporator was washed twice with n-hexane to remove the residual fatty acids from SL. The SL was dried at 40 °C in a vacuum oven. The SL productivity (Y_1) and the SL-to-biomass yield (Y_2) were determined as follows:

$$Y_1(mg\ L^{-1}h^{-1}) = \frac{SL_f - SL_i}{t} \quad (3)$$

$$Y_2(mg_{SL}\ g_{cell}^{-1}) = \frac{SL_f - SL_i}{X_f - X_i} \quad (4)$$

where SL and X are the concentrations of SL and DCW respectively. The subscripts of f and I denote the final and initial values. t is the duration of cultivation.

The residual fatty acids were gravimetrically determined after the removal of n-hexane in a rotary evaporator (60 °C). The residual glucose was spectrophotometrically measured at 540 nm after reacting with 3, 5 dinitro-salicylic acid according to the standard method [9].

The quality of the produced SL was characterized by HPLC and LC-MS analyses. The HPLC system was an Agilent HPLC 1200 equipped with a Kromasil column (100-5-C_{18}). The mobile phase consisted of acetonitrile and water (8:2 vv^{-1}) with a flow rate of 0.5 mL min^{-1} and the eluent was monitored with a UV

detector at 207 nm [10]. The LC-MS analysis was also carried out by an Agilent 1200 series LC system equipped with an Agilent 6410 Triple Quadrupole mass spectrometer. Nitrogen at 300 °C with a rate of 10 L min^{-1} was used as the nebulizer gas. Ionization at the negative mode was applied by the electrospray ionization at 4,000 V [11].

3. Results and Discussion

3.1. Optimization by the one-factor-at-a-time approach

In this study, the raw soapstock obtained from the refinery process of sunflower oil was treated for the removal of hydratable and not-hydratable gums and then used as the hydrophobic carbon source for the production of SL by *C. catenulata*. Several works showed that the production of sophorolipid was modified in the presence of both hydrophilic and hydrophobic carbon sources in the cultivation medium. For instance, the production of SL by *Candida bombicola* was strongly affected by the use of different lipids as the second carbon source [13]. The study showed that the production yields of sophorolipid were 0.84, 0.20, and 0.03 g per gram of hexadecane, soybean oil, and glucose respectively [13]. Further characterization of the produced sophorolipids also indicated that the SL was mostly in the acidic form in the presence of glucose however the application of soybean oil and hexadecane resulted in both acidic and lactone forms [13]. These findings confirm that the produced sophorolipids depending on the presence of nutrients such as the type of carbon and nitrogen sources in the culture are different [14]. In a dual carbon source system, the concentration of each source and the proportion of the sources has affected the performance of the cells in the production of SL. The effect of the initial concentration of glucose was evaluated at 50, 100, and 150 g L^{-1} when the concentration of soapstock was 80 g L^{-1} and the results are presented in Figure 2a. The increase of the concentration of glucose from 50 to 100 g L^{-1}, slightly increased the Y_1 from 50.83 g L^{-1} h^{-1} to 52 g L^{-1} h^{-1}. While the application of higher concentration of glucose decreased the Y_1 to 48.86 mg L^{-1} h^{-1} by *C. catenulata* cells. The SL-to-biomass yield (Y_2) showed a similar trend and the highest Y_2 was about 712.25 mg_{SL} g_{cell}^{-1} obtained at 100 g L^{-1} of the initial concentration of glucose. It is mainly due to the inhibitory role of glucose at a high concentration which negatively affected the production of SL by the yeast cells. [15]. The effects of the initial concentration of soapstock were studied at 60, 80, and 100 g L^{-1} when the initial concentration of glucose was 100 g L^{-1}. It can be seen in Figure 2b, an increase in the concentration of soapstock from 60 to 80 g L^{-1} increased the Y_1 from 49.65 g L^{-1} h^{-1} to 52 g L^{-1} h^{-1}. Further increase of up to 100 g L^{-1} in the concentration of soapstock resulted in a decrease in the Y_1. This finding was consistent with the previous report [16] that showed an increase in the concentration of oleic acid up to 100 g L^{-1} increased the production of SL by *Starmerella bombicola* NRRL Y-17069 while a further increase in the concentration of the substrate decreased the production of SL. This behavior is mainly due to the limitation of the oxygen mass transfer in the culture by increasing the viscosity of the culture at a high concentration of soapstock [15].

Candida genera are able to use organic and inorganic nitrogen sources; however the type and concentration of nitrogen source is a very crucial factor in the fermentation process. Previous works showed that nitrogen limitation is a dominant condition for the synthesis of SL [17, 18]. The effects of nitrogen sources on the growth of sophorolipid-producing yeast, and the effects of *Wickerhamiella domercqiae* on the production and composition of sophorolipids

were studied. Organic nitrogen sources are more favorable for the accumulation of biomass than inorganic ones. The presence of ammonium ions from different inorganic nitrogen sources (except NH_4HCO_3) greatly inhibited the production of lactonic sophorolipids. However, when organic nitrogen sources were used, the production of lactonic sophorolipid was strongly increased. The production of crystalline lactonic sophorolipids from organic/inorganic nitrogen sources was enhanced with the increase in the pH value adjusted by sodium hydroxide or sodium citrate solution [17]. In this study, urea as a cheap nitrogen source in comparison with yeast extract was examined and the results are presented in Figure 2c. With an increase in the concentration of urea from 2.5 g L^{-1} to 10 g L^{-1}, the concentration of biomass was enhanced. It indicated that nitrogen was the growth-limiting nutrient in the medium [19, 20]. Figure 2c represents the dependence of Y_1 and Y_2 on the concentration of urea. The largest value of about 52 g L^{-1} h^{-1} of Y_1 was observed at the concentration of urea of 7.5 g L^{-1}.

To optimize the concentration of the inoculum, different inoculum sizes ranging from 2 to 8 %vv^{-1} have been evaluated and results are presented in Figure 2d. No significant difference in the amount of Y_1 and Y_2 was observed at the inoculum sizes of higher than 4 % vv^{-1}. The results are consistent with previous findings showing that an increase in the inoculum size from 2.5% to 10% did not affect the production of SL by *Starmerella bombicola* NRRL Y-17069 and *Candida bombicola* [16, 21]. In fact, a high cell concentration negatively affected the production of SL by reducing the dissolved oxygen in the medium.

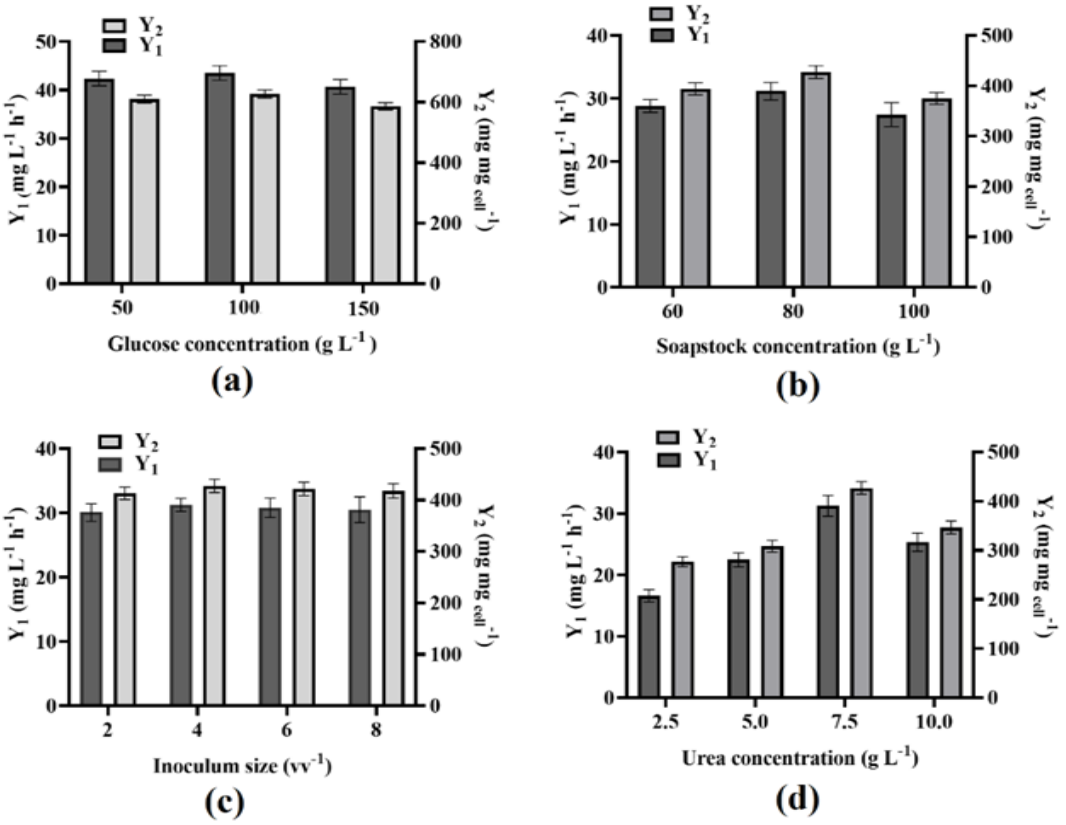

Figure 2. Effects of the cultivation conditions on Y_1 and Y_2. (a) Effect of the initial concentration of glucose, (b) the effect of the initial concentration of soapstock, (c) the effect of the initial concentration of urea, and (d) effect of the inoculum size (Experiments were carried out at 30 °C and 150 rpm for 144 h).

3.2. Optimization by the RSM

The best cultivation conditions which were obtained by the one-factor-at-a-time approach did not consider the interactional effects between the test factors. The RSM is equipped with useful tools that account for the interactions with the lowest experimental effort, because this approach used a special design of experiments for a statistical evaluation of the factors. The other advantages of RSM are the polynomial regression analysis for modeling of the response behavior, and the numerical optimization. The design of experiments according to the Box-Behnken design is presented in Table 1. The experiments were carried out and the results of the Y_1 and Y_2 are shown in Table 1. The value of Y_1 was changed from 26.16 mg L^{-1} h^{-1} at experiment # 2 to 60.36 mg L^{-1} h^{-1} at experiment # 3. However the Y_2 was shifted from 420 mg g_{cell}^{-1} at experiment # 1 to 713 mg g_{cell}^{-1} at experiment # 16. The cultivation conditions at experiments #3 and #7 differed from the best conditions obtained by the one-factor-at-a-time approach. The experimental data were fitted to Eq. (2), and the coefficients of the model were determined by the multi-non-linear regression analysis as follows:

$$Y_1\,(\text{mg L}^{-1}\,\text{h}^{-1}) = 57.40 + 1.89\,X_1 + 5.73\,X_2 + 3.77\,X_3 + 0.76\,X_4 + 2.48\,X_1X_2 + 1.66\,X_1X_3 - 19.51\,X_1^2 - 4.78\,X_2^2 - 1.44\,X_3^2 - 0.60\,X_4^2 \quad (5)$$

$$Y_2\,(\text{mg}_{SL}\,g_{cell}^{-1}) = 617.83 + 32.92\,X_1 + 38.67\,X_2 + 29.33\,X_3 + 8.25\,X_4 - 11.25\,X_1X_2 - 7.50\,X_1X_3 - 125.25\,X_1^2 + 8.13\,X_2^2 + 8.87\,X_3^2 + 6.50X_4^2 \quad (6)$$

The significance of all coefficients in the developed models was determined by the analysis of variance (ANOVA) test at a confidence level of 0.95 (Table 2). The coefficients of β_{14}, β_{23}, β_{24}, and β_{34} were not significant in describing the behavior of both Y_1 and Y_2 and thus removed from Eq. (5) and Eq. (6). The *P*-values of Eq. (5) and Eq. (6) were lower than 0.0001 indicating the models were highly significant and accurate. The estimated coefficient and the corresponding *P*-values confirmed that the test variables played significant roles in the responses. The low CV of the models revealed the reliability of the models. The determination coefficients (R^2) for Y_1 and Y_2 were respectively 0.994 and 0.993 which indicate that the models could describe the variability of the responses. The values of Pred-R^2 confirm a perfect agreement between the experimental data and predicted values of the responses. Also, the values of adequate precision for the models were higher than 4, representing a perfect fit. Figure 3 shows the parity plots comparing the experimental data and relevant predicted values by the developed models. A good agreement between the data and predicted values was observed in Figure 3 for both responses.

Table 1
Experimental design by the RSM and obtained results for the production of SL by *C. catenulata.* Parentheses show the relevant coded values of the factor levels.

Experiment No.	X_1 (g L^{-1})	X_2 (g L^{-1})	X_3 (g L^{-1})	X_4 (% vv^{-1})	Y_1 (mg L^{-1} h^{-1})	Y_2 ($mg_{SL}g_{cell}^{-1}$)
1	50 (-1)	60 (-1)	7.5 (0)	5 (0)	28.41	420
2	150 (+1)	60 (-1)	7.5 (0)	5 (0)	26.16	499
3	50 (-1)	80 (0)	7.5 (0)	5 (0)	35.88	517
4	150 (+1)	100 (+1)	7.5 (0)	5 (0)	43.55	551
5	100 (0)	80 (0)	5.0 (-1)	2 (-1)	50.64	595
6	100 (0)	80 (0)	10.0 (1)	2 (-1)	59.76	655
7	100 (0)	80 (0)	5.0 (-1)	8 (+1)	52.21	605
8	100 (0)	80 (0)	10.0 (+1)	8 (+1)	60.36	662
9	50 (-1)	80 (0)	7.5 (0)	2 (-1)	33.86	447
10	150 (+1)	80 (0)	7.5 (0)	2 (-1)	38.24	531
11	50 (-1)	80 (0)	7.5 (0)	8 (+1)	35.09	485
12	150 (+1)	80 (0)	7.5 (0)	8 (+1)	40.71	541
13	100 (0)	60 (-1)	5.0 (-1)	5 (0)	42.31	569
14	100 (0)	60 (-1)	5.0 (-1)	5 (0)	54.05	646
15	100 (0)	60 (-1)	10.0 (+1)	5 (0)	49.02	619
16	100 (0)	100 (+1)	10.0 (+1)	5 (0)	58.10	713
17	50 (-1)	80 (0)	5.0 (-1)	5 (0)	31.91	431
18	150 (1)	80 (0)	5.0 (-1)	5 (0)	32.22	517
19	50 (-1)	80 (0)	10.0 (+1)	5 (0)	37.21	505
20	150 (+1)	80 (0)	10.0 (+1)	5 (0)	44.19	561
21	100 (0)	60 (-1)	7.5 (0)	2 (-1)	45.29	586
22	100 (0)	100 (+1)	7.5 (0)	2 (-1)	56.98	666
23	100 (0)	60 (-1)	7.5 (0)	8 (+1)	47.07	611
24	100 (0)	100 (+1)	7.5 (0)	8 (+1)	58.45	675
25	100 (0)	80 (0)	7.5 (0)	5 (0)	57.91	621
26	100 (0)	80 (0)	7.5 (0)	5 (0)	58.29	625
27	100 (0)	80 (0)	7.5 (0)	5 (0)	57.11	621
28	100 (0)	80 (0)	7.5 (0)	5 (0)	56.66	612
29	100 (0)	80 (0)	7.5 (0)	5 (0)	57.93	612
30	100 (0)	80 (0)	7.5 (0)	5 (0)	56.51	616

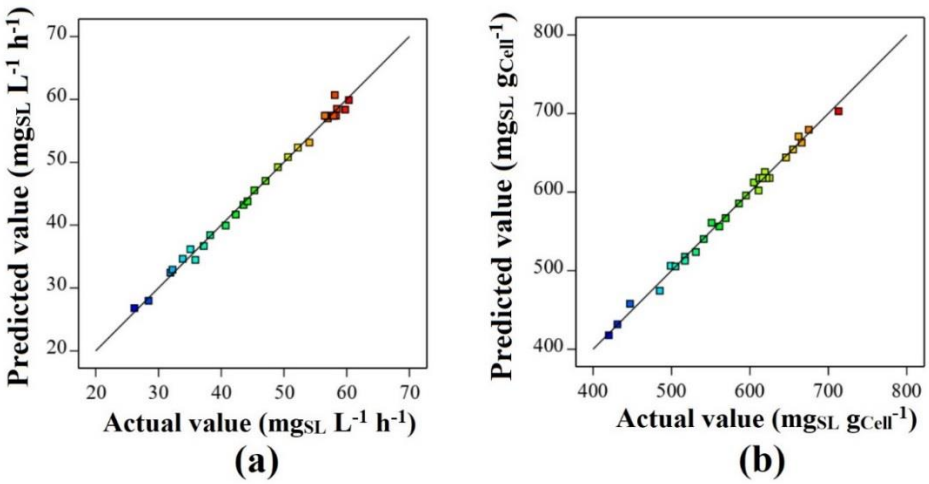

Figure 3. Comparison of the experimental data and relevant prediction values of (a)Y_1 and (b) Y_2 by the RSM.

Table 2
ANOVA of the developed models for Y_1 and Y_2.

Source	Eq. (5)				Eq. (6)			
	SS	**Df**	***F*-value**	***P*-value**	**SS**	**Df**	***F*-value**	***P*-value**
Model	3341.59	10	329.96	< 0.0001	1.615E+05	10	300.77	< 0.0001
X_1	43.03	1	42.49	< 0.0001	13002.08	1	242.10	< 0.0001
X_2	393.63	1	388.69	< 0.0001	17941.33	1	334.06	< 0.0001
X_3	170.86	1	168.72	< 0.0001	10325.33	1	192.26	< 0.0001
X_4	6.94	1	6.85	0.0170	816.75	1	15.21	0.0010
X_1X_2	24.57	1	24.26	< 0.0001	506.25	1	9.43	0.0063
X_1X_3	11.03	1	10.89	0.0038	225.00	1	4.19	0.0548
X_1^2	2609.07	1	2576.29	< 0.0001	1.076E+05	1	2002.97	< 0.0001
X_2^2	156.56	1	154.59	< 0.0001	452.68	1	8.43	0.0091
X_3^2	14.15	1	13.97	0.0014	540.11	1	10.06	0.0050
X_4^2	2.52	1	2.49	0.1312	289.71	1	5.39	0.0315
Residual	19.24	19			1020.42	19		
Lack of fit	16.51	14	2.16	0.2032	877.58	14	2.19	0.1975
Pure error	2.73	5			142.83	5		
Total	3360.83	29			1.626E+05	29		
R^2	0.994				0.993			
Adjusted R^2	0.991				0.990			
Predicted R^2	0.983				0.982			
Mean	46.87				577.13			
SD	1.01				7.33			
CV%	2.15				1.27			
Adeq. Precision	55.602				64.214			

The confidence of β_2 was the biggest coefficient in Eq. (5) which indicates the initial concentration of soapstock was the most influential factor on the Y_1. After the concentration of soapstock (47.16%), concentration of urea (31.03%), concentration of glucose (15.56%), and size of ther inoculum (6.25%) were influential on the Y_1. Another note in Eq. (5) is the significance of the coefficients that showed the interactions of the variables. The coefficient of β_{12} was the biggest interactional effect, which indicates the interaction of glucose and the concentration of soapstock is more important in comparison with another significant interaction (β_{13}). A comparison between Eq. (5) and Eq. (6) showed that the most influential factor on Y_2 was also the initial concentration of soapstock (β_2 = 38.67). The β_{12} which represents the interaction of glucose and the concentration of soapstock is the biggest international confidence in Eq. (6).

To illustrate the interactions between the test factors, the surface plots of Figure 4 are presented. According to the ANOVA in Table 2, the X_1X_2 and X_1X_3 were the only significant interactional confidence in describing behaviors of Eq. (5) and Eq. (6). Thus, the interactions are illustrated in Figure 4. The results indicate that both responses had similar trends by the variation of the test factors. The dependency of Y_1 as a function of X_1 and X_2 is shown in Figure 4a. The effect of initial concentration of glucose on Y_1 is dependent on the concentration of soapstock and thus the interaction is important in the bioprocess. The interaction between the factors is defined as the dependency of the changes of one factor on other factors. Therefore, the changes in the concentration of glucose result in different

shifts in the productivity with the variation of soapstock in this study. The Y_1 increases with an increase in the concentration from 50 to 100 mg L^{-1} and then decreases at further increase in the concentration of glucose for all concentrations of soapstock. A high concentration of glucose resulted in a higher viscosity of the medium. It limits the oxygen transfer in the culture, where the synthesis of SL as an aerobic metabolite needs access to oxygen. On the other hand, the lipase enzyme is among the key enzymatic systems participating in the synthesis of SL to perform esterification of hydroxy fatty acid methyl esters [22-24]. Previous work showed that a high concentration of glucose decreases the activity of this lipase [25]. A ratio of 1:1 of these hydrophobic and hydrophilic carbon sources showed a higher SL productivity in previous work [8]. *Candida* species could convert fatty acids to SL molecules at the following pathway: Firstly, the monooxygenase enzyme converted fatty acids to hydroxy fatty acids. In the next step, glucose is coupled to the hydroxyl group of fatty acids through the action of one glycosyltransferase, and finally, the second glucose is glucosidal linked to the C_2 position of the first part of glucose by glycosyltransferase [26]. With the usage of soapstock as a hydrophobic carbon source, the fatty acids could be directly converted to the SL by the above-described pathway. In Figure 4b, the Y_1 was increased by elevating the concentration of glucose ranging from 50 to 100 g L^{-1} and the concentration of 5 to 10 g L^{-1} of urea. Choosing an appropriate nitrogen source is important for both the cell growth and production of SL. Many references have reported that the production of sophorolipids was initiated by the depletion or limitation of nitrogen sources in the medium [14, 17, 27]. In this study, urea was used as the main nitrogen source for the production of SL. When the initial concentration of urea was increased up to 10 g L^{-1}, the production rate of SL increased as well. It is in agreement with previous findings [19].

In Figure 4c, the SL-to-biomass yield (Y_2) was increased with an increase in the concentration of glucose ranging from 50 to 100 g L^{-1} and the concentration of soapstock of 60 to 100 g L^{-1}. Glucose as a hydrophilic carbon source was consumed from the culture faster than soapstock. Therefore, glucose as a growth support substrate had an influential effect on the growth of the cells however free fatty acids in soapstock as a hydrophobic carbon source were mainly consumed after the depletion of the concentration of glucose. Glucose concentration above 100 g L^{-1} damped the Y_2. It is due to the increase in the cell population which results in oxygen limitation in the culture. In Figure 4d, the Y_2 was increased when the initial concentration of urea and concentration of glucose were 10 g L^{-1} and 100 g L^{-1} respectively. In this case, although a high concentration of urea led to producing a high amount of yeast cells in the medium, at the concentrations of above 100 g L^{-1} of glucose the specific production rate of SL sharply decreased based on the reasons mentioned before.

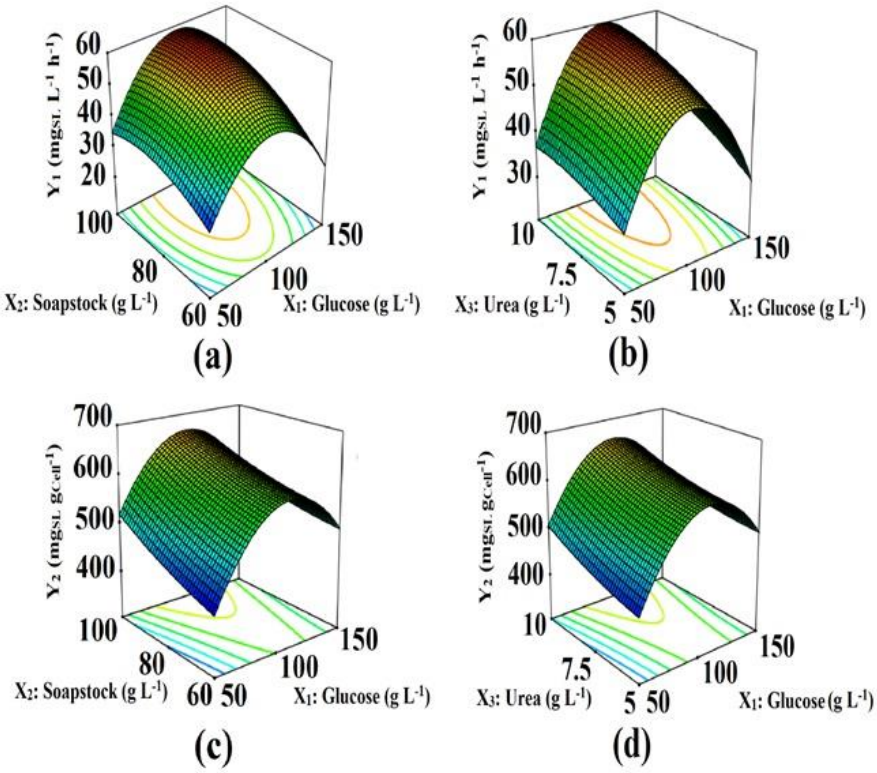

Figure 4. Surface plots show the dependency of responses on operational factors. (a) Y_1 as a function of X_1 and X_2, (b) Y_1 as a function of X_1 and X_2, (c) Y_2 as a function of X_1 and X_2, and (d) Y_2 as a function of X_1 and X_3.

To obtain an optimal setting of the test factors, the desirability function available in the *Design Expert* software is used. The achievement to a simultaneous maximum values of Y_1 and Y_2 was selected as the optimization criterium. Generally, the desirability function shifted from zero to one. A value of one indicates an ideal state to achieve the optimization criteria and the value of zero indicates that there is no achievement of a suitable response within the specified interval. The dependency of the overall desirability on the test factors is presented in Figure 5. The desirability function predicted that at an optimal setting of the initial concentration of 103.4 g L^{-1} of glucose, initial concentration of 98.7 g L^{-1} of soapstock, initial concentration of 9.93 g L^{-1} of urea, and a size of 6.3 % vv^{-1} of the inoculum simultaneous maximum values of Y_1 = 59.18 mg L^{-1} h^{-1} and Y_2 = 709.52 mg_{SL} g_{cell}^{-1} could be obtained. These cultivation conditions were experimentally evaluated and Y_1 = 59.10 mg L^{-1} h^{-1} and Y_2 = 713 mg_{SL} g_{cell}^{-1} were obtained. In comparison with these results, previously the production rate of about 45 mg L^{-1} h^{-1} of SL by *Starmerella bombicola*, a commercial SL producer strain, was reported using 100 g L^{-1} of glucose and 100 g L^{-1} of waste cooking oil as the carbon sources [28]. In order to reduce the peroxide value in waste cooking oil, the waste was treated by an activated earth to reach the peroxide value of less than 5 meq kg^{-1} [28]. Also, *Candida batistae* CBS 8550 was cultivated on a medium containing 50 g L^{-1} of glucose and 50 g L^{-1} of olive oil as the carbon sources to produce acid-forms of SLs at 83 mg L^{-1} h^{-1} [29]. The SL synthesized from glycerol as a cost-effective carbon source for *S. bombicola* ATCC 22214 was studied and

found an optimal concentration of 150 g L^{-1} of glycerol with 100 g L^{-1} of the refined sunflower oil yielded an SL productivity of about 32 mg L^{-1} h^{-1} [30].

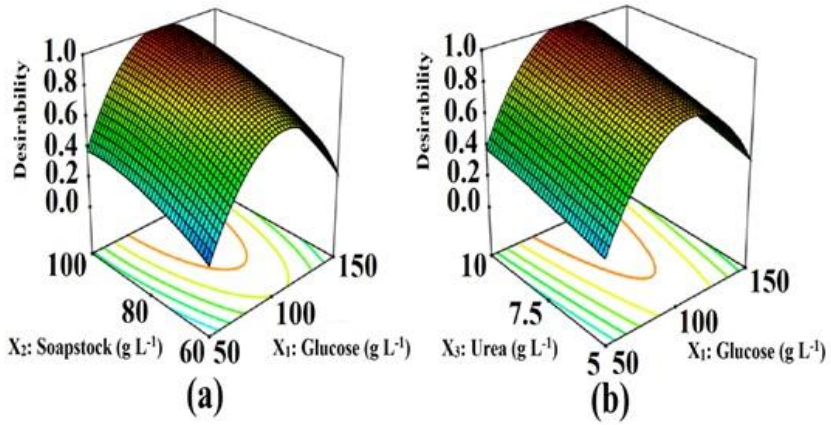

Figure 5. Dependency of the overall desirability to the level of the test factors in the optimization of the production of SL. (a) Dependency of the desirability on X_1 and X_2, and (b) dependency of the desirability on X_1 and X_3.

3.3. Characterization of the produced SL

Sophorolipid produced by *C. catenulata* at the optimum conditions were analyzed by FTIR, HPLC and LC-MS analyses. The FTIR spectrum of the produced SL is presented at Figure 6.. The FTIR spectrum shows a pick at 3414 cm^{-1}, which corresponds to O-H bonds, typically present in the fatty acid moiety of SL molecules [31]. The picks at 1638 cm^{-1} indicate the unsaturated carbon-carbon (C=C) bonds [31]. The peaks at 1440 cm^{-1} correspond to the C–O–H plane bending of carboxylic acid (COOH). The indicated band appearing at 1733 cm^{-1} ascribed to the carbonyl group (C=O) has not been observed in the present study. It indicates the lactonic sophorolipids are not produced in this study [12].

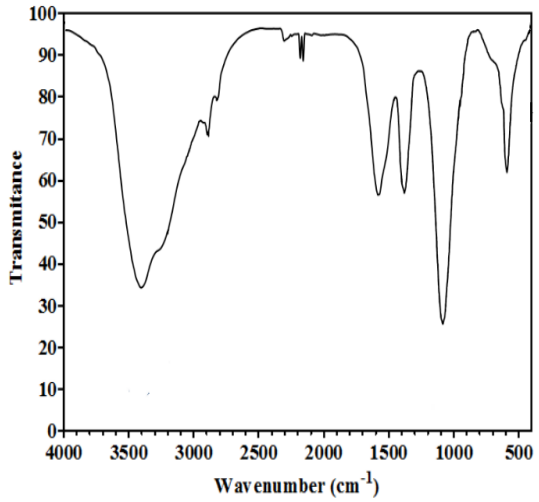

Figure 6. FTIR spectrum of the produced SL.

The results of HPLC and LC-MS analyses are presented in Figure 7. In Figure 7a, the HPLC chromatogram showed a main pick eluted at 5.85 min. The peaks with a retention time ranging from 2 to 4 min, showed the presence of solvents and impurities because the isolated SL without further purification was injected into the HPLC column. Further identification of the main produced SL eluted at 5.85 min was carried out by the LC-MS analysis. The ESI mass spectrum in Figure 7b confirmed the presence of di-acylated C16:1 acidic

sophorolipid anions with an m/z ratio of 679.1 amu. The indentifing fragment in SL structures which demonstrats the position of C=C bond was observed at the m/z of 142.1 amu. Also, it can be seen that the fraction of hexadecanoic acid with a single double bond (C16:1) was revealed at the m/z of 254.2. The fractions of sophorose with 2 acetyl groups were obtained at the m/z of 427.2. The result confirms the production of an acidic SL by *C. catenulata* in this study. In comparison with our work, the analysis of the SLs produced by *C. bombicola* showed an acidic SL [15-L-(2'-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl] oxy)-hexadecanoic acid 6', 6" diacetate] at the same retention time [15]. Previous studies indicated that the acidic SLs due to their lower hydrophobicity in comparison with lactonic SLs eluted from the HPLC column at a lower retention time than 6 min [15]. In agreement with this finding, Figure 7a showed lactonic forms were not produced in this study.

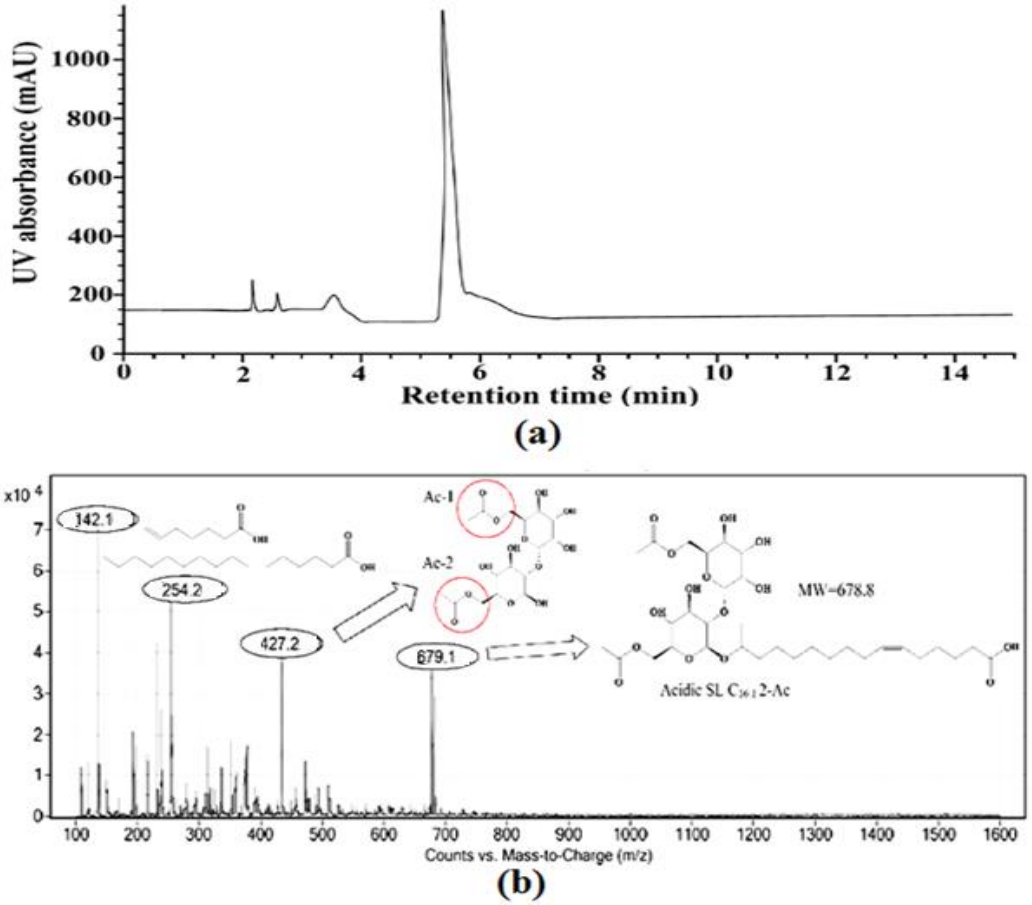

Figure 7. Characterization of the SL produced by *C. catenulata* under the optimum conditions determined by the RSM. (a) PHLC analysis and (b) LC-MS chromatogram of the eluted pick at 5.85 min.

4. Conclusion

In the present study, the culture conditions were optimized by considering the initial concentrations of glucose, degummed sunflower soapstock, urea, and inoculation size by two approaches of the one factor at time and RSM. The RSM due to the consideration of the interactional effects between the variables, predicted a better optimal condition in comparison with the one-factor-at-a-time approach where an 11.6% enhancement in the SL productivity (Y_1) was obtained at the optimal setting of the variables by the RSM. It indicates that the interactions between the parameters of the fermentation process has a crucial impact on the

performance of the living cells, and thus they must be considered in the optimization of the process. However, the other response in the system (Y_2) was similar in both optimization approaches (the best Y_2 was about 713 mg_{SL} g_{cell}^{-1} in RSM and one-factor-at-a-time approaches). The analysis of the produced SL showed an acidic deacetylation sophorolipid molecule with the fatty acid moiety of C16:1 as the main product derived from the degummed sunflower soapstock.

Authors' Contributions: This work was carried out in collaboration between all authors. **Fariba Amiri** gathered the initial data, managed the literature searches, and produced the initial manuscript. **Alireza Habibi** anchored the field study, interpreted the data, and revised the manuscript. **Mohammad Mehdi Nourouzpour** helped with the interpretation of data.

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