

Article

Bioactivity of a Novel Glycolipid Produced by a Halophilic *Buttiauxella* sp. and Improving Submerged Fermentation Using a Response Surface Method

Abdolrazagh Marzban ¹, Gholamhossein Ebrahimipour ² and Abolghasem Danesh ^{1,*}

¹ Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad 91775-1365, Iran; marzban86@gmail.com

² Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Tehran 19839-4716, Iran; G-ebrahimi@sbu.ac.ir

* Correspondence: DaneshA@mums.ac.ir; Tel.: +98-51-3882-3255; Fax: +98-51-3882-3251

Academic Editor: Derek J. McPhee

Received: 29 August 2016; Accepted: 15 September 2016; Published: 22 September 2016

Abstract: An antimicrobial glycolipid biosurfactant (GBS), extracted and identified from a marine bacterium, was studied to inhibit pathogenic microorganisms. Production of the GBS was optimized using a statistical method, a response surface method (RSM) with a central composite design (CCD) for obtaining maximum yields on a cost-effective substrate, molasses. The GBS-producing bacterium was identified as *Buttiauxella* Species in terms of biochemical and molecular characteristics. This compound showed a desirable antimicrobial activity against some pathogens such as *E. coli*, *Bacillus subtilis*, *Bacillus cereus*, *Candida albicans*, *Aspergillus niger*, *Salmonella enterica*. The rheological studies described the stability of the GBS at high values in a range of pH (7–8), temperature (20–60) and salinity (0%–3%). The statistical optimization of GBS fermentation was found to be pH 7, temperature 33 °C, Peptone 1%, NaCl 1% and molasses 1%. The potency of the GBS as an effective antimicrobial agent provides evidence for its use against food and human pathogens. Moreover, favorable production of the GBS in the presence of molasses as a cheap substrate and the feasibility of pilot scale fermentation using an RSM method could expand its uses in food, pharmaceutical products and oil industries.

Keywords: glycolipid biosurfactant; antimicrobial activity; *Buttiauxella*; response surface method

1. Introduction

Biosurfactants, which are synthesized by a variety of living organisms, are amphipathic molecules that have numerous applications in the food, oil, cosmetic and medical industries [1]. In recent years, they have drawn attention from many researchers for use as emulsifying agents in the food processing, oil recovery and pharmaceutical industries [2–4]. GBSs are classified into four categories: (i) Rhamnolipids produced by *pseudomonas* species [5]; (ii) Trehalose lipids released mainly from *rhodococci* [6]; (iii) Xylolipid synthesized by *lactobacilli* [7]; and (iv) Sophorolipids obtained from *candida* species [8].

Biosurfactants as a green alternative are preferred over synthetic surfactants owing to their lower toxicity, higher biodegradability and great stability at different physiochemical conditions [9]. The capability of biosurfactants to reduce surface tension and form stable emulsions is a virtue for countless applications [10]. Besides, biosurfactants have appeared to inhibit some pathogenic organisms. Several studies have suggested some potent biosurfactants with broad spectrum activity against human, plant and food pathogens [8,11,12]. They have attributed the antimicrobial and

anti-adhesive activities to the amphipathic nature of biosurfactants, especially the hydrophobic tail. In line with raising awareness about emerging antibiotic resistance among pathogens, biosurfactants may have surmounted the cumbersome exercises to discover new antimicrobial agents [13].

The present research intends to elucidate the structure of an antimicrobial glycolipid isolated from a marine halotolerant bacterium identified phylogenetically as a *Buttiauxella* species. Optimization of glycolipid production was also conducted by response surface method (RSM) in terms of antimicrobial activity of crude antimicrobial glycolipids under different cultural conditions. In addition, the surface active property, oil spreading efficiency and the emulsification activity of the GBS have been examined.

2. Results

2.1. Bacterial Characterization and Antimicrobial Studies

The isolated bacterium was identified as a halotolerant *Gamma Proteobacterium* belonging to the *Enterobacteriales* family. Biochemical and morphological examinations revealed to be *bacillus* (rod-shaped), gram-negative, motile, catalase-positive and oxidase-negative. The *Buttiauxella* species is one of the members of enterobacteriaceae that could be found in various habitats. The strain had 99% similarity to *Enterobacter* sp. as concluded via DNA BlastN in NCBI Genbank. Accordingly, the isolate was identified as *Buttiauxella* sp. M44 and the associated 16S rRNA sequence was deposited in Genbank with accession number KU350741.

The glycolipid BSs produced by *Buttiauxella* sp. M44 exhibited a different inhibitory effect on the test microorganisms (Table 1). Antimicrobial activity was highly potent against *C. albicans*, *A. niger* and then *E. coli*. A moderate potential was found to inhibit the growth of *S. enteric*, *B. subtilis*, *B. cereus* and *S. aureus* while no zone of inhibition was observed against *P. aeruginosa*.

Table 1. Antagonistic activity of glycolipid BS produced by *Buttiauxella* sp. M44.

Test Organism	MIC ($\mu\text{g/mL}$)	Disc Diffusion (mm)
<i>E. coli</i>	200	18.5 \pm 2.1
<i>S. enterica</i>	250	13.1 \pm 3.3
<i>C. albicans</i>	150	23.5 \pm 2.3
<i>A. niger</i>	100	26.2 \pm 2.7
<i>B. Subtilis</i>	300	14.41 \pm 3.2
<i>B. cereus</i>	250	10.60 \pm 4.5
<i>S. aureus</i>	450	5.8 \pm 1.7
<i>P. aeruginosa</i>	-	-

2.2. Compositional Characterization of by GC-MS

GC-MS analysis shows that a major part of the antimicrobial glycolipid produced by the bacterium is probably related to a glucose-derived sugar linked to a fatty acid moiety such as octadecanoic acid and 9-octadecenoic acid, while the remaining compounds comprise four derivative analogues with the only difference in their fatty acid moieties (Table 2). The GC chromatogram and the corresponding mass spectrum for each compound are presented in Figure 1a,b.

Table 2. Major fractions of GBS identified by GC-MS results.

Compound Name	Formula	Molecular Weight
Tetradecanoic acid methyl ester	C ₁₅ H ₃₀ O ₂	242
9-Hexadecenoic acid methyl ester	C ₁₇ H ₃₂ O ₂	268
Hexadecanoic acid methyl ester	C ₁₇ H ₃₄ O ₂	270
9-Octadecenoic acid methyl ester	C ₁₉ H ₃₆ O ₂	296
Octadecanoic acid methyl ester	C ₁₉ H ₃₈ O ₂	298
9,12-Octadecadienoic acid methyl ester	C ₁₉ H ₃₄ O ₂	294
D-Glucopyranose, 2, 3, 4, 5, 6 pentaacetate	C ₁₆ H ₂₂ O ₁₁	390

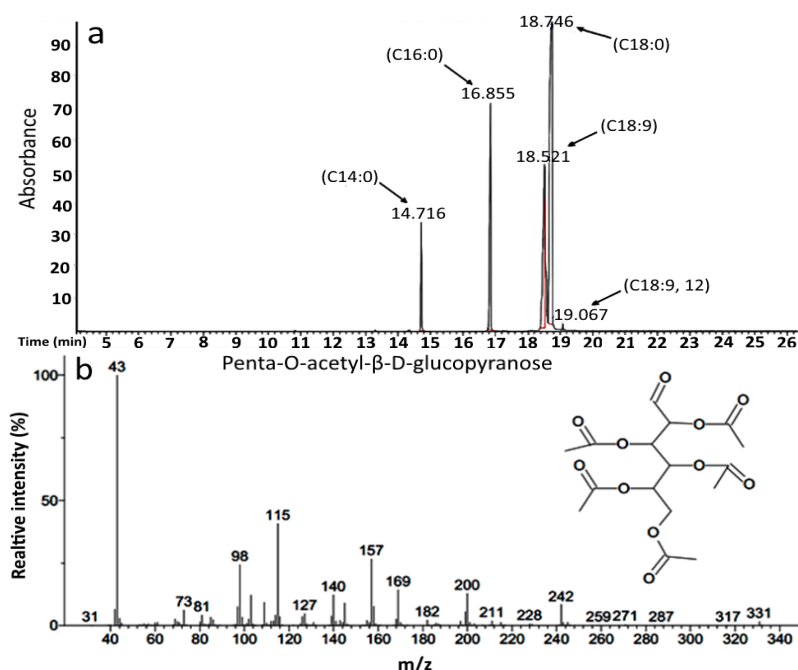


Figure 1. TIC profiles of gas chromatography (a) fatty acid portion showed at least 5 fatty acid derivatives and (b) glycosylic portion of glycolipid produced by *Buttiauxella* sp. M44.

2.3. Characterization of GBS by FT-IR

FT-IR spectrum displays a broad peak at 3435.42 cm^{-1} elucidating OH group (Figure 2). Two stretch signals at 2940.29 and 2877.33 could be associated with CH_2 and CH_3 in the hydrocarbon chain. A lowered peak at 1769.34 cm^{-1} proves the presence of a carbonyl group ($\text{C}=\text{O}$) conjugated with sugar as well as a deformed signal at 1374.82 affected by the carboxyl group. Additionally, three stretch signals appeared at 1213.84 , 1121.24 and 1118.04 cm^{-1} where the former indicates a $\text{C}-\text{O}-\text{C}$ bond and the two latter confirm the presence of $\text{C}=\text{O}$ in the glycolipid structure [14]. The region between 700 and 950 cm^{-1} is considered a carbohydrate fingerprint and often presents some peaks related to anomeric carbon [15].

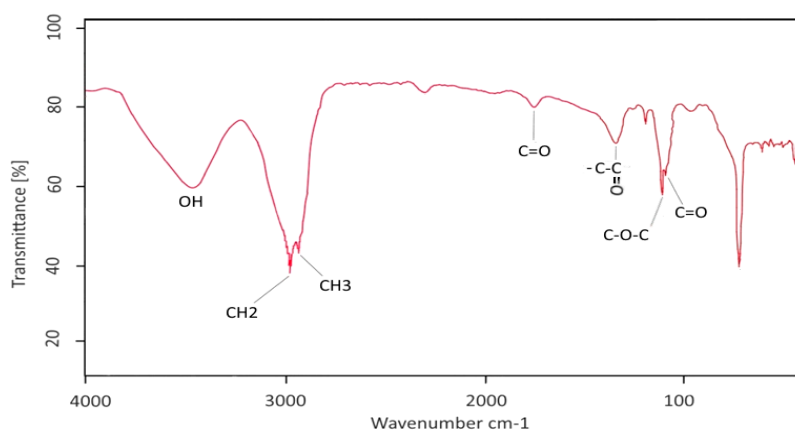


Figure 2. Fourier transform infrared (FTIR) spectrum of glycolipid biosurfactant.

Carbon and hydrogen NMR (^{13}C - and ^1H -NMR) were performed to elucidate the structure of the purified GBS. Table 3 describes the ^{13}C - and ^1H -NMR spectra of GBS, D-glucopyranose and octadecanoic acid. The chemical shift of the signals related to sugar and lipid moiety as compared

to corresponding peaks of D-glucose and octadecanoic acid denotes the molecular structure of GBS. The major peaks (as shown by star) indicate how fatty acids are linked to the sugar moiety. On comparison to ^{13}C -NMR spectrum of octadecanoic acid, it can be confirmed that a marked shift away from about 181.7 ppm has occurred and the related peak has been transferred to 172.8 ppm, indicating that the carboxyl group in fatty acids are covalently attached to OH-C6 from the sugar moiety [16,17]. Moreover, lack of ^1H -NMR peaks related to OH-C6 from glucopyranose and COOH from fatty acid indicates that an ester bond has been created between OH-C6 of the sugar and COOH of fatty acid in GBS. The ^1H -NMR profile for GBS structure displays multiple peaks at 3.3 to 4 ppm, while lacking carboxylic group peak at 10–12 ppm, indicating lipid-to-sugar ester conjugation [17].

Table 3. ^1H -NMR and ^{13}C -NMR interpretation of GBS structure compared with those of D-glucose and octadecanoic acid.

Functional Group		D-Glucose		Glycolipid		Octadecanoic Acid	
		^1H -NMR	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -NMR	^{13}C -NMR
Sugar moiety	C-1, H-1	4.92	92.6	4.55	95.1	-	-
	C-2, H-2	3.53	73.5	3.13	72.8	-	-
	C-3, H-3	3.5	73.8	3.16	74.5	-	-
	C-4, H-4	3.15	70.8	3.13	69.8	-	-
	C-5, H-5	3.53	72.6	3.58	71.5	-	-
	C-6, (6-Hydroxyl)	1.29	61.6	**	65.2	-	-
Lipid moiety	1' (Carboxyl)	-	-	**	172.8 *	11.00	180.5 *
	2' (CH ₂)	-	-	2.26	34.2	2.35	35.4
	3' (CH ₂)	-	-	1.56	26.3	1.64	24.7
	-CH ₂ -(C'4-C'16)	-	-	1.25–1.27	29.0–33.1	1.26–1.32	33.9
	C'17 (CH ₂)	-	-	1.23	23.7	0.93	25.1
	C'18 (CH ₃)	-	-	0.86	14.0	0.88	15

* Meaningful chemical shift; ** remove of peaks related to glycosylic bond.

2.4. Effect of pH, Temperature, and Salinity on GBS Activity

The rheological parameters of GBS after treating for 1 h in the range of pH, temperature and salt have been presented in the Table 4. Crude GBS exhibited a considerable ability to reduce surface tension (ST) in the range of pH 7–8, temperature of 20–60 °C and salinity of 0%–3%. The maximum bioemulsification activity (E24) was observed in the pH range of 7–8, which was about 47%. The effect of temperature was not significant on the E24 value produced by the bioactive compound from 20 to 100 °C. Overall, the increase in NaCl concentration decreased E24 produced by the bioactive compound. Oil spreading activity remained around 11 mm for all treatments and no change was observed in different conditions.

Table 4. Stability of GBS treated in different physical conditions.

Properties	pH			
	4	7	8	12
ST (mN/m)	18.4 ± 2.1	48.2 ± 3.8	50.5 ± 6.4	34.7 ± 4.0
OSD (mm)	10.1 ± 1.7	12.4 ± 1.2	12.6 ± 1.5	11.0 ± 2.2
E24 (%)	32.1 ± 3.3	46.4 ± 4.2	48.4 ± 5.4	22.7 ± 1.7
Properties	Temperature (°C)			
	20	35	60	100
ST (mN/m)	46.6 ± 4.8	53.5 ± 6.7	50.7 ± 6.5	10.0 ± 1.6
OSD (mm)	10.6 ± 2.5	9.7 ± 1.0	11.3 ± 2.3	12.5 ± 1.2
E24 (%)	48.3 ± 6.3	50.2 ± 7.1	54.8 ± 8.5	53.9 ± 6.6
Properties	NaCl (%)			
	0	3	6	10
ST (mN/m)	51.2 ± 7.6	48.4 ± 6.4	31.7 ± 5.2	28.6 ± 4.3
OSD (mm)	11.5 ± 1.7	12.2 ± 2.7	9.5 ± 0.8	8.6 ± 1.1
E24 (%)	44.7 ± 3.8	47.2 ± 6.5	33.5 ± 1.7	24.2 ± 3.0

2.5. Optimization of Glycolipid Production by RSM

Antimicrobial activity was determined by plotting diameters of zones of inhibition against reciprocates of the dilution factors followed by calculating the area under the dilution curve (AUDC) as response. Based on the experimental data in which those insignificant terms removed using a stepwise regression and the output formula achieved from RSM with CCD, the following quadratic model was proposed.

$$Y = +6.74 + 1.19X_1 - 0.32X_2 + 0.41X_1X_2 + 0.44X_1X_5 - 0.71X_2X_4 - 0.53X_3X_5 - 0.23X_1^2 - 1.40X_2^2 + 0.33X_4^2$$

Experimental data in 32 trials are shown in Table 5 as actual and predicted values. Table 6 represents the results of the ANOVA relative to the quadratic model. The model was significant, indicating the ability and accuracy of the model in predicting responses ($p < 0.0001$). In addition, an insignificant lack of fit ($p \gg 0.05$) for the model indicated that the model fits the experimental data perfectly. The predicted R squared of 0.7476 agreed with the adjusted R squared demonstrating strong correlation between experimental and predicted values. Finally, adequate precision of 21.158, evaluating the signal-to-noise ratio, revealed a strong signal compared to noise. A value above 4 (signal-to-noise ratio > 4) denotes adequate precision of the model. The influence of the variables on antimicrobial activity as linear coefficients was found to be strongly significant for pH (X_1) and temperature (X_2). Interactive coefficients were significant for X_1X_2 , X_1X_5 , X_2X_4 and X_3X_5 . The quadratic coefficients for the model were significant for X_1^2 , X_2^2 and X_4^2 . As seen in Table 6, coefficient of variation (C.V. = 7.91) suggests that the model is reliable and the response values are repeatable.

Table 5. Experimental runs and results, actual and predicted, along with amounts of physical and nutritional variables.

Run	pH	Temperature (°C)	Peptone (g/L)	NaCl (%)	Molasses (%)	Actual Response	Predicted Response
1	7	35	0.5	3	1	11.34	11.08
2	6	30	0.75	4	1.5	15.06	15.75
3	6	30	1.25	2	1.5	12.67	13
4	6	20	0.75	2	1.5	4.42	3.59
5	6	30	0.75	2	1.5	14.49	13.49
6	5	25	1	1	1	10.4	10.78
7	8	30	0.75	2	1.5	16.39	16.45
8	6	30	0.75	2	1.5	12.17	13.49
9	5	35	1	3	1	8.28	7.89
10	6	40	0.75	2	1.5	0	1.02
11	6	30	0.75	0	1.5	17.07	16.57
12	7	35	1	3	2	10.84	10.26
13	5	25	1	3	2	8.47	8.34
14	7	35	0.5	1	2	16.95	16.78
15	6	30	0.75	2	1.5	14.22	13.49
16	6	30	0.75	2	0.5	15.33	15
17	6	30	0.75	2	1.5	14.05	13.49
18	7	25	0.5	3	2	15.81	15.81
19	5	25	0.5	1	2	8.38	8.66
20	6	30	0.75	2	1.5	13.78	13.49
21	7	35	1	1	1	16.24	16.16
22	5	35	0.5	1	1	8.21	8.24
23	5	35	1	1	2	7.06	6.76
24	6	30	0.25	2	1.5	13.4	13.25
25	5	35	0.5	3	2	6.25	5.78
26	4	30	0.75	2	1.5	6.81	6.92
27	7	25	1	1	2	12.54	12.72
28	7	25	0.5	1	1	9.13	9.64
29	6	30	0.75	2	1.5	12.41	13.49
30	6	30	0.75	2	2.5	13	13.51
31	5	25	0.5	3	1	12.38	12.58
32	7	25	1	3	1	14.6	14.69

Table 6. Statistical analysis (ANOVA) for evaluating the significance of variables.

Source	Terms	p-Value	Source	Terms	p-Value
Model	quadratic	<0.0001 *	X ₂ X ₄	Interactive	<0.0001 *
pH (X ₁)	Linear	<0.0001 *	X ₂ X ₅	Interactive	0.6633
Temperature (X ₂)	Linear	0.0057 *	X ₃ X ₄	Interactive	0.0772
Peptone (X ₃)	Linear	0.7495	X ₃ X ₅	Interactive	0.0008 *
NaCl (X ₄)	Linear	0.2946	X ₄ X ₅	Interactive	0.1217
Molasses (X ₅)	Linear	0.0730	X ₁ ²	squared	0.0225 *
X ₁ X ₂	Interactive	0.0044 *	X ₂ ²	Squared	<0.0001 *
X ₁ X ₃	Interactive	0.5965	X ₃ ²	squared	0.6003
X ₁ X ₄	Interactive	0.3503	X ₄ ²	Squared	0.0024 *
X ₁ X ₅	Interactive	0.0030 *	X ₅ ²	squared	0.2817
X ₂ X ₃	Interactive	0.8642	Lack of Fit	-	0.6260
Parameter	Value		Parameter	Value	
Std. Dev.	0.46		R-Squared	0.9814	
Mean	5.81		Adj R-Squared	0.9475	
C.V. %	7.91		Pred R-Squared	0.7476	
PRESS	31.51		Adeq Precision	21.158	

* Denotes significant terms.

The fitted three dimensional plots show the effects of the independent factors with significant interactions with the variable pairs (Figure 3). The shape of these plots could be helpful to understand the model and the status of interaction between the independent variables. The optimum predicted responses are shown at highlighted points based on interactive effects between each pair of independent factors. As per the model, the maximum predicted response for antimicrobial activity could be estimated at 17.26 at the desirable conditions, including pH 7, temperature 33 °C, peptone 1%, NaCl 1% and molasses 1% (*w/v*).

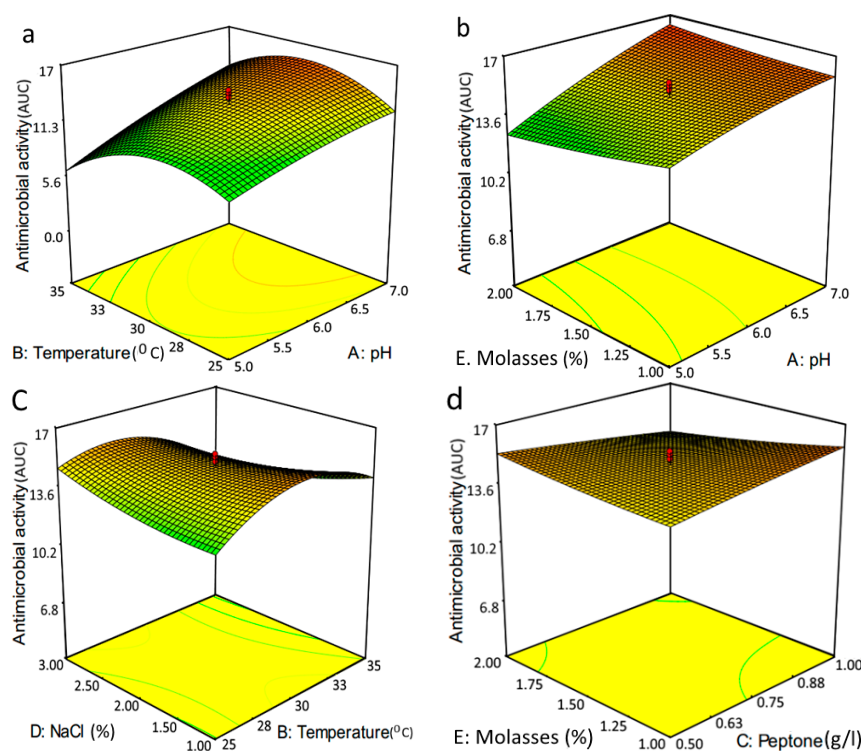


Figure 3. Three-dimensional surface plots. Five significant terms between variables: (a) interactive effects between temperature and pH exhibited a polynomial curvature; (b) molasses and pH have no curvature, but affected significantly polynomial; (c) NaCl and Temperature have significant polynomial curvature and (d) molasses and peptone have interactive polynomial effect, but no curvature.

2.6. Time Course Study of Bacterial Growth and GBS Production

The experiment was conducted according to the predicted parameters of RSM for optimum antimicrobial activity. The antimicrobial and emulsification (E24) activities accord with bacterial growth (OD 600), which increased up to 42 h post-incubation (Figure 4). The antimicrobial activity remained constant during the following hours of the incubation, while emulsification activity decreased along with bacterial growth. Moreover, the antimicrobial activity reached around the predicted response, denoting the reliability of RSM prediction. The time course study shows a maximum inhibitory effect around 15.5 AUDC, meaning that it presents a significant correlation with the predicted value (AUDC 17.5) calculated by the RSM statistical algorithm. Besides, this difference between the RSM predicted and time course responses may be due to unknown and unpredictable conditions.

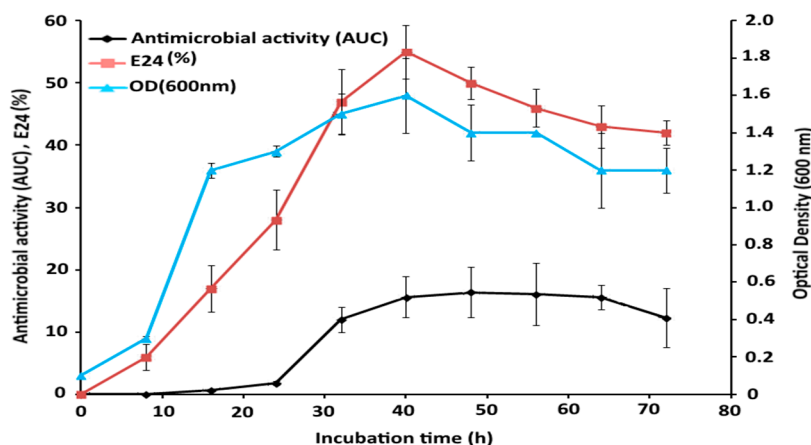


Figure 4. Time course for production of the glycolipid by the bacterium. Antimicrobial activity, bacterial growth and emulsification activity were determined during 72-h incubation at the optimized conditions suggested based on RSM design.

3. Discussion

Buttiauxella species is one of the members of enterobacteriaceae found in various habitats. Up to now, there have been a few reports on the antimicrobial activity of *Buttiauxella* species. Furthermore, the antimicrobial activity of other enterobacteriaceae has been reported in the literature. It has been reported that *Serratia marcescens* variants produced bioemulsifying compounds with antimicrobial activity [18]. Dusane et al. [19] described a glycolipid bioemulsifier with anti-biofilm property produced by a marine strain of *Serratia marcescens*. In the current investigation, it was found that the *Buttiauxella* (one of the nearest species to *Enterobacter* subspecies.) could produce a set of glycolipids with concomitant antimicrobial and bioemulsifying activity, where the glycolipids consist of a glucose linked to varying C14, C16 and C18 fatty acid moieties [14]. To our knowledge, this is the first time that the molecular structure of the potential bioemulsifying compounds (GBSs) have been scrutinized with the methods discussed in the “Experimental” section. Given the variation in the carbon length of the molecules, it is plausible for the GBS to manifest antimicrobial activity against a broader spectrum of microorganisms with superior activity as compared to other GBSs [20]. It was, therefore, noteworthy that we achieved the optimum conditions for GBS production, given cultural factors that may affect the production [21].

The optimization of a fermentation process is a common action leading to low-cost production, especially when the substrate for the microorganism growth and productivity is economically cost-effective [22]. In this regard, statistical design methods could contribute to producing conclusive optimum conditions for the production. Response surface methods have been substantiated to have a practical advantage over conventional methods for optimizing a fermentation process [5]. In the current investigation, a molasses batch was utilized as an inexpensive energy and carbon source for

bacterial growth and bioemulsifier production [23,24]. Given the optimum conditions achieved by the RSM, it was found that the bacteria could produce the maximum yield when the temperature was set at 33 °C, which is amenable for application in large-scale production without additional cooling equipment or consuming energy. Since bacterial growth and metabolism results in elevating the temperature of the incubating system, the mentioned temperature could be spontaneously achieved in the fermentation system [25,26]. Moreover, the AUDC of the antimicrobial activity in different dilutions was applied as a rough indicator of the production of the more relevant GBSs with high antimicrobial activity [27]. It seems plausible that the optimum conditions are also optimal for the production of the GBSs that exhibit superior antimicrobial activity as compared to other GBSs produced by the bacterium. Profiling the GBS production when the bacterium faces different cultural conditions would be an interesting issue that merits further investigation. The same study also merits consideration with respect to other species producing multiple emulsifiers with antimicrobial activity. On the other hand, the extracellular entity of the GBSs is regarded as an advantage in the purification steps of the GBSs from the bacteria with high feasibility in the food processing industry [28]. Substituting peptone, used as the nitrogen and protein source in the RSM study, with other less expensive alternative sources such as whey might further reduce the cost of the GBSs production [29]. The current GBSs, having different hydrophobic lengths, might also be suited for oil remediation, as it suggests that they might have different hydrophobic–lipophilic balances, suitable for emulsifying diverse hydrocarbons found in oil [30]. In situ exploitation of the bacteria, together with gross GBSs in field practice, might be highly promising in terms of oil-remediation efficiency [31,32].

In conclusion, due to the versatility of the GBSs in antimicrobial and emulsifying activities, the total GBS might be a promising alternative to chemical emulsifiers for application in various industries, including food processing and oil industries.

4. Experimental Section

4.1. Bacterial Isolation and Characterization

Soil and sediment samples were collected from mangrove forest, Qeshm Island, south of Iran (N 26°57'51" / E 56°27' to E 55°16' of eastern longitude). The samples were dissolved in sterile seawater and then, bacterial strains were cultured on Muller Hinton agar made in seawater. Then, they were screened for antimicrobial activity using cross streak culture method [33] and an isolate with superior antimicrobial activity against *E. coli* was selected for further detailed examinations. Biochemical, morphological and 16S rRNA sequence analysis were conducted to identify the isolate. To amplify the 16S rRNA gene fragment, universal primers Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rd1 (5'-AGGAGGTGATCCAGCC-3') were used and PCR process was programmed as described by Ebrahimipour et al. [9]. The PCR product was sequenced by Sinagene Company (Tehran, Iran) and nucleotide sequence was edited by BioEdit software version 5.0.6 (Abbott company, Calsbad, CA, USA). RNA sequence was analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

4.2. Media Preparation and Antimicrobial Activity

For preparation of antimicrobial compounds, a self-synthetic medium was designed based on Zobel marine broth containing ($\text{g}\cdot\text{L}^{-1}$) the following: glucose (10), peptone (5), yeast extract (5), K_2HPO_4 (1), $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ (0.2), Na_2CO_3 (1) in sterile water with an initial pH of 7.0 ± 0.1 [34]. A loop of overnight inoculum was transferred into a 250 mL Erlenmeyer flask with 100 mL broth medium and incubated on a rotary shaker (100 rpm) at 35 °C for 72 h.

After centrifugation of the media at 5000 (rpm) for 15 min, the cell free supernatant was tested for antimicrobial activity against indicator pathogens such as *Escherichia coli* (ATCC25922), *Salmonella enteric* (ATCC13076), *Bacillus Subtilis* (ATCC465), *Bacillus cereus* (ATCC11778), *Candida albicans* (ATCC10231), *Pseudomonas aeruginosa* (ATCC85327) and *Staphylococcus aureus*

(ATCC25923), *Aspergillus niger* (isolated from spoiled grape) using disc diffusion method. Additionally, the minimum inhibitory concentration (MIC) was determined [35].

4.3. Antimicrobial GBs Purification and Partial Characterization

The crude antimicrobial compound was decanted from the supernatant with equal volume of solvents such as ethyl acetate, chloroform and dichloromethane. The solvents were evaporated in a rotary vacuum evaporator and the dried powder was tested for detailed analysis. To further purify the sample, the resultant powder was passed through a silica gel column chromatography (1.5 × 30 cm; silica G-60; Merck, Darmstadt, Germany). The column was eluted with a mixture of chloroform/methanol/water by volume ratio of 65:25:5. Based on the disc diffusion results, the active fractions were taken and combined together. This process was repeated several times to reach maximum purity. These fractions were examined for the purity and preliminary determination of the GBSs, using thin layer chromatography (TLC). The developing phase for TLC was chloroform/methanol/water (70:10:2).

4.4. Instruments and Analytical Method

4.4.1. Gas Chromatography-Mass Spectrometry (GC-MS)

The purified compound composition was determined through splitting it into lipophilic and hydrophilic (sugar) moieties followed by their related analysis by GC-MS. The lipophilic moiety was converted to fatty acid methyl esters (FAME) using anhydrous solutions of HCl/methanol (5% *w/v*) at 100 °C for 1 h in a boiling water bath [36]. FAME was extracted with n-hexane and the remaining phase was taken for derivatizing sugars into acetylated versions. The sugar moiety was converted to acetylated derivative [37]. FAMES and sugar derivative were analyzed by a gas chromatograph (Agilent Technologies Inc., New York, NY, USA) equipped with a HP-5MS fused silica capillary column (60 cm × 0.25 mm ID × 0.25 film thickness, Agilent Technologies) with injector and detector temperatures of 280 °C and 300 °C, respectively, coupled to a mass spectrometer for mass scanning at a scan rate of 1.2 per second. The voltage of the detector was adjusted to 350 V. The oven temperature program was started from 130 °C and increased to 220 °C at a rate of 2 °C per minute. The carrier gas was nitrogen at a flow rate of 1 mL·min⁻¹ and a split ratio of 50:1 [38].

4.4.2. Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR spectroscopy was carried out on a spectrometer (BRUKER, Karlsruhe, Germany) by KBr pellet method. The pellet was analyzed by measuring in the range of 4000–400 cm⁻¹.

4.4.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

The bioactive compound was used for NMR spectroscopy by a BRUKER DRX-300 Avance spectrometer (BRUKER). The sample was dissolved in CDCl₃ and Tetramethylsilane (TMS) was used as an internal standard. The ¹H- and ¹³C-NMR spectrums were depicted at 400 and 150 MHz, respectively.

4.5. Physical Stability Studies of the Bioactive Compound

The purified glycolipid was examined for rheological properties such as surface tension reduction (STR) activity, emulsification activity (E24 index) and oil spreading diameter (OSD). STR activity was measured by a Fisher Scientific tensiometer (Fisher Scientific Co., Pittsburgh, PA, USA). E24 index and OSD were also determined as described by Ebrahimipour et al. [9]. GBS dissolved in distilled water (1 g·L⁻¹) and was subjected to the following conditions: pH (4–12), temperatures (20–120 °C) and NaCl concentrations (0%–15%) for 1 h. The rheological properties of the treatment were then inspected using above-mentioned tests.

4.6. Optimization of GBS Production by Response Surface Method

Medium optimization for antimicrobial activity was carried out by a statistical design experiment, RSM. Five variables with significant effect, i.e., pH, temperature, peptone, NaCl and molasses concentration were selected to attain optimum condition for GBS production. The bacteria were grown in 250 mL flasks containing 100 mL broth medium shaken at 100 rpm and incubated for 72 h. As response, the emulsification activity related to each experimental run was determined followed by antimicrobial activity.

A half-milliliter of the cell free supernatant was taken, two-fold diluted serially and aliquots of 100 μ L were added to sterile blank discs (6 mm). Upon drying, disc diffusion was performed against *E. coli* as reference strain. Then, the antimicrobial activity for each run was determined by plotting clear zone diameters against reciprocates of the dilution factors followed by calculating the area under the dilution curve (AUDC) as a response. The reason for employing such an approach is to avoid unknown external interferences related to difference in medium compositions resulted in varying viscosity of the media.

Subsequently, Design-expert 7.0 software was employed to find an appropriate model based on the central composite design (CCD). The experimental plan was drafted in 32 trials and all the experiments were conducted in triplicate. Their average was taken for calculating emulsification and antimicrobial activity value. The RSM responses were fitted by the following equation in terms of second-order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j$$

where Y is the predicted response value, β_0 is the intercept term, β_i , β_{ii} and β_{ij} pertain to linear, quadratic and interaction coefficients for x_i , x_i^2 and x_{ij} respectively.

4.7. Time-Course Study of GBS Production

The production of GBS was carried out in 250 mL Erlenmeyer flasks containing 100 mL of the optimal medium according to the RSM prediction with the following composition ($\text{g}\cdot\text{L}^{-1}$): Peptone (1%), yeast extract (0.5%), glucose (1%), NaCl (1%), K_2HPO_4 (0.1%), $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ (0.02%) and Na_2CO_3 (1%). The best physical conditions for GBS production were achieved at pH 7, 33 $^\circ\text{C}$ and 100 rpm. Bioemulsification and antimicrobial activities as well as bacterial growth were measured during 72 h incubation at 8-h intervals.

Acknowledgments: The data presented in this report was a part of A. Marzban's Ph.D. thesis (Grant number: 911025) supported by the Biotechnology Research Center, Mashhad University of Medical Sciences (MUMS) and Department of Microbiology, Faculty of Biological Sciences of Shahid Beheshti University. We would like to thank Manouchehr Teymouri for his help in writing without any expectations.

Author Contributions: Experiment design and supervision of all experiments and results: Abolghasem Danesh and Abdolrazagh Marzban. Analysis of the results and manuscript drafting: Gholamhossein Ebrahimipour. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interests.

References

1. Morya, V.K.; Ahn, C.; Jeon, S.; Kim, E.K. Medicinal and cosmetic potentials of sophorolipids. *Mini Rev. Med. Chem.* **2013**, *13*, 1761–1768. [[CrossRef](#)] [[PubMed](#)]
2. Barin, R.; Talebi, M.; Biria, D.; Beheshti, M. Fast bioremediation of petroleum-contaminated soils by a consortium of biosurfactant/bioemulsifier producing bacteria. *Int. J. Environ. Sci. Technol.* **2014**, *11*, 1701–1710. [[CrossRef](#)]
3. Campos, J.M.; Montenegro Stamford, T.L.; Sarubbo, L.A.; de Luna, J.M.; Rufino, R.D.; Banat, I.M. Microbial biosurfactants as additives for food industries. *Biotechnol. Prog.* **2013**, *29*, 1097–1108. [[CrossRef](#)] [[PubMed](#)]

4. Gilavand, F.; Marzban, A.; Ebrahimipour, G.; Karkhane, M. Investigation of hydrocarbon bio-removal by the indigenous bacteria isolated from crude oil contaminated soils. *J. Microbiol. Biotechnol. Food Sci.* **2015**, *5*, 212–215. [[CrossRef](#)]
5. Deepika, K.V.; Kalam, S.; Sridhar, P.R.; Podile, A.R.; Bramhachari, P.V. Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium *Pseudomonas aeruginosa* KVD-HR42 using response surface methodology. *Biocatal. Agric. Biotechnol.* **2016**, *5*, 38–47. [[CrossRef](#)]
6. Franzetti, A.; Gandolfi, I.; Bestetti, G.; Smyth, T.J.; Banat, I.M. Production and applications of trehalose lipid biosurfactants. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 617–627. [[CrossRef](#)]
7. Saravanakumari, P.; Mani, K. Structural characterization of a novel xylolipid biosurfactant from *Lactococcus lactis* and analysis of antibacterial activity against multi-drug resistant pathogens. *Bioresour. Technol.* **2010**, *101*, 8851–8854. [[CrossRef](#)] [[PubMed](#)]
8. Kim, K.; Yoo, D.; Kim, Y.; Lee, B.; Shin, D.; Kim, E.K. Characteristics of sophorolipid as an antimicrobial agent. *J. Microbiol. Biotechnol.* **2002**, *12*, 235–241.
9. Ebrahimipour, G.; Gilavand, F.; Karkhane, M.; Kavyanifard, A.; Teymouri, M.; Marzban, A. Bioemulsification activity assessment of an indigenous strain of halotolerant *Planococcus* and partial characterization of produced biosurfactants. *Int. J. Environ. Sci. Technol.* **2014**, *11*, 1379–1386. [[CrossRef](#)]
10. Kaya, K.; Morrison, L.F.; Codd, G.A.; Metcalf, J.S.; Sano, T.; Takagi, H.; Kubo, T. A novel biosurfactant, 2-acyloxyethylphosphonate, isolated from waterblooms of *Aphanizomenon flos-aquae*. *Molecules* **2006**, *11*, 539–548. [[CrossRef](#)] [[PubMed](#)]
11. Alit-Susanta, W.G.N.; Takikawa, Y. Phenotypic characterization of *Pseudomonas fluorescens* PfG32R and its spontaneous *gacS* mutants and biocontrol activity against bacterial wilt disease of tomato. *J. Gen. Plant Pathol.* **2006**, *72*, 168–175. [[CrossRef](#)]
12. Sinanoglou, V.J.; Zoumpoulakis, P.; Heropoulos, G.; Proestos, C.; Ćirić, A.; Petrovic, J.; Glamoclija, J.; Sokovic, M. Lipid and fatty acid profile of the edible fungus *Laetiporus sulphureus*. Antifungal and antibacterial properties. *J. Food Sci. Technol.* **2015**, *52*, 3264–3272. [[CrossRef](#)] [[PubMed](#)]
13. Das, P.; Mukherjee, S.; Sen, R. Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. *J. Appl. Microbiol.* **2008**, *104*, 1675–1684. [[CrossRef](#)] [[PubMed](#)]
14. Jadhav, M.; Kagalkar, A.; Jadhav, S.; Govindwar, S. Isolation, characterization, and antifungal application of a biosurfactant produced by *Enterobacter* sp. MS16. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 1347–1356. [[CrossRef](#)]
15. Fusconi, R.; Maria Nascimento Assunção, R.; de Moura Guimarães, R.; Rodrigues Filho, G.; Eduardo da Hora Machado, A. Exopolysaccharide produced by *Gordonia polyisoprenivorans* CCT 7137 in GYM commercial medium and sugarcane molasses alternative medium: FT-IR study and emulsifying activity. *Carbohydr. Polym.* **2010**, *79*, 403–408. [[CrossRef](#)]
16. Arata, S.; Kurosu, H.; Kuroki, S.; Ando, I. Structural characterization of stearic acid in the crystalline state by the cross polarization in solid state ¹³C NMR. *J. Mol. Struct.* **1999**, *513*, 133–138. [[CrossRef](#)]
17. Gurst, J.E. NMR and the Structure of D-glucose. *J. Chem. Educ.* **1991**, *68*, 1003. [[CrossRef](#)]
18. Wei, Y.-H.; Lai, H.-C.; Chen, S.-Y.; Yeh, M.-S.; Chang, J.-S. Biosurfactant production by *Serratia marcescens* SS-1 and its isogenic strain SMΔR defective in *SpnR*, a quorum-sensing LuxR family protein. *Biotechnol. Lett.* **2004**, *26*, 799–802. [[CrossRef](#)] [[PubMed](#)]
19. Dusane, D.H.; Pawar, V.S.; Nancharaiah, Y.V.; Venugopalan, V.P.; Kumar, A.R.; Zinjarde, S.S. Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of *Serratia marcescens*. *Biofouling* **2011**, *27*, 645–654. [[CrossRef](#)] [[PubMed](#)]
20. Dalsoo, Y.; Kim, K.; Kim, Y.; Kim, E. Antimicrobial Activity of Biosurfactants. *J. Soc. Cosmet. Sci. Korea* **2001**, *27*, 57–58.
21. Pazzetto, R.; Ferreira, S.B.S.; Santos, E.J.S.; Moriwaki, C.; Guedes, T.A.; Matioli, G. Preservation of *Bacillus firmus* Strain 37 and Optimization of Cyclodextrin Biosynthesis by Cells Immobilized on Loofa Sponge. *Molecules* **2012**, *17*, 9476–9488. [[CrossRef](#)] [[PubMed](#)]
22. Zohdi, N.K.; Amid, M. Optimization of Extraction of Novel Pectinase Enzyme Discovered in Red Pitaya (*Hylocereus polyrhizus*) Peel. *Molecules* **2013**, *18*, 14366–14380. [[CrossRef](#)] [[PubMed](#)]
23. Shahriari Moghadam, M.; Ebrahimipour, G.; Abtahi, B.; Ghassempour, A.; Hashtroudi, M.S. Biodegradation of polycyclic aromatic hydrocarbons by a bacterial consortium enriched from mangrove sediments. *J. Environ. Health Sci. Eng.* **2014**, *12*, 1–9. [[CrossRef](#)] [[PubMed](#)]

24. Banat, I.M.; Satpute, S.K.; Cameotra, S.S.; Patil, R.; Nyayanit, N.V. Cost effective technologies and renewable substrates for biosurfactants' production. *Front. Microbiol.* **2014**, *5*, 697. [[CrossRef](#)] [[PubMed](#)]
25. Wei, Y.-H.; Wang, L.-F.; Changy, J.-S.; Kung, S.-S. Identification of induced acidification in iron-enriched cultures of *Bacillus subtilis* during biosurfactant fermentation. *J. Biosci. Bioeng.* **2003**, *96*, 174–178. [[CrossRef](#)]
26. Joshi, S.; Bharucha, C.; Jha, S.; Yadav, S.; Nerurkar, A.; Desai, A.J. Biosurfactant production using molasses and whey under thermophilic conditions. *Bioresour. Technol.* **2008**, *99*, 195–199. [[CrossRef](#)] [[PubMed](#)]
27. Ćavar, S.; Maksimović, M.; Vidic, D.; Parić, A. Chemical composition and antioxidant and antimicrobial activity of essential oil of *Artemisia annua* L. from Bosnia. *Ind. Crops Prod.* **2012**, *37*, 479–485. [[CrossRef](#)]
28. Das, P.; Mukherjee, S.; Sen, R. Substrate dependent production of extracellular biosurfactant by a marine bacterium. *Bioresour. Technol.* **2009**, *100*, 1015–1019. [[CrossRef](#)] [[PubMed](#)]
29. Dubey, K.; Juwarkar, A. Distillery and curd whey wastes as viable alternative sources for biosurfactant production. *World J. Microbiol. Biotechnol.* **2001**, *17*, 61–69. [[CrossRef](#)]
30. Gatard, S.; Nasir, M.N.; Deleu, M.; Klai, N.; Legrand, V.; Bouquillon, S. Bolaamphiphiles Derived from Alkenyl L-Rhamnosides and Alkenyl D-Xylosides: Importance of the Hydrophilic Head. *Molecules* **2013**, *18*, 6101–6112. [[CrossRef](#)] [[PubMed](#)]
31. Dadrasnia, A.; Ismail, S. Biosurfactant production by *Bacillus salmalaya* for lubricating oil solubilization and biodegradation. *Int. J. Environ. Res. Public Health* **2015**, *12*, 9848–9863. [[CrossRef](#)] [[PubMed](#)]
32. Anaukwu, C.G.; Ezemba, C.C.; Anakwenze, V.N.; Agu, K.C.; Okeke, B.C.; Awah, N.S.; Ekwealor, I.A. Effect of biosurfactant produced by *Citrobacter murlinae* AF025369 and a synthetic surfactant on degradation of crude oil. *Edorium J. Microbiol.* **2016**, *2*, 1–6.
33. Velho-Pereira, S.; Kamat, N. Antimicrobial screening of actinobacteria using a modified cross-streak method. *Indian J. Pharm. Sci.* **2011**, *73*, 223–228. [[PubMed](#)]
34. Simidu, U.; Hasuo, K. An improved medium for the isolation of bacteria from marine fish. *Microbiology* **1968**, *52*, 355–360. [[CrossRef](#)]
35. Ebrahimipour, G.; Moradi, A.; Mehrdad, M.; Marzban, A.; Alaei, H. Evaluation of antimicrobial substance produced by a bacterium isolated from *Parmacella iberica*. *Jundishapur J. Microbiol.* **2011**, *4*, 131–140.
36. Ichihara, K.I.; Fukubayashi, Y. Preparation of fatty acid methyl esters for gas-liquid chromatography. *J. Lipid Res.* **2010**, *51*, 635–640. [[CrossRef](#)] [[PubMed](#)]
37. Jerkovic, I.; Mastelic, J. GC-MS Characterization of Acetylated D-glucopyranosides: Transglucosylation of Volatile Alcohols Using Almond D-glucosidase. *Croat. Chem. Acta* **2004**, *77*, 529–535.
38. Metcalfe, L.; Schmitz, A.A.; Pelka, J. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* **1966**, *38*, 514–515. [[CrossRef](#)]

Sample Availability: Samples of the compounds produced by *Buttiexella* sp. M44 are available from the authors.



© 2016 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (<http://creativecommons.org/licenses/by/4.0/>).