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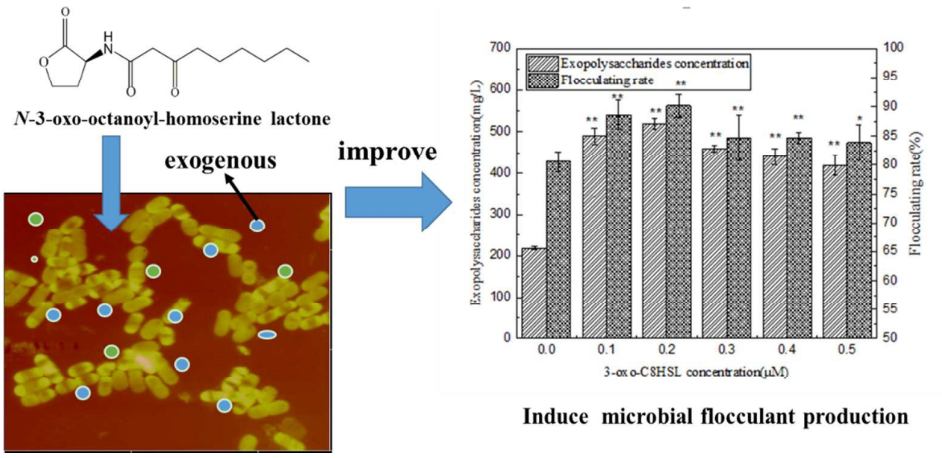
Graphic abstract



Induce microbial flocculant production

N-3-oxo-octanoyl-homoserine lactone as a promotor to improve the microbial flocculant production by an exopolysaccharide bioflocculant-producing bacterium *Agrobacterium tumefaciens* F2

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Abstract

This study showed that *Agrobacterium tumefaciens* F2 can produce *N*-3-oxo-octanoyl-homoserine lactone (3-oxo-C8HSL), a type of the *N*-acyl-homoserine lactone (AHL) class of microbial quorum-sensing signaling molecule. After the addition of exogenous 3-oxo-C8HSL, the exopolysaccharide concentration of microbial flocculants improved by 1.4 times. Fermentation conditions for adding 3-oxo-C8HSL were further optimized by response surface methodology. The optimal fermentation conditions were 0.22 μM 3-oxo-C8HSL at 30.36 °C and initial pH of 8.11. The corresponding exopolysaccharide concentration reached 556.49 mg/L, and the flocculating rate was 91.54%. The microbial flocculant production increased by 1.55 times and flocculation efficiency was increased by 10.96% under these fermentation conditions. Results demonstrated that the microbial flocculant production was regulated by AHL-mediated quorum sensing mechanisms in *A. tumefaciens* F2.

Keywords: Microbial flocculants, Quorum sensing, *N*-acyl-homoserine lactone, Exopolysaccharides production, Production efficiency

1. Introduction

Quorum sensing (QS) can coordinate gene expression and regulate different bacterial population density-dependent behaviors.[1] This process is performed through the detection of concentration of auto-inducers, which are extracellular signaling molecules produced by bacteria. A wide range of behavior is affected by *N*-acyl-homoserine lactone (AHL)-mediated QS regulation, including swarming motility, biofilm formation, stress survival, horizontal DNA transfer, and the synthesis of colonization and exopolysaccharides.[2] Exopolysaccharide production of some bacterial species was initiated by auto-inducers, and their production demonstrated AHL-mediated QS dependence.[3,4] The formation of extracellular polymeric substances, such as exopolysaccharides, was induced by three auto-inducers, namely, *N*-3-oxohexanoyl-homoserine lactone (3-oxo-C6HSL), *N*-hexanoyl-homoserine lactone (C6-HSL), and *N*-3-oxo-octanoyl-homoserine lactone (3-oxo-C8HSL).[5] Tan et al.[6] observed that exopolysaccharide production was increased by add-back AHLs and induced in a signal concentration-dependent manner. Therefore, exopolysaccharide production is directly related to AHL-mediated QS and can be improved by the induction of signaling molecules.

Microbial flocculants are environment-friendly functional materials used to treat domestic sewage and industrial wastewater. However, the low yield and weak activities of such microbial flocculants restrict their practical application.[7] Microbial flocculants produced by microorganisms are classified as biodegradable polymers, containing proteins, polysaccharides, and lipids. The concentration of the main active ingredient of these flocculants is of great importance to their yield and activities.[8] However, few reports exist on improving the production of microbial flocculants through microbial activity regulation.

Agrobacterium tumefaciens F2 in our laboratory is an exopolysaccharide bioflocculant-producing bacterium with high flocculating activity in a variety of wastewater. Several studies on *A. tumefaciens* have demonstrated that AHL-mediated QS mechanisms could regulate the bacterial fitness and pathogenesis, and this AHL-mediated QS was strongly induced by 3-oxo-C8HSL in *A. tumefaciens*.[9,10] However, few studies exist on the regulation of AHL-mediated QS upon microbial flocculant production by *A. tumefaciens*. Through the genome sequence of *A. tumefaciens* F2, the genes related to the LuxR-family regulators were found in the genome of strain F2, which play important roles in AHL-mediated QS.[11] This evidence preliminarily proves that the phenotypes of *A. tumefaciens* F2 are regulated by AHL-mediated QS.

In this study, 3-oxo-C8HSL as an auto-inducer was used to produce microbial flocculants. The role of 3-oxo-C8HSL in microbial flocculant production by strain F2 was confirmed. Furthermore, the optimal fermentation to add 3-oxo-C8HSL was determined. Overall, a novel fermentation method to improve the production of microbial flocculants was provided.

2. Materials and methods

2.1 Fermentation conditions

Microbial flocculant-producing bacterium *A. tumefaciens* F2 was isolated from soil and deposited in the China General Microbiological Culture Collection Center (Accession no. 10131). The main active components of the microbial flocculants produced by strain F2 were exopolysaccharides.

Seed liquid was created by inoculating *A. tumefaciens* F2 into 100 mL of flocculation medium in a shaker at 140 rpm for 30 h at 30 ℃ based on our previous report.[12] Afterward, 5 mL of seed liquid was fermented into the flocculation medium

under the same conditions. The flocculation medium was composed of the following ingredients: 0.0556 mol/L glucose, 0.0287 mol/L K_2HPO_4, 0.0147 mol/L KH_2PO_4, 0.0017 mol/L NaCl, 0.0083 mol/L urea, 0..0028 mol/L yeast extract, and 0.0008 mol/L $MgSO_4 \cdot 7H_2O$ at pH 7.2 to 7.5.

2.2 Analysis of production efficiency

After 30 h, 20 mL of fermentation broth was centrifuged at 12,000 ×*g* for 5 min to remove cell pellets. Up to 40 mL of cold ethanol (4 °C) was added to the supernatant. Crude microbial flocculant precipitate was extracted after 2 h and redissolved in 20 mL of water.

To investigate the efficiency of the microbial flocculant production, crude-fermented liquid extract was used to analyze the exopolysaccharide concentration and flocculation efficiency. The exopolysaccharide concentration was determined by phenol–sulfuric acid assay with glucose as standard.[13] The flocculation efficiency was evaluated using the modified method of Kurane et al.[14] Up to 10 mL of crude fermented liquid extract and 1.5 mL of 10% $CaCl_2$ solution were added into a 5 g/L kaolin clay suspension. The pH of this suspension was adjusted to 7.5 based on our previous studies 12, and the mixture was stirred at 160 rpm for 120 s and then 40 rpm for 120 s. The solution was allowed to settle for 20 min, and the turbidity of the sample was subsequently determined using a spectrophotometer at 550 nm. A control test (without added microbial flocculants) was performed under the same conditions. The flocculation efficiency was calculated using Eq. (1):

$$\frac{A-B}{A} \times 100\% \quad (1)$$

where A is the absorbance of the control group at 550 nm, and B is the absorbance of the sample at 550 nm.

2.3 AHL detection

Agrobacterium tumefaciens F2 was grown to the early stationary phase. Cells were removed from 1000 mL of fermentation medium by centrifugation at 12,000 ×*g* for 10 min. AHLs were then extracted from the fermentation broth supernatants with three equal volumes of ethyl acetate.[15] The extracts were dried using a pressure-blowing concentrator and redissolved in 2 mL of acetonitrile (50%) before analysis by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). To determine whether *A. tumefaciens* F2 can produce signal molecule 3-oxo-C8HSL, the synthetic 3-oxo-C8HSL (>96%, sigma) dissolved in 2 mL of acetonitrile (100%) was analyzed by UPLC–MS/MS as the standard monitoring profile.

Waters ACQUITY UPLC instrument (Waters Corporation, Milford, MA, USA) was used to chromatograph the samples. Solvent B (water with 0.5% formic acid) and solvent A (acetonitrile) were used as mobile phases at a flow rate of 0.15 mL/min. The gradient was increased linearly from 50% (v/v) acetonitrile/50% (v/v) water–formic acid to 20% (v/v) acetonitrile/80% (v/v) water–formic acid, followed by 5% (v/v) acetonitrile/95% (v/v) water–formic acid. Waters ACQUITY TQD mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with electrospray ionization was employed with MassLynx MS software version 4.1. Positive-ion polarity mode was used to ionize the compound, and multi-reaction monitoring mode was employed to perform specific analyses.[16]

2.4 Experimental matrix

The effects of temperature and pH on microbial flocculant production with added 3-oxo-C8HSL was studied, and the fermentation conditions was designed based on the optimal fermentation conditions of strain F2 in our previous studies. Exogenous 3-oxo-C8HSL dissolved in dimethyl sulphoxide was added to the flocculation

medium with final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 μM. A control sample without added exogenous 3-oxo-C8HSL was prepared. Samples with 0.2 μM exogenous 3-oxo-C8HSL were fermented at 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C to determine the effects of temperature on microbial flocculant production with added 3-oxo-C8HSL. Samples without added 3-oxo-C8HSL were also fermented at different temperatures and used as controls. The initial pH of the samples was 7.5. The pH values of the samples with 0.2 μM exogenous 3-oxo-C8HSL were adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 to determine the effect of pH on microbial flocculant production with 3-oxo-C8HSL. Samples without 3-oxo-C8HSL at different pH values were used as controls. All samples were incubated at 30 °C.

After all the samples were fermented for 30 h, the exopolysaccharide concentration and flocculation efficiency of the samples were determined in triplicate.

2.5 Optimization of fermentation conditions

Response surface methodology (RSM) was employed to optimize further the fermentation conditions with added 3-oxo-C8HSL. The optimum variables were culture temperature, initial pH of flocculation medium, and added 3-oxo-C8HSL concentration. The levels of these three parameters were determined in accordance with previous single-factor experiments. Box-Behnken Design (BBD) was used to design 17 groups of independent experiments, and the maximum exopolysaccharide concentration was predicted by Design-Expert.8.05b Trial Version. The relationships between the independent variables and response, as well as between the coded and actual values were calculated using previously described equations.[17]

$$Y_i = b_0 + \sum_1^4 b_i X_i + \sum_1^4 b_{ii} X_i^2 + \sum_1^4 i \sum_1^4 j b_{ij} X_i X_j \quad (2)$$

where Y_i is the predicted response, X_i, X_j are input variables; b_0 is the offset term; b_i is the *i*th linear coefficient; b_{ii} is the quadratic coefficient and b_{ij} is the *ij*th interaction

coefficient.

2.6 Statistical analysis

Data were statistically analyzed through one-way ANOVA in SPSS version 17.0, and significant differences were evaluated through least significant difference test. Differences were considered significant at $p<0.01$.

3. Results and Discussion

3.1 Identification of AHLs

Analysis by UPLC–MS/MS was performed on extracted fermentation broth supernatants from *A. tumefaciens* F2 in comparison with that of synthetic 3-oxo-C8HSL standard. As shown in Fig. 1, the chromatographic retention times of the 3-oxo-C8HSL standard and extracted samples were consistent with the same *m*/*z* of 242.[15] The results showed that 3-oxo-C8HSL was detected in extracted fermentation broth supernatants, and *A. tumefaciens* F2 can produce 3-oxo-C8HSL as an auto-inducer. This results definitely proves that the phenotypes of *A. tumefaciens* F2 can be regulated by AHL-mediated QS.

3.2 Effects of 3-oxo-C8HSL dosage

Various 3-oxo-C8HSL dosages were used to study the influence of 3-oxo-C8HSL on microbial flocculant production. Exopolysaccharide concentration and flocculation efficiency were measured to evaluate the efficiency of microbial flocculant production. Fig. 2 shows a significant improvement in exopolysaccharide concentration and flocculation efficiency ($p<0.01$) in all dosages compared with the control, suggesting a key role of the exogenous 3-oxo-C8HSL in microbial flocculant production. The exopolysaccharide concentration and flocculation efficiency reached the maximum values (519.36 mg/L, 90.11%) by adding 0.2 μM 3-oxo-C8HSL. The exopolysaccharide concentration and flocculation efficiency improved by 1.4 times

and 10.11%, respectively, compared with the control. Therefore, the microbial flocculant production of *A. tumefaciens* F2 can be induced by 3-oxo-C8HSL, and a large increase in microbial flocculant production efficiency of *A. tumefaciens* F2 occurred with added exogenous 3-oxo-C8HSL.

QS can regulate different bacterial population density-dependent behaviors of some bacterial species and this process is performed through the detection of concentration of auto-inducers, which are extracellular signaling molecules produced by bacteria. The regulation of bacterial population density-dependent behaviors was induced when the auto-inducers reached a threshold concentration. Therefore, the microbial flocculant production can be induced with the increase of concentration of auto-inducer through the addition of exogenous 3-oxo-C8HSL, and the exopolysaccharide concentration and flocculation efficiency was improved in different dosages. In our study, microbial flocculant production was induced when the added exogenous C6-HSL reached a threshold concentration (0.2 μM). However, microbial flocculant production can be induced by all 3-oxo-C8HSL dosages because the threshold concentration of 3-oxo-C8HSL was lower than 0.1 μM.

3.3 Effects of pH and temperature

The effects of fermentation conditions with added 3-oxo-C8HSL are observed in cell growth and stability of signal molecules.[18,19] The effects of initial fermentation pH and fermentation temperature on microbial flocculant production with added 3-oxo-C8HSL were analyzed. Fig. 3 shows various levels of increase in the microbial flocculant production under different fermentation temperatures. Compared with the control, the exopolysaccharide concentration was improved with added 3-oxo-C8HSL between 20 °C and 35 °C (p<0.01). In particular, the exopolysaccharide concentration and flocculation efficiency reached their highest values when the temperature was

30 °C (p<0.001). The result showed that the improvement on the microbial flocculant production with added 3-oxo-C8HSL reached the highest value when the temperature was 30 °C through SPSS analysis.

Our previous studies demonstrated that exopolysaccharide concentration with added C6-HSL no longer significantly improved (p>0.01) at 35 °C because AHLs were more prone to hydrolysis at high temperatures.[20,21] The concentration of auto-inducer was below the threshold concentration (0.2 μM) when the C6-HSL was hydrolysis at 35 °C. Therefore, the exopolysaccharide production was not induced at 35 °C. However, Delalande et al. found that short-chain AHLs underwent hydrolysis easier than long-chain AHLs with temperature changes.[22] Moreover, the threshold concentration of 3-oxo-C8HSL was so low (below 0.1 μM) that the concentration of auto-inducer may still reach the threshold concentration when the 3-oxo-C8HSL was hydrolysis in part at 35 °C. Therefore, the microbial flocculant production was improved with added 3-oxo-C8HSL at high temperatures (35 °C).

The microbial flocculant production showed various degrees of increase at different pH values (Fig. 4). Compared with the control, samples with added 3-oxo-C8HSL showed significantly improved (p<0.001) exopolysaccharide concentration and flocculation efficiency in the pH range of 5.0 to 8.0. However, this promotion was weakened at pH 9.0 (p<0.01) through SPSS analysis. The main reason is that AHLs are unstable in alkaline environment.[23] The highest values of exopolysaccharide concentration and flocculation efficiency with added 3-oxo-C8HSL were observed when the pH value was 8.0.

3.4 RSM analysis

As an auto-inducer, 3-oxo-C8HSL was used to ferment microbial flocculants and improve their yield. To optimize the fermentation conditions, BBD was used to

maximize the exopolysaccharide concentration of microbial flocculants produced by strain F2. The BBD plan in the coded levels of the three independent variables is shown in Table 1, and the experimental data are listed in Table 2.

Model accuracy was verified by ANOVA (Table 3). ANOVA of the quadratic model shows the model *F*-value of 10.17 and model *P*-value of 0.0029, indicating that the model was significant. The coefficient of determination (R^2) was 0.9290, which signified that the quadratic model was reliable; a variation of almost 93% can be explained by this model. Moreover, the value of adjusted determination coefficient (adjusted $R^2 = 0.8376$) indicated that the value of exopolysaccharide concentration was in accordance with the experimental value. The exopolysaccharide concentration (mg/L) *Y* can be explained by the following second-order polynomial equation:

$$Y = 540.71 + 12.14A + 15.04B + 40.17C - 12.79AB - 4.86AC + 15.33BC - 21.43A^2 - 41.51B^2 - 38.99C^2$$

As shown in Table 3, the variables C, B^2, and C^2 were significant factors ($p<0.05$). The concentration of added 3-oxo-C8HSL (factor C) was the most significant factor for the exopolysaccharide concentration ($p<0.001$). This finding demonstrated the importance of the addition of 3-oxo-C8HSL in microbial flocculant production.

When all other variables were maintained at their central levels, the relative effects and interactions of the various components of the fermentation conditions can be analyzed, and the exopolysaccharide concentration can be predicted by 3D response surfaces and contour plots (Fig. 5). As shown in Fig. 5, the interaction between the various components of the fermentation conditions was negligible. The temperature–pH contour plot (Fig. 5a) showed that the optimum exopolysaccharide concentration occurred when the temperature ranged from 29 °C to 31 °C and the

initial fermentation pH was 8.0 to 8.25. The increase in exopolysaccharide concentration also occurred when the concentrations of the addition of 3-oxo-C8HSL and initial fermentation pH were between 0.15 and 0.225 μM and between 7.5 and 8.25, respectively (Fig. 5b). As shown in Fig. 5c, the maximum exopolysaccharide concentration occurred when the concentrations of added 3-oxo-C8HSL and the fermentation temperatures were between 0.20 and 0.25 μM and between 30 °C and 31 °C, respectively.

3.5 Experimental verification

The quadratic model predicted that the maximum exopolysaccharide concentration was 553.86 mg/L, and the optimal fermentation conditions were initial pH of 8.11, 30.36 °C temperature, and 0.22 μM 3-oxo-C8HSL concentration. An experimental verification was performed in triplicate and independently three times, and the corresponding exopolysaccharide concentration was 556.49±22.54 mg/L. The relative deviation between the predicted values and experimental values was 0.33% indicating a good agreement between the predicted and experimental results. Microbial flocculant production increased by 1.55 times compared with the control under these conditions. At this condition, the flocculation efficiency was 91.54%, which increased by 10.96% compared with the control. However, the flocculation efficiency hardly increased compared with the result of single-factor experiment, because flocculation activity increases as exopolysaccharides concentration increases to a certain limit.[20] Overall, the active ingredient concentration of microbial flocculants and microbial flocculant production were significantly improved by adding 3-oxo-C8HSL after the optimization of fermentation conditions.

4. Conclusions

A. tumefaciens F2 can produce 3-oxo-C8HSL as a signal molecule to regulate

287 microbial activity. Microbial flocculant production was improved by adding
288 exogenous 3-oxo-C8HSL because microbial flocculant production of strain F2 was
289 reduced by exogenous 3-oxo-C8HSL. The exopolysaccharide concentration was
290 significantly improved by adding 3-oxo-C8HSL between 20 ℃ and 35 ℃ and at the
291 pH range of 5.0 to 9.0. Long-chain AHLs were stable as the temperature and pH
292 changed. The microbial flocculant production efficiency significantly increased at
293 optimal fermentation conditions. Overall, the results demonstrated the existence of QS
294 mechanisms in *A. tumefaciens* F2, as well as the important role of AHLs in microbial
295 flocculant production. This fermentation method of adding 3-oxo-C8HSL under
296 optimal fermentation conditions will provide significance in the application of
297 microbial flocculants.

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List of Figure

Fig. 1. LC-MS chromatogram of the synthetic 3-oxo-C8HSL standards (a) and extracted sample from *A. tumefaciens* strain F2 (b)

Fig. 2. Effect of different concentration of 3-oxo-C8HSL on exopolysaccharides concentration and flocculating rate

One-way ANOVA was performed to compare each experimental group to the control group where significant differences are indicated as follows: *p<0.01 and **p<0.001.

Fig. 3. Effect of fermentation temperature on exopolysaccharides concentration and flocculating rate

One-way ANOVA was performed to compare each experimental group to the control group respectively where significant differences are indicated as follows: *p<0.01 and **p<0.001.

Fig. 4. Effect of initial fermentation pH on exopolysaccharides concentration and flocculating rate

One-way ANOVA was performed to compare each experimental group to the control group respectively where significant differences are indicated as follows: *p<0.01 and **p<0.001.

Fig. 5. The response surface plot and contour plot of exopolysaccharides concentration:

(a) show the effects of initial fermentation pH (factor A) and fermentation temperature (factor B); (b) show the effects of initial fermentation pH (factor A) and the concentration of addition 3-oxo-C8HSL (factor C) and; (c) show the effects of fermentation temperature (factor B) and the concentration of addition 3-oxo-C8HSL (factor C) on exopolysaccharides concentration; .

372 # Tables

373 Table 1 Conditions and levels of the optimization design

Symbol	Factor	Unit	-Levels	Center	+Levels
A	initial fermentation pH	—	-1(7.5)	0(8.0)	1(8.5)
B	fermentation temperature	℃	-1(28)	0(30)	1(32)
C	the concentration of addition of 3-oxo-C8HSL	μM	-1(0.15)	0(0.2)	1(0.25)

374

375

376 Table 2 The second design with experimental values and predicted values of
377 exopolysaccharides concentration

No.	Factor A	Factor B	Factor C	Exopolysaccharides concentration(mg/L)	
				Experimental	Predicted
1	-1	-1	0	452.3	437.8
2	1	-1	0	487.3	487.67
3	-1	1	0	493.83	493.46
4	1	1	0	477.65	492.16
5	-1	0	-1	400.65	423.12
6	1	0	-1	449.52	457.13
7	-1	0	1	520.78	513.18
8	1	0	1	550.20	527.73
9	0	-1	-1	428.30	420.34
10	0	1	-1	441.87	419.76
11	0	-1	1	447.91	470.02
12	0	1	1	522.78	530.75
13	0	0	0	540.57	540.71
14	0	0	0	538.70	540.71
15	0	0	0	541.78	540.71
16	0	0	0	539.29	540.71
17	0	0	0	543.22	540.71

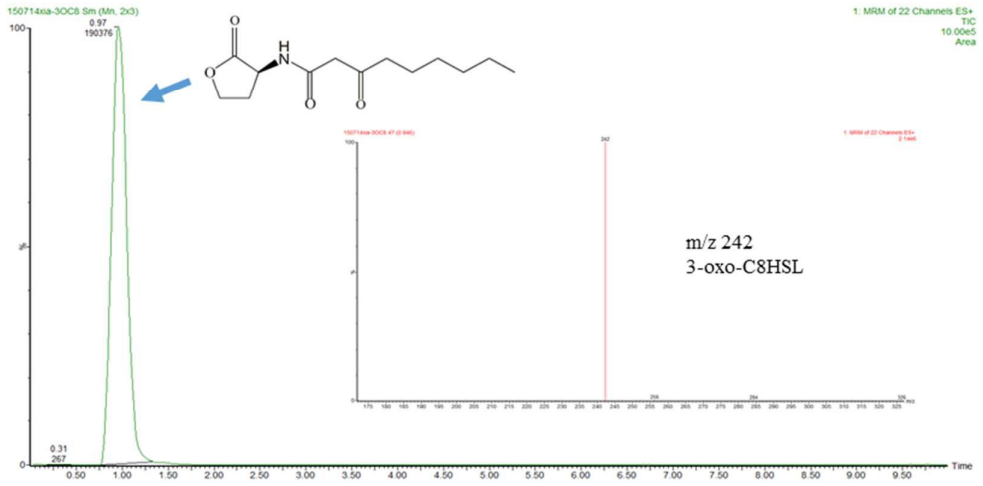
378
379

380 Table 3 ANOVA for the experiments.

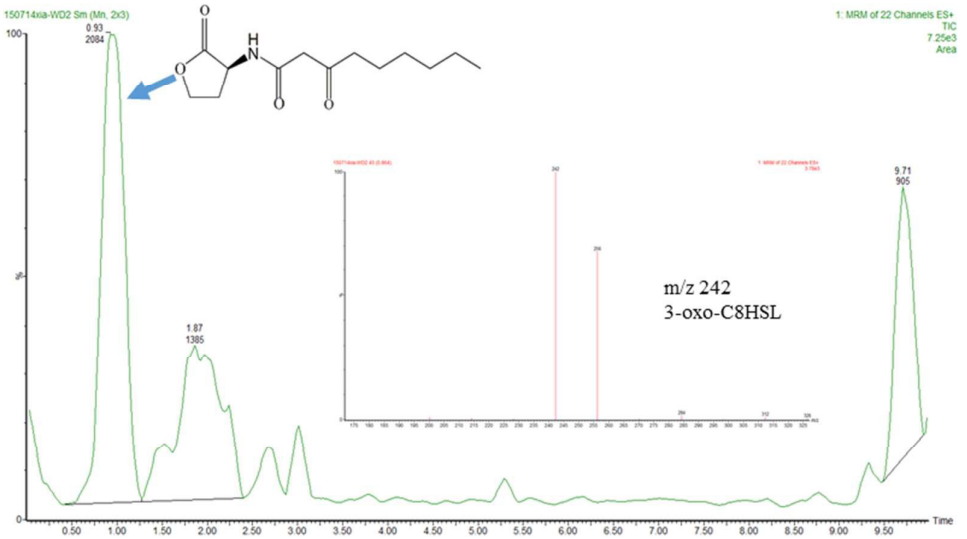
Source	D.F.	SS	Mean square	*F*-Value	*P*-Value
Model	9	34836.47	3870.72	10.17	0.0029
A	1	1178.87	1178.87	3.10	0.1218
B	1	1809.19	1809.19	4.75	0.0656
C	1	12906.70	12906.70	33.91	0.0006
AB	1	654.69	654.69	1.72	0.2310
AC	1	94.60	94.60	0.25	0.6334
BC	1	939.55	939.55	2.47	0.1601
A^2	1	1934.29	1934.29	5.08	0.0588
B^2	1	7253.41	7253.41	19.06	0.0033
C^2	1	6400.10	6400.10	16.82	0.0046
Residual	7	2664.07	380.58		
Total	16	3500.54			

381 D.F.: degrees of freedom; SS: sum of squares.

382



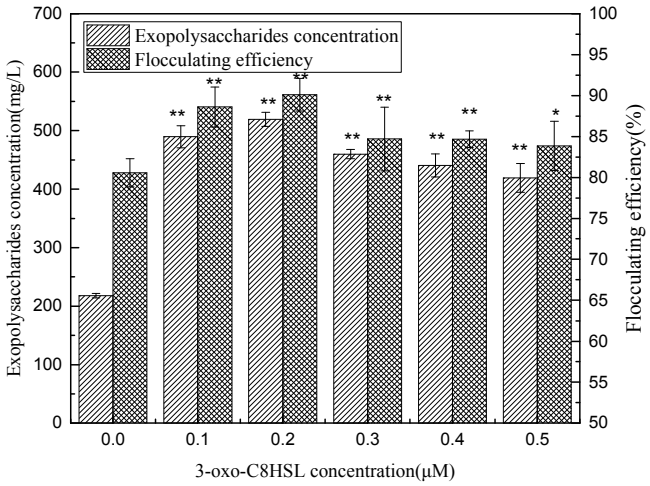
(a)



(b)

Fig. 1

391



392

393

394 **Fig. 2**

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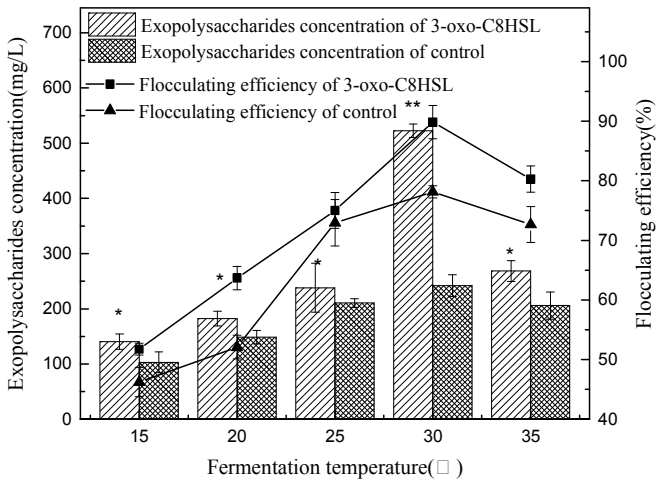
396

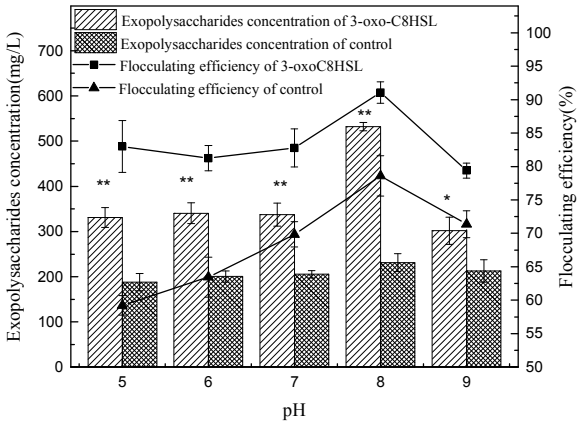


397

398 **Fig. 3**

399

400

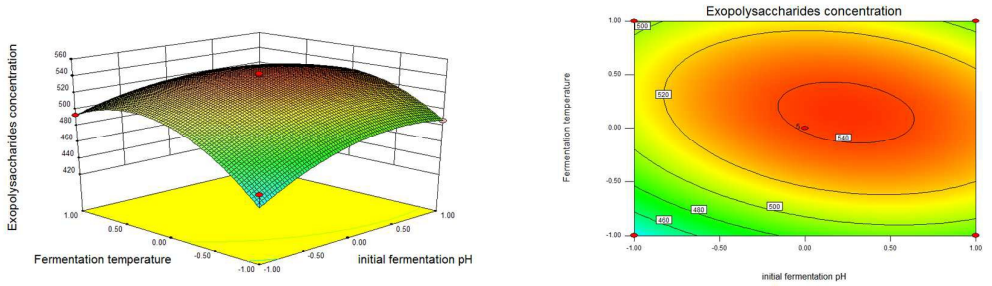


401

402 **Fig.4**

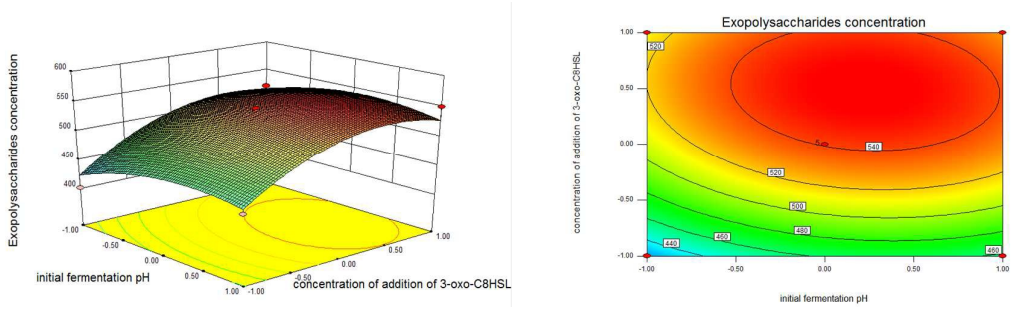
403

404



405

406 (a)



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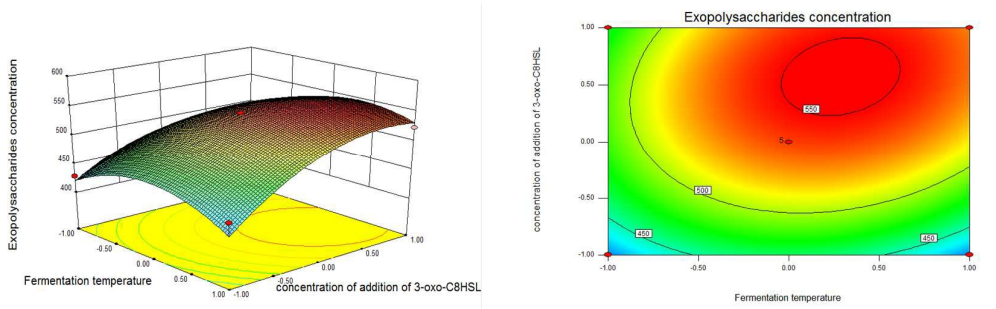
408 (b)



409

410 (c)

411 **Fig.5**

412