Carbohydrate Polymers 115 (2015) 694–700

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimization of culture medium compositions for gellan gum production by a halobacterium *Sphingomonas paucimobilis*

Jun Zhang [a,b], Ya-chen Dong [a,b], Lin-lin Fan [a,b], Zhi-hua Jiao [a,b], Qi-he Chen [a,b,*]

[a] Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310058, China
[b] Food Microbiology Research Key Laboratory of Zhejiang Province, Hangzhou 310058, China

ARTICLE INFO

Article history:
Received 27 July 2014
Received in revised form
10 September 2014
Accepted 11 September 2014
Available online 23 September 2014

Keywords:
Halobacterium *Sphingomonas paucimobilis*
Gellan gum
Culture medium optimization
Fractional factorial design (FFD)
Response surface methodology (RSM)

ABSTRACT

The effect of culture medium compositions on gellan gum production produced by fermentation with a halobacterium *Sphingomonas paucimobilis* QHZJUJW CGMCC2428 was studied. In this work, a fractional factorial design was applied to investigate the main factors that affected gellan gum production by *S. paucimobilis* QHZJUJW CGMCC2428. Sucrose was the best carbon source for gellan gum and peptone displayed better inducing effect. Central composite design and response surface methodology were adopted to derive a statistical model for optimizing submerged culture medium composition. These experimental results showed that the optimum culture medium for producing gellan gum was composed of 40.00 (w/v) sucrose, 3.00% peptone (w/v), $MgSO_4$ (w/v), 9.20% KH_2PO_4 (w/v), 7.50% Na_2HPO_4 (w/v), 4.30% K_2SO_4 (w/v), pH 6.8–7.0. The maximal gellan gum was 19.89 ± 0.68 g/L, which was agreed closely with the predicated value (20.12 g/L). After incubated for 72 h under the optimized culture medium in 5-L bioreactor, the gellan gum fermentation reached about 19.90 ± 0.68 g/L, which was higher than that in the initial cultivation medium.

1. Introduction

Gellan gum is an anionic, extracellular and water-soluble bacterial polysaccharide which is composed of tetrasaccharide repeating units of two residues of β-D-glucose, one of β-D-glucuronic and one of α-L-rhamnose (Per Erik, Bengt, & Paul, 1983; Banerjee, Ravi, & Bhattacharya, 2013). The repeat units have *O*-acetyl and L-glyceryl moieties on the D-glucosyl residue adjacent to the D-glucuronyl residue as the side chain (O'Neill, Selvendran, & Morris, 1983). Compared with other extracellular microbial polysaccharides, gellan gum has many advantages such as high transparency, high strength, perfect formation, good taste, thermal stability, acid-resistant enzymes, etc. Thus, it has been widely applied in food industry as thickening, binding, stabilizing, emulsifying, texturing and gelling agent (Sutherland, 1998; Morris, 1990) and light chemical industries such as solidifying microbial media (Shangu, Valiant, & Tutlane, 1983; Lin & Casida, 1984), coating materials, medicine, and cosmetics (Banerjee et al., 2013; Banik, Kanari, & Upadhyay, 2000; Nuno et al., 2012; Prajapati, Jani, Zala, & Khutliwala, 2013).

Gellan gum was firstly discovered by U.S. scientists in 1978, and then was reported by Kang, Veeder, and Kaneko (1982) to be successfully produced from *Pseudomonas* species on a laboratory scale (Shangu et al., 1983). In consideration of its great commercial value, it is necessary to improve the production by breeding high producing strains (West, 2002), optimizing the culture conditions (Banik, Santhiagu, & Upadhyay, 2007; Bajaj & Saudagar, 2006) and developing other approaches such as using whey and corn starch as substrates (Dlamini & Peiris, 1997; Madhavan, Reeta, Sabarinath, & Pandey, 2003), adding nonionic surfactants (Tween 80, Tween 40 and Triton X-100) (Santhiagu & Banik, 2008) and other strategies (Kanari, Banik, & Upadhyay, 2002; Madhavan et al., 2003; Wang et al., 2006; Banik & Santhiagu, 2007; Zhu, Sheng, & Tong, 2013). Good efficiency in production improvement was achieved by all these approaches.

Previous studies have shown that response surface methodology (RSM) is a successful approach to optimize the product synthesis by fermentation. RSM overcomes some limitations of single parameter optimization and is applied successfully for the optimization of medium constituents and other critical reaction parameters (Ye & Jiang, 2011; Box, Hunter, & Hunter, 1978; Xiong et al., 2004; Gan & Latiff, 2011; Sun, Liu, & Kennedy, 2010). In our work, an endophyte halobacterium *Sphingomonas paucimobilis* QHZJUJW that previously isolated from Qinghai Plateau was found to produce gellan gum during the liquid cultivation. The objective

* Corresponding author at: Department of Food Science and Nutrition, Zhejiang University, Yuhangtang Rd. 866, Hangzhou 310058 China. Tel.: +86 571 86984316.
E-mail address: chenqh@zju.edu.cn (Q.-h. Chen).

http://dx.doi.org/10.1016/j.carbpol.2014.09.029
0144-8617/

of this study was to conduct an optimized production protocol which designed by defining the most important components in the medium makeup, and then to investigate the critical subset of submerged medium components for higher gellan gum production.

2. Materials and methods

2.1. Microorganism and culture medium

S. paucimobilis QHZJUJW was used in this study. This strain was preserved at Zhejiang University and deposited in the China General Microbiological Culture Collection Center (*S. paucimobilis* CGMCC2428). This bacterium showed good performance for cell growth and gellan gum formation at the culture environment containing 3% NaCl, it was determined to be a halobacterium. The initial slant medium (LB) was composed of 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L agar (if necessary) per at pH 7.0. The seed culture medium contained 5 g/L sucrose, 5 g/L tryptone, 1 g/L yeast extract, 3 g/L beef extract, 5 g/L NaCl (initial pH 7.0). The liquid fermentation medium was comprised of 30–40 g/L sucrose, 2.0–4.0 g/L tryptone, 7.0–9.0 g/L KH_2PO_4, 7.0–9.0 g/L Na_2HPO_4, 2.0–4.0 g/L K_2SO_4, 2.0–4.0 g/L $MgSO_4$ and the initial pH was adjusted to 6.8–7.0.

2.2. Culture conditions

A loopfull cells of *S. paucimobilis* QHZJUJW from LB slant culture were inoculated into 50 mL of the fresh seed medium in 250 mL flasks and aerobically incubated for 18–24 h at 30 °C, 200 rpm on a rotary shaker. Then, 10% (v/v) of the seed culture was transferred to 50 mL fermentation medium in 250-mL Erlenmeyer flasks followed by incubation on a 200 rpm rotary shaker at 28–30 °C, for 72 h. The fermentation media were varied based on the experimental design.

Batch fermentations were studied in a 5.0-L stirred bioreactor (Biostat B plus, Sartorius Stedim Biotech Inc., Germany) with a 3.5-L working volume. The process set parameters were pH, 7.0 (±0.1); temperature, 30 °C; agitation speed, 200 r/min; and aeration rate, 1.2 vvm. Both agitation and aeration were kept constant throughout the entire process.

2.3. Optimization of fermentation medium using one factor-at-a-time method

2.3.1. Effect of different carbon source

Different carbon sources (soluble starch, glucose, lactose, maltose, sucrose, and soybean oil with the proportion of fatty acids: sinoleic acid 50.62%, oleic acid 32.45%, palmitic acid 11.12%, octadecanoic acid 4.82%) at 3% (w/v) were used independently in the fermentation media to study the effects on gellan production. The culture flasks were inoculated with 10% (v/v) culture seed in cell growth of 24 h and then incubated as described earlier. Samples were checked for gellan formation after 72 h cultivation.

2.3.2. Effect of nitrogen source

The cultivation the media were respectively prepared with different nitrogen sources to illustrate the effect on gellan formation. The organic nitrogen sources used in this work were urea, tryptone, peptone, and the control flask contained yeast extract as it is mentioned above. The inorganic nitrogen sources were $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2PO_4$, NH_4Cl, NH_4NO_3 and KNO_3. They were supplemented in the fermentation medium at the concentration of 3.0 g/L. The culture flasks were inoculated (10% v/v, 24 h) and incubated as previously mentioned. Samples were assayed after 72 h for gellan gum formation.

2.4. Analytical methods

2.4.1. Determination of cell dry weight and cell biomass

At required time intervals, at least three 20 mL fermentive broth were withdrawn aseptically from the respective flask and then were harvested by centrifugation. The precipitates were washed with distilled water and were dried at 60 °C for 12 h to determine the dry cell weight (DCW). The cell dry weight was then determined gravimetrically and expressed in g/L. The cell free supernatant was used for the recovery of gellan and for the estimation of sucrose. OD_{600} was also adopted to reflect the biomass density qualitatively using a spectrophotometer after incubated culture. The blank control is distilled water.

2.4.2. Recovery of native gellan and quantification

The method reported by Fialho et al. (2008) was used for the recovery of gellan from the fermentation broth. After fermentation, the culture broth was immersed in 90–95 °C water-bath for 20 min and then was centrifuged at 3000 rpm for 30 min to separate completely the biomass. A measured volume of the cell free supernatant (10 mL) was added into three volumes of ice-cold alcohol followed by keeping overnight at 4 °C to precipitate gellan gum. The precipitate formed was then recovered by centrifuging at 3000 rpm for 30 min. The precipitated gellan was then dried to a constant weight by keeping in a hot air oven (60 °C, 12 h). The gellan produced was thus determined gravimetrically and expressed in g/L.

2.4.3. Residual sucrose estimation

Residual sucrose estimation was investigated by measuring the OD_{290} value of sucrose hydrolysis product. 0.03 mL of sample was mixed with 2.0 mL of sucrose hydrolyzate. The solution was topped up with distilled water to 5 mL, and heated for 8 min in a boiling water bath, then cooled by water. The sucrose concentration was calculated according to the standard curve.

2.5. Experimental design

2.5.1. Fractional factorial designs (FFD)

Factorial design is useful in identifying the important nutrients and interactions between two or more nutrients in relatively less experiments as compared to the one-factor at a time technique. The number of experiments can be reduced by using FFD without loss of information about the main effects (Li, Bai, Cai, & Ouyang, 2002). To identify most important ingredients in the culture medium, a 6 fractional factorial design leading to 20 sets of experiments was used to verify the most significant factors affecting the production of gellan gum. All the experiments were performed in triplicate. The variables were coded according to the following equation:

$$xi = \frac{X_i - X_0}{\Delta X_i}$$

where xi is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and $\triangle X_i$ is the step change value. The range and the levels of the variables with both coded values and natural values investigated in this study are given in Table 1. The production of gellan gum was considered as the dependent variable or response (Yi).

The next experiment was then carried out along the path of steepest ascent.

2.5.2. Central composite design (CCD)

In order to describe the nature of the response surface in the optimum region, a central composite design with five coded

Table 1
Range of values for FFD.

Independent variables	Variable name	Levels[a]		
		−1	0	+1
X_1 (g/100 mL)	Sucrose	3	4	5
X_2 (g/100 mL)	Peptone	0.15	0.30	0.45
X_3 (g/100 mL)	$MgSO_4$	0.22	0.32	0.42
X_4 (g/100 mL)	KH_2PO_4	0.62	0.82	1.02
X_5 (g/100 mL)	Na_2HPO_4	0.55	0.75	0.95
X_6 (g/100 mL)	K_2SO_4	0.20	0.30	0.40

[a] $x_1 = (X_1 - 1)/0.5$; $x_2 = (X_2 - 1)/0.5$; $x_3 = (X_3 - 0.5)/0.3$; $x_4 = (X_4 - 0.3)/0.2$; $x_5 = (X_5 - 0.05)/0.05$; $x_6 = (X_6 - 0.1)/0.1$.

levels was performed. The quadratic model for predicting the optimal point was expressed according to following equation:

$$y = b0 + \sum bixi + \sum biixi2 + \sum bijxixj \quad (1)$$

where y was the response variable, $b0$, bi, bii, bij were the regression coefficients variables, for intercept, linear, quadratic and interaction terms, respectively, and x_i and x_j were independent variables. Data were analyzed using the response surface regression (RSREG) procedure (SAS Institute Inc, Cary, NC, USA) and x is the coded level of the independent variable.

3. Results and discussion

3.1. Effects of different carbon source and its concentration on gellan gum production

The production culture medium basically composed of sources and salt solution was preferably used for gellan production. Fig. 1 showed the effect of different carbon sources on the formation of native gellan. The maximum gellan gum (17.85 g/L) was obtained after 48 h of incubation time using sucrose. Similarly, 30 g/L or 40 g/L sucrose was found to be the optimal amount for gellan production. In contrast, soybean vegetable oil showed the worse effect on inducing gellan formation. Even though gellan gum production was promoted by increased carbon source, the cost-effectiveness of

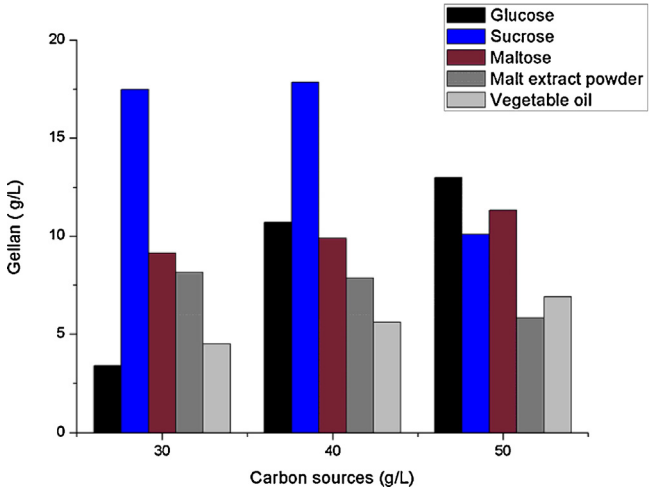

Fig. 1. Effects of different carbon sources and their concentration on gellan gum production by halobacterium *S. paucimobilis* QHZJUJW. The fermentation was performed in 250 mL flasks containing 50 mL of the fermentation medium under the same conditions (agitation 200 rpm, 30 °C). The fermentation medium contained 3.0 g/L tryptone, 8.2 g/L KH_2PO_4, 7.5 g/L Na_2HPO_4, 3.0 g/L K_2SO_4, 3.2 g/L $MgSO_4$ and the initial pH was adjusted to 7.0. The concentrations of each carbon source (glucose, sucrose, maltose, malt extract powder, vegetable oil) were 30 g/L, 40 g/L, 50 g/L, respectively.

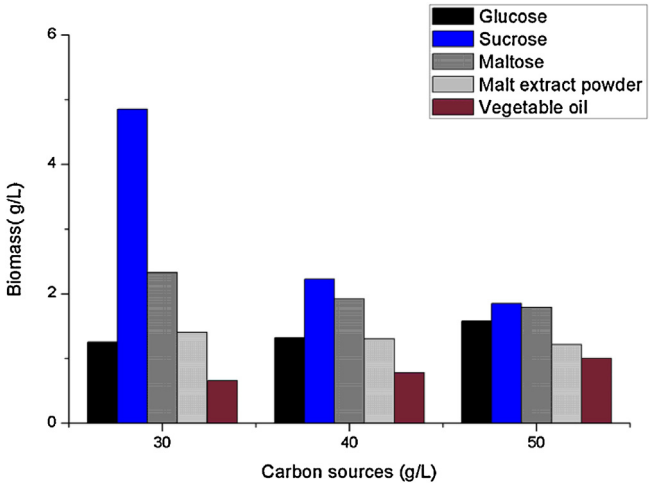

Fig. 2. Effects of different carbon sources and their concentration on biomass accumulation by halobacterium *S. paucimobilis* QHZJUJW. The fermentation was performed in 250 mL flasks containing 50 mL of the fermentation medium under the same conditions (agitation speed 200 rpm, 30 °C). The fermentation medium contained 3.0 g/L tryptone, 8.2 g/L KH_2PO_4, 7.5 g/L Na_2HPO_4, 3.0 g/L K_2SO_4, 3.2 g/L $MgSO_4$ and the initial pH was adjusted to 7.0. The carbon sources (glucose, sucrose, maltose, malt extract powder, vegetable oil) were 30 g/L, 40 g/L, 50 g/L.

using glucose must also be considered. Biomass variation was indicated in Fig. 2, and it was found that biomass accumulation was in accordance with the gellan formation. 30 g/L sucrose showed the best effect on cell biomass accumulation. The previous research reported that carbon source concentration was one of the most important factors in the high-level production of gellan (Bajaj & Saudagar, 2006). In addition, a lower carbon source concentration favored cell growth, but resulted in a lower gellan gum production. Interestingly, in this study higher carbon source concentration did not lead to beneficial effect on cell growth. There was thus a competition between gellan biosynthesis and cell growth.

In general, plentiful secretion of microbial exopolysaccharides is observed when the producing bacteria are supplied with an abundant carbon source and minimal nitrogen (Kim, Lee, Ko, Rhee, & Park, 1999). The C/N ratio rather than the carbon or nitrogen source concentration was the major factor affecting xanthan gum production in *Xanthomonas campestris* (Yang, Lo, & Min, 1996). Thus, we conducted the investigation of nitrogen sources and its concentration on gellan gum formation.

3.2. Effects of different nitrogen source and its concentration on gellan gum production

In general, the type and concentration of nitrogen source in the culture medium influence either biomass or product formation. With the minimal nitrogen used, maximum exopolysaccharide was observed. The choice of nitrogen source has a strong effect on gellan gum characteristics (Prajapati et al., 2013). In view of six nitrogen sources investigated, peptone and corn residue powder were found to be the most effective ones in improving gellan gum production from *S. paucimobilis* QHZJUJW (Fig. 3). This result that yeast extract supported the maximum gellan gum production was consistent with the report by Bajaj and Saudagar (2006). By contrast, as shown in Fig. 4 that yeast extract actually resulted in the worst performance on cell accumulation, and only corn residue powder demonstrated a favorable effect. Nevertheless, the use of corn residue powder led to the difficulty of isolation of gellan gum from culture broth. Combined the product formation and cell growth, we considered peptone as the better nitrogen source for gellan fermentation. Clearly, the impacts of carbon source and nitrogen source on

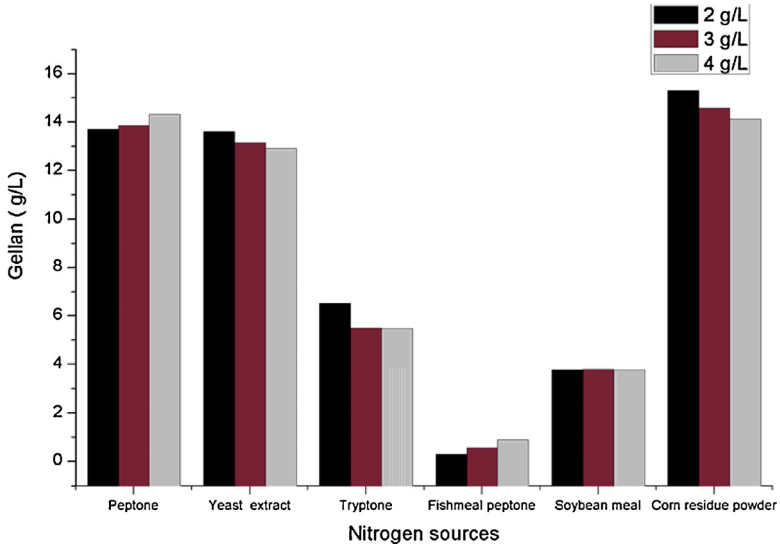

Fig. 3. Effects of nitrogen sources on gellan production by a halobacterium *S. paucimobilis* QHZJUJW. The fermentation was performed in 250 mL flasks containing 50 mL of the culture medium under the same conditions (agitation speed 200 rpm, 30 °C). The fermentation medium contained 30 g/L sucrose, 8.2 g/L KH_2PO_4, 7.5 g/L Na_2HPO_4, 3.0 g/L K_2SO_4, 3.2 g/L $MgSO_4$ and the initial pH was adjusted to 7.0. The nitrogen sources (peptone, yeast extract, trytone, fishmeal peptone, soybean meal, corn residue powder) were 3.0 g/L.

cell accumulation and product formation were with different regulatory roles. In the present work, the production of gellan gum was found to be cell growth-related closely with carbon sources, nitrogen sources and their ratio.

3.3. Optimization of culture media compositions for gellan gum production

3.3.1. The screening stage: The half fractional factorial design

The screening experiments were designed to evaluate the impacts of six factors which were concentrations of sucrose, peptone, $MgSO_4$, KH_2PO_4, Na_2HPO_4, K_2SO_4. A two-level half fractional factorial design was employed and the result of the fractional factorial design is shown in Tables 1 and 2. The production of gellan gum referred to as g/L varied markedly in a range from 5.0 g/L to 15.0 g/L. The lowest production of gellan gum was obtained when minimal levels of tryptone, $MgSO_4$, KH_2PO_4 and K_2SO_4 were used. Clearly, these variables significantly affected the production of gellan gum.

As can be seen from Table 3, the factors of KH_2PO_4 (x_4) and K_2SO_4 (x_6) were found to be significant at the probability level of 95% for gellan gum production, which demonstrated that the two factors had significant effects on the formation of gellan gum. Likewise, the factor peptone (x_2) was significant at the probability level of 90%. The main effects of these variables were positive. Moreover, there were interactions among x_1 and x_2, x_1 and x_5 factors ($P < 0.05$). Other factors demonstrated insignificant effect on the production of gellan gum. The effects of sucrose, peptone, $MgSO_4$, KH_2PO_4, Na_2HPO_4, K_2SO_4 on biomass accumulation were also analyzed by multiple regression techniques.

However, based on the results of *P*-test for variance between the averages of observation of two-level experiment and center point showed that the difference was not significant ($p > 0.05$) (Table 3). This data indicated that optimum point was not in the domain of our experiment. The experiment of steepest ascent path was thus

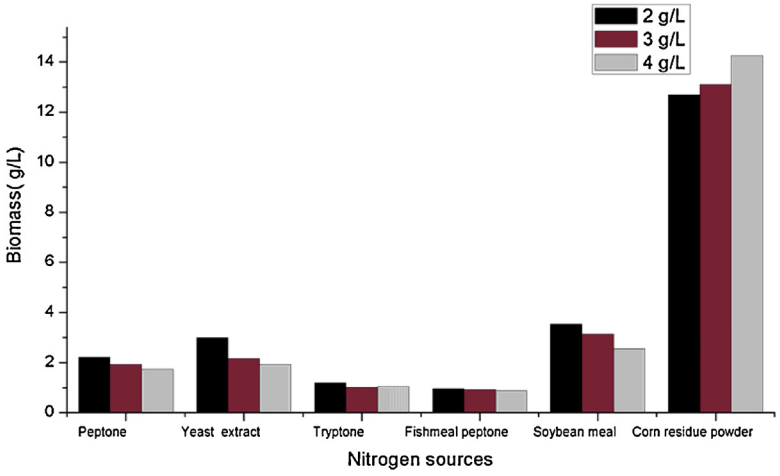

Fig. 4. Effects of different nitrogen sources on biomass accumulation by halobacterium *S. paucimobilis* QHZJUJW. The fermentation was performed in 250 mL flasks containing 50 mL of the fermentation medium under the same conditions (agitation speed 200 r/min, 30 °C). The fermentation medium contained 30 g/L sucrose, 8.20 g/L KH_2PO_4, 7.50 g/L Na_2HPO_4, 3.00 g/L K_2SO_4, 3.20 g/L $MgSO_4$ and the initial pH was adjusted to 7.0. The nitrogen sources (peptone, yeast extract, trytone, fishmeal peptone, soybean meal, corn residue powder) were 3.0 g/L.

Table 2
Experimental designs and the results of the FFD.

Run	x_1	x_2	x_3	x_4	x_5	x_6	Y_1 (gellan gum, g/L)	Y_2 (CDW, g/L)
1	−1	−1	−1	1	−1	1	10.63	3.10
2	−1	1	−1	−1	1	1	11.47	4.22
3	−1	−1	1	1	1	−1	13.52	2.17
4	1	−1	1	1	−1	−1	10.28	5.44
5	1	−1	1	−1	−1	1	10.25	4.44
6	0	0	0	0	0	0	10.77	5.68
7	0	0	0	0	0	0	10.92	6.29
8	1	1	−1	1	−1	−1	10.68	4.98
9	1	−1	−1	1	1	1	12.20	4.11
10	−1	1	1	−1	−1	−1	12.60	7.33
11	−1	−1	1	−1	1	1	11.23	3.74
12	−1	−1	−1	−1	−1	−1	8.70	3.35
13	1	1	1	−1	1	−1	11.27	5.8
14	0	0	0	0	0	0	10.95	6.19
15	−1	1	−1	1	1	−1	15.08	2.54
16	0	0	0	0	0	0	10.56	6.22
17	1	1	1	1	1	1	12.73	6.99
18	−1	1	1	1	−1	1	14.95	4.93
19	1	1	−1	−1	−1	1	9.77	4.90
20	1	−1	−1	−1	1	−1	5.07	2.83

required to reach optimum domain. KH_2PO_4 (x_4) and K_2SO_4 (x_6) were then determined to be the main factors for further optimization.

3.3.2. The steepest ascent experiment

The steepest ascent is a method which uses the magnitude and sign of linear effects to make sure the direction toward predictive higher responses. The path begins at the center of the current design space and stretches well outside the design space (Chen, He, & Ali, 2002). A sequence of equally spaced locations along the path is then selected to design a set of experiments. Thus, KH_2PO_4 (x_4) and K_2SO_4 (x_6) concentrations were increased in order to improve the formation of gellan gum (Y_1). Other factors (x_1, x_2, x_3, x_5) were fixed at zero level. The values of gellan gum production obtained in these experiments were summarized in Table 4. It can be seen that the data fell into our expectations with the increase of gellan gum values. The highest yield of gellan gum (16.57 ± 0.36 g/L) was achieved in the seventh step, yet this expolysaccharide could not be increased in further experimentation. It indicated that the experimentation had approached the neighborhood of the optimum culture medium.

3.3.3. Optimization of KH_2PO_4 and K_2SO_4 for the maximal production of gellan gum

Response surface methodology using central composite design (CCD) was employed to determine the optimal levels of two selected factors (KH_2PO_4 and K_2SO_4) that influenced gellan gum production. Table 5 shows the experimental CCD and its corresponding results. Regression analysis was performed to fit the response function with the experimental data. The statistical significance of the second-order model equation was checked by an F-test (ANOVA) with data shown in Table 6. The fit value, termed R^2 (determinant coefficient), of the polynomial model was calculated to be 0.91, indicating that 91% of the variability in the response could be explained by the second-order polynomial prediction equation given below (Eq. (2)). The ANOVA results showed that this model was appropriate. It also suggested that the production of gellan gum by a halobacterium *S. paucimobilis* QHZJUJW was primarily determined by the linear and quadratic terms of KH_2PO_4 (x_4) and K_2SO_4 (x_6) concentrations of the model and no significant

Table 4
The steep ascent design for gellan gum formation by halobacterium *S. paucimobilis* QHZJUJW.

Run	X_4 (KH_2PO_4)	X_6 (K_2SO_4)	Gellan gum (g/L)
1	8.2	3.0	13.63 ± 0.23
2	8.5	3.2	14.42 ± 0.21
3	8.8	3.4	13.32 ± 0.17
4	9.1	3.6	14.51 ± 0.22
5	9.4	3.8	15.08 ± 0.26
6	9.7	4.0	15.95 ± 0.31
7	10.0	4.2	16.57 ± 0.36
8	10.3	4.4	16.24 ± 0.31
9	10.6	4.6	16.10 ± 0.28

Table 3
Results of the FFD regression analysis for gellan gum production (Y_1).

Source	Coefficient estimate	Mean square	F value	Prob > F
Model	11.21	14.69	6.40	0.0042
X_1	−0.52	4.25	1.85	0.2033
X_2	0.70	7.94	3.46	0.0926
X_4	1.74	48.55	21.15	0.0010
X_6	1.03	17.04	7.42	0.0214
$X_1 \times X_2$	−1.06	17.83	7.77	0.0192
$X_1 \times X_3$	0.44	3.12	1.36	0.2704
$X_1 \times X_5$	0.91	13.38	5.83	0.0364
$X_2 \times X_6$	0.58	5.46	2.38	0.1539
Curvature		0.10	0.04	0.8365
Residual		2.30		
Lack of fit		2.83	2.69	0.2235
Pure error		1.05		
Cor total				

* $P > 0.3$, the results are not shown. $R^2 = 0.8367$.

Table 5
Experimental design and results of the central composite design.

Run	x_4	x_6	Gellan gum (g/L) observed[a]	Gellan gum (g/L) predicted
1	1	−1	14.39	16.44
2	0	0	16.47	14.94
3	0	−1.414	14.62	20.13
4	1	1	17.03	16.42
5	−1	−1	15.44	20.37
6	0	0	16.25	16.70
7	−1	1	14.12	13.60
8	1.414	0	16.59	17.26
9	0	1.414	16.64	16.63
10	−1.414	0	16.51	16.64
11	0	0	16.64	16.63
12	0	0	16.17	16.64
13	0	0	16.34	16.62

[a] $x_4 = (X_4 - 0.97)/0.5$; $x_6 = (X_6 - 0.4)/0.3$. Mean ± standard deviation ($n = 3$).

Table 6
ANOVA data of the central composite design.

Source	Sum of squares	DF	Mean square	*F* value	Prob > *F*
Model	38.11	5	7.62	13.45	0.0018
x_4	13.50	1	13.50	23.82	0.0018
x_6	13.34	1	13.34	23.54	0.0019
x_4^2	6.34	1	6.34	11.19	0.0123
x_6^2	2.50	1	2.50	4.40	0.0740
$x_4 \times x_6$	1.22	1	1.22	2.14	0.1865
Residual	3.97	7	0.57		
Lack of fit	3.38	3	1.18	7.72	0.0386
Pure error	0.58	4	0.15		
Cor total	42.07	12			

* $R^2 = 0.91$ ($n = 3$).

interaction existed between the two factors. The equation for the gellan gum showed positive linear effect and negative quadratic effect.

$$Y_1 = 16.65 - 1.30x_4 + 1.29x_6 + 0.95x_4^2 - 0.60x_6^2 - 0.55x_4 \times x_6 \quad (2)$$

The three-dimensional graph obtained from the calculated response surface is shown in Fig. 5. Three-dimensional response surface plots of KH_2PO_4 (x_4) and K_2SO_4 (x_6) against gellan gum production (Y_1) can explain the results of the statistical and mathematical analyses. It is evident from the plot that Y_1 reached its maximum at a combination of coded level −0.99(x_4), +1(x_6). The predicted maximum production of gellan gum is 20.19 g/L when KH_2PO_4 (x_4) is 9.20 g/L, K_2SO_4 (x_6) 4.30 g/L. This theoretical maximum value is 32.25% higher than the yield from the control fermentation without optimization (13.15 ± 0.72 g/L).

3.4. Experimental validation of the optimized culture conditions

In order to validate the adequacy of this model, verification experiments were carried out at the predicted optimal culture media. The mean concentration of the obtained gellan gum from triplicate trials in shake flasks was 19.91 ± 1.25 g/L, which was much near the predicted valve (20.13 g/L). Furthermore, the suitability of the model was investigated by batch fermentation of gellan gum using a halobacterium *S. paucimobilis* QHZJUJW in a 5.0-L bioreactor. The sucrose consumption, cell growth and the production of gellan gum were monitored during the submerged cultivation (Fig. 6). As shown in Fig. 6, the highest concentration

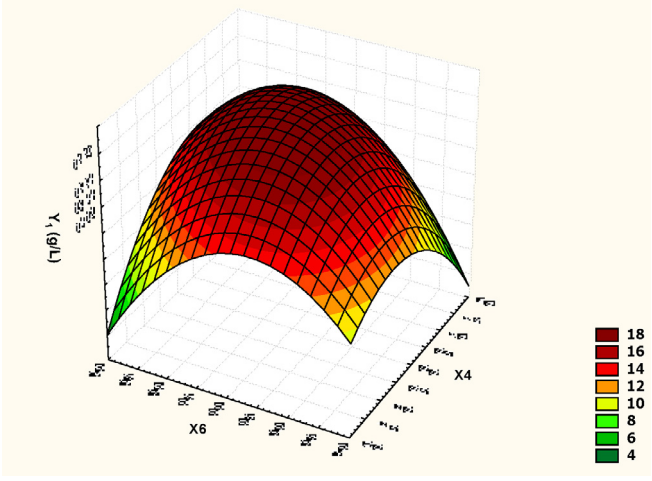

Fig. 5. Response surface plot of batch fermentation in 5-L bioreactor for KH_2PO_4 9.20 g/L, K_2SO_4 against gellan gum production by halobacterium *S. paucimobilis* QHZJUJW under the optimized condition. The optimized fermentation medium contained 40 g/L sucrose, 9.20 g/L KH_2PO_4, 7.50 g/L Na_2HPO_4, 4.30 g/L K_2SO_4, 3.20 g/L $MgSO_4$ and the initial pH was adjusted to 7.0 (agitation speed 200 r/min, 30 °C).

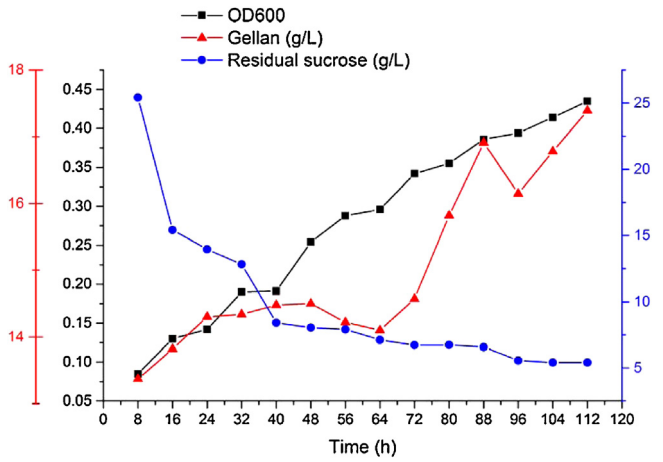

Fig. 6. Time course of batch fermentation in 5-L bioreactor for gellan gum production by a halobacterium *S. paucimobilis* QHZJUJW under the optimized condition. The optimized fermentation medium contained 40 g/L sucrose, 9.20 g/L KH_2PO_4, 7.50 g/L Na_2HPO_4, 4.30 g/L K_2SO_4, 3.20 g/L $MgSO_4$ and the initial pH was adjusted to 7.0. agitation speed 200 r/min, aeration rate 1.2 vvm, 30 °C.

of 19.90 ± 0.68 g/L gellan gum was achieved with an initial concentration of 40 g/L sucrose. The good correlation between these results confirmed that the response model was adequate to reflect the expected optimization.

Previously demonstrated, a C/N ratio above 20.7 had no significant effect on gellan gum production (Huang et al., 2012). In the present study, we also found that low nitrogen is favorable for gellan gum formation. Besides, inorganic salts were found to cause to significant effects on gellan gum production. Interestingly, it is revealed that high inorganic salts concentration in the optimized culture medium seemly favored gellan formation when compared to other reports of *S. paucimobilis*. The regulatory mechanism that high inorganic salts induced gellan gum formation should be paid attention. Zhu et al. (2013) indicated that the best sucrose concentration and temperature for cell growth and gellan gum accumulation were different. A two-stage strategy with controlling sucrose concentration and culture temperatures simultaneously was constructed. Thus, it is worth investigating the effect of culture conditions such as temperature, aeration rate, pH on gellan gum production in the future research.

4. Conclusions

In summary, the effect of culture medium compositions on exopolysaccharide gellan gum production by a newly identified halobacterium *S. paucimobilis* QHZJUJW was studied with one-factor one-level and response surface methodology design. Response surface methodology proved to be a powerful tool in optimizing the culture medium for halobacterium *S. paucimobilis* QHZJUJW. The experimental results clearly showed that the gellan gum by *S. paucimobilis* QHZJUJW was dependent mainly on the concentrations of KH_2PO_4 and K_2SO_4, as well as peptone. The high concentration of KH_2PO_4 and K_2SO_4 had the most significant effects on the increase of gellan gum. Through the statistically designed optimization, the gellan gum production by *S. paucimobilis* QHZJUJW could be increased from an average of 13.52 g/L in the initial medium to an average of 19.91 g/L in the optimized medium, resulting in an approximately 47.26% enhancement. The reported fermentative production of gellan gum by *Sphingomonas paucimobilis* ATCC 31461 had different data of 43.6 g/L (Bajaj and Saudagar, 2006), 17.71 g/L (Huang et al., 2012), 13.81 g/L (Banik et al., 2007), 22.61 g/L (Zhu et al., 2013). Additionally, the constitutive expression of Vitreoscilla hemoglobin

in *Sphingomonas elodea* had a maximum yield of 16.82 g/L (Wu et al., 2010). In this study, the optimized liquid culture medium for producing gellan gum by a halobacterium *S. paucimobilis* QHZJUJW was composed of 40.00 g/L sucrose, 3.00 g/L peptone, 3.20 g/L $MgSO_4$, 7.50 g/L Na_2HPO_4, 9.20 g/L KH_2PO_4, 4.30 g/L K_2SO_4, at pH 7.00. The high content of salts included in the culture medium was a typical characteristics for gellan gum formation by *S. paucimobilis* QHZJUJW. The validated experiment of fermentation process was also conducted under the 5.0-L bioreactor. Further optimization of culture conditions and the use of gellan gum in the food processing are required to conduct in the future study.

Acknowledgements

This work was financially supported by Nature Science Foundation of Zhejiang (LY13H300003) and by the Foundation of Fuli Institute of Food Science, Zhejiang University, China (KY201302).

References

Bajaj, I. B., & Saudagar, P. S. (2006). Statistical approach to optimization of fermentative production of gellan gum from *Sphingomonas paucimobilis* ATCC, 31461. *Journal of Bioscience and Bioengineering*, *102*, 150–156.

Banerjee, S., Ravi, R., & Bhattacharya, S. (2013). Textural characterisation of gellan and agar based fabricated gels with carrot juice. *LWT—Food Science and Technology*, *53*, 255–261.

Banik, R. M., & Santhiagu, A. (2007). Improvement in production and quality of gellan gum under high dissolved oxygen tension levels. *Biotechnology Letters*, *28*, 1347–1350.

Banik, R. M., Kanari, B., & Upadhyay, S. N. (2000). Exopolysaccharide of the gellan family: Prospects and potential. *World Journal of Microbiology and Biotechnology*, *16*, 407–414.

Banik, R. M., Santhiagu, A., & Upadhyay, S. N. (2007). Optimization of nutrients for gellan gum production by *Sphingomonas paucimobilis* ATCC-31461 in molasses based medium using response surface methodology. *Bioresource Technology*, *98*, 792–797.

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*. New York, NY: Wiley.

Chen, Q. H., He, G. Q., & Ali, M. A. M. (2002). Optimization of medium composition for the production of elastase by *Bacillus* sp. EL31410 with response surface methodology. *Enzyme and Microbial Technology*, *30*, 667–672.

Dlamini, A. M., & Peiris, P. S. (1997). Production of exopolysaccharide by *Pseudomonas* sp. ATCC 31461 (*Pseudomonas elodea*) using whey as fermentation substrate. *Applied Microbiology and Biotechnology*, *47*, 52–57.

Fialho, A. M., Moreira, L. M., Granja, A. T., Popescu, A. O., Hoffmann, K., & Sa-Correia, I. (2008). Occurrence, production, and applications of gellan: current state and perspectives. *Applied Microbiology and Biotechnology*, *79*, 889–900.

Gan, C. Y., & Latiff, A. A. (2011). Extraction of antioxidant pectic-polysaccharide from mangosteen (*Garcinia mangostana*) rind: Optimization using response surface methodology. *Carbohydrate Polymers*, *83*, 600–607.

Huang, J., Jiang, S., Xu, X. Q., Wu, H. S., Zhu, X. C., Ke, Z. M., et al. (2012). Effects of carbon/nitrogen ratio, dissolved oxygen and impeller type on gellan gum production in *Sphingomonas paucimobilis*. *Annals of Microbiology*, *62*, 299–305.

Kanari, B., Banik, R. M., & Upadhyay, S. N. (2002). Effect of environmental factors and carbohydrate on gellan gum production in batch mode. *Applied Biochemistry and Biotechnology*, *102*, 129–140.

Kang, K. S., Veeder, G. T., & Kaneko, T. (1982). Agar like polysaccharide produced by pseudomona species: Production and basic properties. *Applied Environmental Microbiology*, *43*, 1086–1091.

Kim, M. K., Lee, I. Y., Ko, J. H., Rhee, Y. H., & Park, Y. H. (1999). Higher intracellular levels of uridine monophosphate under nitrogen-limited conditions enhance the metabolic flux of curdlan synthesis in *Agrobacterium* species. *Biotechnology and Bioengineering*, *62*, 317–323.

Li, C., Bai, J. H., Cai, Z. L., & Ouyang, F. (2002). Optimization of a cultural medium for bacteriocin production by *Lactococcus lactis* using response surface methodology. *Journal of Biotechnology*, *93*(1), 27–34.

Lin, C. C., & Casida, L. E. (1984). Gelrite as a gelling agent in media for the growth of thermophilic microorganisms. *Applied and Environmental Microbiology*, *47*, 427–429.

Madhavan, N. K., Reeta, R. S., Sabarinath, C., & Pandey, A. (2003). Fermentative production of gellan using *Sphingomonas paucimobilis*. *Process Biochemistry*, *38*, 1513–1519.

Morris, V. J. (1990). Biotechnically produced carbohydrate with functional properties for use in food system. *Food Biotechnology*, *4*, 45–47.

Nuno, A. S., Micheal, J. C., Roger, Y. T., Nuno, S., Antonio, J., Salgado, R. L., et al. (2012). The effects of peptide modified gellan gum and olfactory ensheathing glia cells on neural stem/progenitor cell fate. *Biomaterials*, *33*, 6345–6354.

O'Neill, M. A., Selvendran, R. R., & Morris, V. J. (1983). Structure of the acidic extracellular gelling polysaccharide produced by *Pseudomonas elodea*. *Carbohydrate Research*, *124*, 123–133.

Per Erik, J., Bengt, L., & Paul, A. S. (1983). Structural studies of gellan gum, an extracellular polysaccharide elaborated by *Pseudomonas elodea*. *Carbohydrate Research*, *156*, 135–139.

Prajapati, V. D., Jani, G. K., Zala, B. S., & Khutliwala, T. A. (2013). An insight into the emerging exopolysaccharide gellan gum as a novel polymer. *Carbohydrate Polymers*, *93*, 670–678.

Santhiagu, A., & Banik, R. M. (2008). Optimization of gellan gum production by *Sphingomonas paucimobilis* ATCC 31461 with nonionic surfactants using central composite design. *Bioscience and Bioengineering*, *105*(3), 204–210.

Shangu, D., Valiant, M., & Tutlane, V. (1983). Gelrite as an agar substitute in bacteriological media. *Applied and Environmental Microbiology*, *46*, 840–847.

Sun, Y., Liu, J., & Kennedy, J. F. (2010). Application of response surface methodology for optimization of polysaccharides production parameters from the roots of *Codonopsis pilosula* by a central composite design. *Carbohydrate Polymers*, *80*, 949–953.

Sutherland, I. W. (1998). Novel and established applications of microbial polysaccharides. *Trends in Biotechnology*, *16*, 41–46.

Wang, X., Xu, P., Yuan, Y., Liu, C. L., Zhang, D. Z., Yang, Z. T., et al. (2006). Modeling for gellan gum production by *Sphingomonas paucimobilis* ATCC 31461 in a simplified medium. *Applied and Environmental Microbiology*, *72*, 3367–3374.

West, T. P. (2002). Isolation of mutant strain of *Pseudomonas* sp. ATCC31461 exhibiting elevated polysaccharide production. *Journal of Industrial Microbiology and Biotechnology*, *29*, 185–188.

Wu, X. C., Chen, Y. M., Li, Y. D., Li, O., Zhu, L., Qian, C. D., et al. (2010). Constitutive expression of Vitreoscilla haemoglobin in *Sphingomonas elodea* to improve gellan gum production. *Journal of Applied Microbiology*, *110*, 422–430.

Xiong, Y. H., Liu, J. Z., Song, H. Y., & Ji, L. N. (2004). Enhanced production of extracellular ribonuclease from *Aspergillus niger* by optimization of culture conditions using response surface methodology. *Biochemical Engineering Journal*, *21*, 27–32.

Yang, S. T., Lo, Y. M., & Min, D. B. (1996). Xanthan gum fermentation by *Xanthomonas campestris* immobilized in a novel centrifugal fibrous-bed bioreactor. *Biotechnology Progress*, *12*, 630–637.

Ye, C. L., & Jiang, C. J. (2011). Optimization of extraction process of crude polysaccharides from *Plantago asiatica* L. by response surface methodology. *Carbohydrate Polymers*, *84*, 495–502.

Zhu, G. L., Sheng, L., & Tong, Q. Y. (2013). A new strategy to enhance gellan production by two-stage culture in *Sphingomonas paucimobilis*. *Carbohydrate Polymers*, *98*, 829–834.