

Statistical Optimization of Medium Composition for Alginate Lyase Production by *Pseudoalteromonas tetraodonis* Strain QZ-4 using Response Surface Methodology

Haiqing Tang^{1,2}, Chongrong Ou³ and Xiaodong Zheng^{1*}

¹College of Biosystems Engineering and Food Science, Zhejiang University, Zhejiang Hangzhou - 310 029, China.

²Ningbo Entry-exit Inspection and Quarantine Bureau, Zhejiang Ningbo - 315 012, China.

³School of Marine Sciences, Ningbo University, Zhejiang Ningbo - 315 211, China.

(Received: 10 January 2013; accepted: 02 March 2013)

The effect of medium composition on alginate lyase production, by a lineage of *Pseudoalteromonas tetraodonis*, was investigated using response surface methodology. The concentrations of inorganic nitrogen and salts in culture medium, such as di-ammonium hydrogen phosphate, sodium chloride, ferrous sulfate, magnesium sulphate and potassium di-hydrogen phosphate, were changed to obtain the optimal production. The result showed that di-ammonium hydrogen phosphate, sodium chloride and magnesium sulphate had significant effect on alginate lyase production using a two-level fractional factorial design (FFD). The maximum yield of alginate lyase was attained in a medium containing (g/L): Alginate sodium 5, (NH₄)₂HPO₄ 11.6, NaCl 32.9, FeSO₄ 0.03, MgSO₄ 0.08 and K₂HPO₄ 0.4, using the surface response methodology and central composite design (CCD). The model prediction of alginate lyase yield at 145.45 U/mL was experimentally verified.

Key words: Alginate lyase, Fermentation, Response surface methodology (RSM), Two-level fractional factorial design (FFD), Central composite design (CCD).

Alginate polysaccharide produced by marine brown algae and certain Gram-negative bacteria is a linear anionic binary copolymer of β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues¹. Alginate lyase (EC 4.2.2.3; EC 4.2.2.11), also known as alginase or alginate depolymerase, catalyzes the degradation of alginate at the non-reducing terminus by β -elimination mechanism, forming unsaturated oligosaccharides with double bonds². Since the original description of alginate lyases about 50 years ago³, more than 50 enzymes have been characterized from a variety of algae, marine invertebrates, terrestrial and marine microorganisms⁴. As a tool enzyme to degrade alginate biologically, alginate lyase has been

explored and utilized in broad fields. It has been reported that alginate lyase or its crude extract can play great role in decomposing brown seaweeds⁵, producing alginate oligosaccharides, analyzing the fine structure of alginate, preparing protoplasts of seaweed⁶, and reducing viscosity of alginate biofilm building up in the lungs of cystic fibrosis sufferers⁷. To elucidate the unique properties of alginate lyase, great efforts have so far been made around enzyme production, purification, characterization, and gene recombinant, and so on⁸⁻¹⁰.

The genus *Pseudoalteromonas* has also attracted interests from researchers for its biological characteristics and potential commercial use. It shows great diversity and adaptivity via synthesizing biologically active molecules¹¹. Strains of the genus have been reported with abilities to produce anti-bacterial compounds, polysaccharides and extracellular enzymes¹². And

* To whom all correspondence should be addressed.
Phone: 086-0574-86817759; Fax: 086-0574-86815386;
E-mail: zheng.xiaodong@yahoo.cn

various seaweed polysaccharides-degrading enzymes have been found among the species, such as agarase, cellulase, laminarinase, and carrageenase, as well as alginate lyase¹³⁻¹⁵. Alginate lyases from *P. elyakovii* IAM 14594, *P. citrea* KMM 3297 and *P. atlantica* AR06 were studied primarily. These marine bacteria capable of producing alginate lyase were isolated from either coastal water or decaying brown seaweeds^{15,16}. Although it is verified that most of marine bacteria secrete alginate lyase only at the presence of alginate⁴, there are numbered reports available on culture media optimization of alginate lyase production in submerged fermentation. The nutrition demand for nitrogen source and inorganic salts of *Pseudoalteromonas* sp. strains still remain unclear and the yield of alginate lyase can be improved further.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for modeling and evaluating the effect of factors on desirable responses. It has been applied to identify critical factors and study interactions among various parameters which have significant influence on the yield of final product¹⁷. Also it has been widely used in optimization of culture media in enzyme production bioprocesses^{18,19}. In the present work, we use RSM to demonstrate the optimization process of alginate lyase production in flask by the wild strain *P. tetraodonis* QZ-4, attempting to characterize the condition of growth and enzyme production and enhance the yield of alginate lyase.

MATERIALS AND METHODS

Culture and growth medium

P. tetraodonis QZ-4 strain (GenBank accession number HM130919) was isolated from the sea water collected from Yellow Sea, China by food laboratory of Zhejiang University. The bacterial culture was grown on seed culture slants (2216E marine medium with 0.5% alginate) and maintained in 20% glycerol at -20°C.

Fermentation media and Batch fermentation

Prior to each experiment, seed culture was prepared by growing single colony in 100-ml flasks containing 10 ml seed culture medium (2216E marine medium with 0.5% alginate). The flask was shaken at 28 °C for 12h at 200 rpm. For the batch

fermentation process, a volume (0.3 ml) of the seed culture was inoculated into a 250-ml Erlenmeyer flask containing 30 ml of fermentation medium. The flasks were incubated in a rotary shaker at 28 °C for 16h at 200 rpm. The original fermentation medium was previously obtained by single factor optimization process, which contained following composition (g/L): alginate sodium 5.0; (NH₄)₂HPO₄ 9.0; NaCl 30.0; FeSO₄ 0.03; MgSO₄ 0.3 and K₂HPO₄ 0.4 (pH 7.4).

Assay for alginate lyase activity

The alginate lyase activity was detected by measuring the absorbance of unsaturated uronate fractions at 235nm²⁰. The culture supernatants (0.1 mL) were mixed with 0.9 ml of sodium phosphate buffer (10 mmol/L, pH 7.4) containing 0.1% (w/v) alginate. The progress of the reaction incubating at 40 °C for 10 min was monitored with a temperature-controlled spectrophotometer (SpectraMax® Plus384, USA), measuring the increase in absorbance at 235 nm. One unit of lyase activity (U/mL) was defined as the amount of enzyme that increases the absorbance at 235 nm to 0.01 for 1 min.

Two-level fractional factorial design

A 2⁵⁻¹ FFD with four centre points was used for the optimization of medium. Statistical analysis was carried out to identify the variables that had significant effect on the response. The independent variables are coded for statistical calculations according to Eq.(1);

$$X_i = \frac{U_i - U_o}{\Delta U} \quad \dots(1)$$

X_i is the independent variable coded value; U_i , the real value of the independent variable; U_o , real value of the independent variable on the centre point and ΔU is the step change. In this study, the concentrations of (NH₄)₂HPO₄, NaCl, FeSO₄, MgSO₄ and K₂HPO₄ were varied as parameters. The composition of original culture medium was taken as the centre point. The alginate lyase yield, which was defined as the enzyme activity per mL broth, was used as the response variable. All treatments were processed in triplicate for the entire experiment. The data were analyzed using the Design Expert software (version 7.0, Stat-Ease Inc., Minneapolis, USA).

Steepest ascent experiment

In an attempt to get closer to the optimum,

data from previous FFD step were used to move through the experimental region using steepest ascent²¹. In the method of steepest ascent, one starts with an initial design, e.g., a 2^N factorial design centered about an initial guess of the optimum. A regression model is then fit to the data from this initial design in order to determine which direction on the design space is likely to yield the most improvement in the response. One moves from the center point in the design along the path of steepest ascent. The path is then followed in steps of equal size until a decline or constant response is observed²².

Central composite design

A 2^3 full factorial central composite design (CCD) was employed to determine the optimal conditions of the three screened factors in FFD. And the value of centre point was decided by previous steepest ascent experiment. And six replicates were run for the centre point. The independent variables are coded for statistical calculations as Eq.(1). Each variable was designed at five levels (-1.681, -1, 0, 1, 1.681) and the concentrations of optimal factors were varied as these parameters. The alginate lyase yield was used as the response variable. The data were analyzed using the Design Expert software.

RESULTS AND DISCUSSION

Two-level fractional factorial design

The FFD is used to screen the important factors among the components of the culture medium by using a set of orthogonal contrasts with -1 and +1 coefficients optimization. The influence of inorganic nitrogen source and other inorganic salts to the production of alginate lyase were studied here. Alginate was chosen as carbon source in this experiment, served as exogenous substrate for alginate lyase production at the same time. Considering the high viscosity and low solubility of alginate, the concentration of alginate in the culture medium was adjusted in previous single factor optimization and kept constant as 5.0 g/L in the following steps. Alginate lyase yield was measured after 16h of fermentation. The design and results of FFD are shown in Table 1. And the analysis of variance (ANOVA) of FFD is shown in Table 2.

As shown in Table 2, the Model F-value of 61.40 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicates that model terms are significant. In this case, X_1 , X_2 , X_4 , and X_1X_2 are significant model terms. Values greater than 0.10 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "Curvature F-value" of 54.79 implies there is significant curvature in the design space. There is only a 0.01% chance that a "Curvature F-value" this large could occur due to noise. The "Lack of Fit F-value" of 3.25 implies the Lack of Fit is not significant relative to the pure error. There is an 18.03% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. Final equation in terms of coded factors is as follows:

$$Y = 82.09 + 12.99X_1 + 16.71X_2 - 7.47X_4 + 10.13X_1X_2 \dots (2)$$

Table 1. The design and results of FFD

Run	X_1^a	X_2^b	X_3^c	X_4^d	X_5^e	Y(U/mL)
1	-1	-1	-1	-1	1	61.8
2	1	-1	-1	-1	-1	77.3
3	-1	1	-1	-1	-1	85.1
4	1	1	-1	-1	1	130.5
5	-1	-1	1	-1	-1	72.2
6	1	-1	1	-1	1	68.3
7	-1	1	1	-1	1	89.4
8	1	1	1	-1	-1	131.9
9	-1	-1	-1	1	-1	57.0
10	1	-1	-1	1	1	57.6
11	-1	1	-1	1	1	54.8
12	1	1	-1	1	-1	111.0
13	-1	-1	1	1	1	59.1
14	1	-1	1	1	-1	69.8
15	-1	1	1	1	-1	73.4
16	1	1	1	1	1	114.3
17	0	0	0	0	0	105.6
18	0	0	0	0	0	108.2
19	0	0	0	0	0	105.2
20	0	0	0	0	0	113.4

^{a, b, c, d, e} X_1 , X_2 , X_3 , X_4 and X_5 represent the independent variable coded value of $(\text{NH}_4)_2\text{HPO}_4$, NaCl, FeSO_4 , MgSO_4 and K_2HPO_4 , respectively; $X_1 = (U_1 - 9) / 2$, $X_2 = (U_2 - 30) / 5$, $X_3 = (U_3 - 0.3) / 0.2$, $X_4 = (U_4 - 0.04) / 0.2$, $X_5 = (U_5 - 0.4) / 0.2$.

The result indicates that the variation of the concentration of FeSO_4 (X_3) and KH_2PO_4 (X_5) has little influence on the production of alginate lyase. And the three statistical significant variables are nitrogen source ($(\text{NH}_4)_2\text{HPO}_4$, (X_1)), sodium (NaCl (X_2)) and magnesium (MgSO_4 , (X_4)). The positive coefficient of X_1 and X_2 , and the negative one of X_4 showed in Eq.(2) indicate that increases in the concentration of $(\text{NH}_4)_2\text{HPO}_4$ and NaCl may give positive effect on production of alginate lyase, while increase of MgSO_4 may reduce the enzyme yield.

Steepest ascent experiment

As to the result above, it indicates that increasing $(\text{NH}_4)_2\text{HPO}_4$ and NaCl , decreasing FeSO_4 as the same time, might have a positive effect on the production. Thus a steepest ascent experiment was designed to pursue the region for optimal point. The basic steps for increasing and reducing concentration of each component were (g/L): $(\text{NH}_4)_2\text{HPO}_4$, + 0.5, NaCl + 1.25, FeSO_4 - 0.05. Keeping the concentration of FeSO_4 (X_3) and KH_2PO_4 (X_5) as constant as center point, for both of them have little influence on enzyme production.

Table 2. ANOVA for selected factorial model

Source	SS	MS	F	P
Model	9701.77	2425.44	61.40	<0.0001
X_1	2701.40	2701.40	68.39	<0.0001
X_2	4465.58	4465.58	113.05	<0.0001
X_4	892.52	892.52	22.59	0.0003
X_1X_2	1642.28	1642.28	41.57	<0.0001
Curvature	2164.24	2164.24	54.79	<0.0001
Residual	553.04	39.50		
Lack of Fit	510.28	46.39	3.25	0.1803
Pure Error	42.76	14.25		
Cor Total	12419.05			

Table 3. Designs and results of steepest ascent experiment

Run	X_1	X_2	X_4	Y(U/mL)
0	9.0	30.0	0.40	111.0
1	9.5	31.25	0.35	120.8
2	10.0	32.5	0.30	134.8
3	10.5	33.75	0.25	129.7
4	11.0	35.0	0.20	122.1
5	11.5	36.25	0.15	100.1
6	12.0	37.5	0.10	96.4
7	12.5	38.75	0.05	92.5
8	13.0	40.0	0.00	90.4

The design of steepest ascent experiment and experimental data are shown in Table 5. The concentration of treatment 2 was the maximum and chosen as the center point in the following CCD experiment.

Table 4. Experimental designs and the results of CCD for statistical calculations

Run	X_1^a	X_2^b	X_4^c	Y(U/mL)
1	-1.000	-1.000	-1.000	67.4
2	1.000	-1.000	-1.000	116.2
3	-1.000	1.000	-1.000	87.9
4	1.000	1.000	-1.000	128.2
5	-1.000	-1.000	1.000	98.6
6	1.000	-1.000	1.000	95.2
7	-1.000	1.000	1.000	70.0
8	1.000	1.000	1.000	125.0
9	-1.682	0.000	0.000	83.4
10	1.682	0.000	0.000	108.6
11	0.000	-1.682	0.000	83.0
12	0.000	1.682	0.000	90.5
13	0.000	0.000	-1.682	82.3
14	0.000	0.000	1.682	78.2
15	0.000	0.000	0.000	139.4
16	0.000	0.000	0.000	148.4
17	0.000	0.000	0.000	150.0
18	0.000	0.000	0.000	140.0
19	0.000	0.000	0.000	145.4
20	0.000	0.000	0.000	136.0

^{a, b, c} X_1 , X_2 , and X_4 represent the independent variable coded value of $(\text{NH}_4)_2\text{HPO}_4$, NaCl and MgSO_4 , respectively; $X_1 = (U_1 - 10) / 2$, $X_2 = (U_2 - 32.5) / 5$, $X_4 = (U_4 - 0.3) / 0.15$

Table 5. Results of CCD regression analysis for alginate lyase production

Source	SS	MS	F	P
Model	14161.88	1573.54	6.13	0.0045
X_1	2456.84	2456.84	9.57	0.0114
X_2	158.35	158.35	0.62	0.4504
X_4	23.50	23.50	0.092	0.7684
X_1^2	2927.49	2927.49	11.40	0.0070
X_2^2	4423.33	4423.33	17.23	0.0020
X_4^2	5654.71	5654.71	22.03	0.0009
X_1X_2	311.37	311.37	1.21	0.2966
X_1X_4	176.62	176.62	0.69	0.4262
X_2X_4	122.49	122.49	0.48	0.5054
Residual	2567.09	256.71		
Lack of Fit	1563.90	312.78	1.56	0.3190
Pure Error	1003.19	200.64		
Cor Total	16728.97			

Central composite design

According to the above experiments, the key factors and their central points chosen for CCD were (g/L): $(\text{NH}_4)_2\text{HPO}_4$ 10.0, NaCl 32.5 and MgSO_4 0.30, respectively. The lowest and the highest concentrations were (g/L): $(\text{NH}_4)_2\text{HPO}_4$, 6.64 and 13.36; NaCl, 24.09 and 40.91, and FeSO_4 , 0.048 and 0.55, respectively. Table 4 shows the CCD design and responses for the production of alginate lyase. And the ANOVA of CCD is shown in Table 5. The

Model F-value of 6.12 implies the model is significant. There is only a 0.46% chance that a "Model F-Value" this large could occur due to noise. X_1 , X_1^2 , X_2^2 , and X_4^2 are significant model terms. Final equation in terms of coded factors is as followed, describing the relationship between the production (Y) and the test variables in coded factors (the concentrations of $(\text{NH}_4)_2\text{HPO}_4$, NaCl and MgSO_4 , respectively):

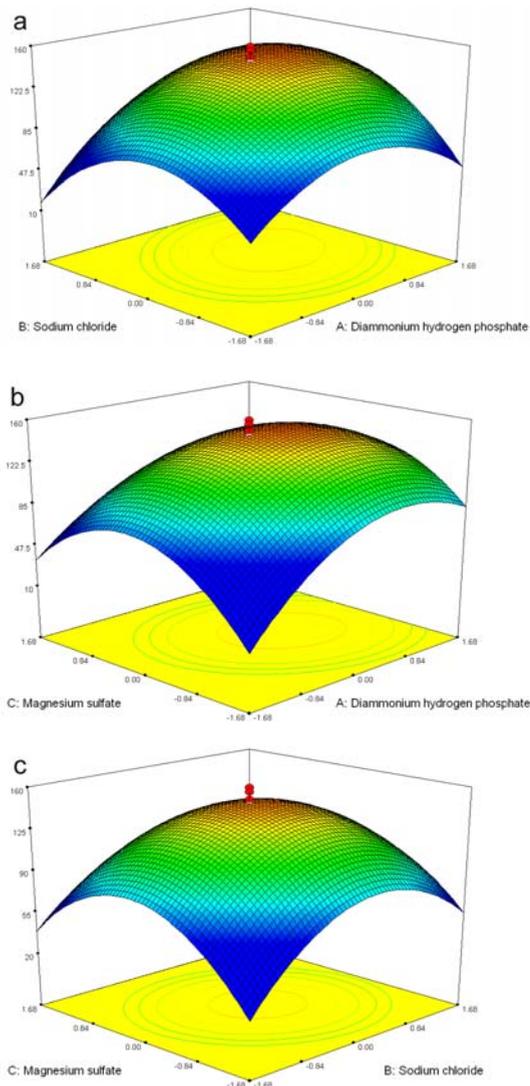
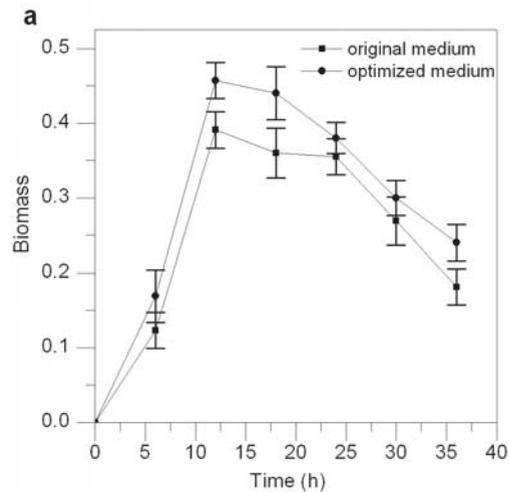
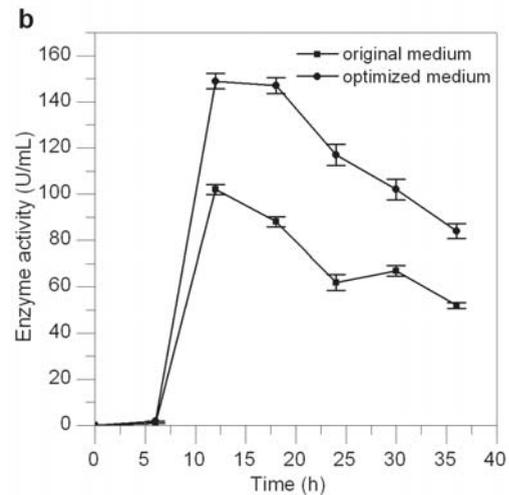


Fig. 1. Three dimensional response surface plots showing the effect of three factors on alginate lyase production. (a) interaction between NaCl and $(\text{NH}_4)_2\text{HPO}_4$, (b) interaction between MgSO_4 and $(\text{NH}_4)_2\text{HPO}_4$, (c) interaction between MgSO_4 sulfate and NaCl



(a) curve of growth,



(b) curve of alginate lyase production

Fig. 2. Curve of growth and enzyme activity of strain QZ-4 in a 36h fermentation period.

$$Y = 144.45 + 13.41X_1 + 3.41X_2 - 1.31X_4 - 14.25X_1^2 - 17.52X_2^2 - 19.81X_4^2 + 6.24X_1X_2 - 4.70X_1X_4 - 3.91X_2X_4 \dots (3)$$

The coefficient of each square item in Eq.(3) is negative, indicating that the parabola opens downwards and has a maximum value. When Y equals the maximum value, the coded values are: X_1 0.531, X_2 0.204, X_4 -1.077. That is, when the corresponding values of variables are (g/L): $(\text{NH}_4)_2\text{HPO}_4$ 11.6, NaCl 32.9, MgSO_4 0.08, respectively, the theoretical maximum of enzyme production is 144.45 U/mL. As shown in Figure 1a-c, the response surface plots obtained represent the interaction between each pair of three factors and indicate their effect on alginate lyase yield.

Three steps above were applied to find the optimal conditions for the design factors. A FFD was used to determine the effect of each constituent on production of alginate lyase. Then steepest ascent experiment was used to approach the optimum condition to produce alginate lyase. Finally, a CCD was applied to estimate the true model for the response and adjust the levels of media constituents. The model prediction of alginate lyase yield was verified repeatedly by batch fermentation using the optimized medium on the flask scale (Figure 2a,b), and also in a 10-L fermentation tank later. As shown in Figure 2b, the observed yield of alginate lyase (149.5 U/mL) is in agreement with the predicted value and increases distinctly in the logarithmic phase compared with former data using original culture medium. However the biomass of both culture are relatively consistent (Figure 2a). It is shown that the optimized culture medium enhances the yield of alginate lyase by improving secretion and/or production of alginate lyase rather than encouraging cell growth. It is assumed that the production of extracellular enzymes from marine bacteria is affected by the concentration of NaCl. As to the marine strain, *P. tetraodonis* QZ-4, the results reveal its high nutrient demand for $(\text{NH}_4)_2\text{HPO}_4$ (11.6 g/L) and NaCl (32.9 g/L) to produce alginate lyase and maintain cell growth, whereas most of alginate lyase producing bacteria prefer $(\text{NH}_4)_2\text{SO}_4$ to be inorganic nitrogen source. Also the salt tolerance of the strain QZ-4 far exceeds the original salinity of the ocean water sample, which is 25.5‰ (w/w) in the sampling season according to statistics.

CONCLUSION

The two or three-step optimization is a valuable tool for rapid evaluation of the effects of different medium constituents. It indicates the importance of each constituent and the trend of its affection, and determines the exact quantity of each constituent required for the medium²³. In this paper, we found that $(\text{NH}_4)_2\text{HPO}_4$, NaCl and MgSO_4 are the three most important factors in the medium, and the medium containing 5 g/L of Alginate sodium, 11.6 g/L $(\text{NH}_4)_2\text{HPO}_4$, 32.9 g/L NaCl, 0.03 g/L FeSO_4 , 0.08 g/L MgSO_4 and 0.4 g/L K_2HPO_4 should be used to maximize alginate lyase yield under the conditions studied. This medium composition resulted in an enzyme yield of 149.5 U/mL, which is 50 percent higher than the result obtained using previous culture medium after single factor optimization.

ACKNOWLEDGMENTS

The research was supported by the National Sparking plan project (2012GA701063) and QianJiang Personnel Plan (2011R10072). The authors are also thankful for the support received from Dr. Ting Yu, Dr. Chenggang Cai and Dr. Yifei Wang.

REFERENCES

1. Haug, A., Larsen, B., Smidsr, O. Studies on the Sequence of Uronic Acid Residues in Alginic Acid. *Acta Chem. Scand.*, 1967; **21**: 691-704.
2. Murata, K., Inose, T., Hisano, T., Abe, S., Yonemoto, Y., Yamashita, T., Takagi, M., Sakaguchi, K., Kimura, A., et al. Bacterial alginate lyase: enzymology, genetics and application. *J. Ferment. Bioeng.*, 1993; **76**(5): 427-37.
3. Preiss, J., Ashwell, G. Alginic acid metabolism in bacteria. I. Enzymatic formation of unsaturated oligosaccharides and 4-deoxy-L-erythro-5-hexoseulose uronic acid. *J. Biol. Chem.*, 1962; **237**: 309-16.
4. Wong, T.Y., Preston, L.A., Schiller, N.L. Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. *Annu. Rev. Microbiol.*, 2000; **54**: 289-340.
5. Liu, G, Yue, L., Chi, Z., Yu, W., Chi, Z., Madzak, C. The Surface Display of the Alginate Lyase on

- the Cells of *Yarrowia lipolytica* for Hydrolysis of Alginate. *Mar. Biotechnol.*, 2009; **11**(5): 619-26.
6. Inoue, A., Kagaya, M., Ojima, T. Preparation of protoplasts from *Laminaria japonica* using native and recombinant abalone alginate lyases. *J. Appl. Phycol.*, 2008; **20**(5): 633-40.
 7. Alkawash, M.A., Soothill, J.S., Schiller, N.L. Alginate lyase enhances antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. *Apmis*, 2006; **114**(2): 131-8.
 8. Yue, M., Ding, H., Qiao, Y. Expression of *Pseudomonas aeruginosa* alginate lyase gene (algL) in *Pichia pastoris* and characterization of the enzyme. *China Agr. Sci.*, 2008; **41**(4): 1192-8.
 9. Kim, D.E., Lee, E.Y., Kim, H.S. Cloning and Characterization of Alginate Lyase from a Marine Bacterium *Streptomyces* sp. ALG-5. *Mar. Biotechnol.*, 2009; **11**(1): 10-6.
 10. Chaki, T., Baba, T., Hiura, N., Kobayashi, M. Purification and characterization of alginate lyase from *Pseudoalteromonas* sp. strain No. 1786. *J. Appl. Glycosci.*, 2008; **55**(2): 81-8.
 11. Nevot, M., Deroncele, V., Montes, M.J., Mercade, E. Effect of incubation temperature on growth parameters of *Pseudoalteromonas antarctica* NF₃ and its production of extracellular polymeric substancesdagger. *J. Appl. Microbiol.*, 2008; **105**(1): 255-63.
 12. Gomez, D., Espinosa, E., Bertazzo, M., Lucas-Elio, P., Solano, F., Sanchez-Amat, A. The macromolecule with antimicrobial activity synthesized by *Pseudoalteromonas luteoviolacea* strains is an L-amino acid oxidase. *Appl. Microbiol. Biot.*, 2008; **79**(6): 925-30.
 13. Nakatani, Y., Lamont, I.L., Cutfield, J.F. Discovery and Characterization of a Distinctive Exo-1,3/1,4-b-Glucanase from the Marine Bacterium *Pseudoalteromonas* sp. Strain BB1. *Appl. Environ. Microb.*, 2010; **76**(20): 6760-8.
 14. Zhou, M.H., Ma, J.S., Li, J., Ye, H.R., Huang, K.X., Zhao, X.W. A κ -carrageenase from a newly isolated *pseudoalteromonas*-like bacterium, WZUC10. *Biotechnol. Bioproc. Eng.*, 2008; **13**(5): 545-51.
 15. Matsushima, R., Watanabe, R., Tsuda, M., Suzuki, T. Analysis of Extracellular Alginate Lyase (alyA) Expression and its Regulatory Region in a Marine Bacterial Strain, *Pseudoalteromonas atlantica* AR06, Using a *gfp* Gene Reporter System. *Mar. Biotechnol.*, 2012; DOI 10.1007/s10126-012-9488-6.
 16. Alekseeva, S.A., Bakunina, I.Y., Nedashkovskaya, O.I., Isakov, V.V., Mikhailov, V.V., Zvyagintseva, T.N. Intracellular alginolytic enzymes of the marine bacterium *Pseudoalteromonas citrea* KMM 3297. *Biochemistry-moscow*, 2004; **69**(3): 262-9.
 17. Choudhury, A.R., Bhattacharyya, M.S., Prasad, G.S. Application of response surface methodology to understand the interaction of media components during pullulan production by *Aureobasidium pullulans* RBF-4A3. *Biocatal. Agr. Biotechnol.*, 2012; **1**(3): 232-7.
 18. Walia, A., Mehta, P., Chauhan, A., Shirkot, C.K. Optimization of cellulase-free xylanase production by alkalophilic *Cellulosimicrobium* sp. CKMX1 in solid-state fermentation of apple pomace using central composite design and response surface methodology. *Ann. Microbiol.*, 2012; DOI 10.1007/s13213-012-0460-5.
 19. Song, D., Chen, Y., Zhu, M., Gu, Q. Optimization of Bacteriocin Production by a New *Lactobacillus* Strain, ZJ317, using Response Surface Methodology. *J. Pure Appl. Microbiol.*, 2012; **6**(4): 1507-15.
 20. Yoon, H.J., Hashimoto, W., Miyake, O., Okamoto, M., Mikami, B., Murata, K. Overexpression in *Escherichia coli*, purification, and characterization of *Sphingomonas* sp. A1 alginate lyases. *Protein Expres. Purif.*, 2000; **19**(1): 84-90.
 21. Scott, M.K., Connie, M.B., Douglas, C.M. A Modified Path of Steepest Ascent for Split-Plot Experiments. *J. Qual. Technol.*, 2005; **37**(1): 75-83.
 22. Murray, R.S.(ed): Principles of Experimental Design for the Life Sciences, 1st edn. Boca Raton: CRC Press, 1996; pp 116.
 23. Rajendhran, J., Krishnakumar, V., Gunasekaran, P. Optimization of a fermentation medium for the production of Penicillin G acylase from *Bacillus* sp. *Lett. Appl. Microbiol.*, 2002; **35**: 523-7.