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Production of enzymes by *Alteromonas* sp. A321 to degrade polysaccharides from *Enteromorpha prolifera*

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ABSTRACT

Polysaccharides from *Enteromorpha prolifera* (PE) are becoming increasingly popular due to its bioactivity and abundant source. Screening novel microorganisms which could secrete enzymes to degrade PE efficiently for oligosaccharides production is a promising solution to improve its application. In this study, a marine bacterium that can produce enzymes to degrade PE specifically was selected. It was identified as *Alteromonas* sp. A321, based on the biochemical properties and 16S rDNA gene sequencing. In order to maximize the activity of degradase for polysaccharides from *E. prolifera* (DPE), the effects of medium composition and culture conditions were investigated. The highest DPE production was obtained in the medium consisting of K_2HPO_4 0.15%, PE 0.9%, $NaNO_3$ 0.4%, NaCl 1.0% and $MgSO_4$ 0.05%. The degradase activity was enhanced from original 0.391 U/ml to 0.744 U/ml. DPE show high efficiency and substrate specificity to PE with 63.53% of reducing sugar production in the 7 h hydrolysis.

1. Introduction

Enteromorpha prolifera is distributed worldwide from the intertidal to the upper subtidal zones, which is one of the most common fouling green algae (Lin, Shen, & Wang, 2008; Zhao et al., 2011). *E. prolifera* is effective for eliminating inflammation and clearing away heat and detoxifying (Jiao et al., 2009). In the past, it was utilized for edible and medical uses by residents of the coastal districts in many countries such as China, Japan, USA, France, and Chile (Aguilera-Morales, Casas-Valdez, Carrillo-Domínguez, González-Acosta, & Pérez-Gil, 2005).

E. prolifera has been shown to consist of 43.4–60.2% as carbohydrate, 16–22.1% as protein, 12.4–18.7% as ash in the component of its dry matter (Mamatha, Namitha, Senthil, Smitha, & Ravishankar, 2007). Therefore, carbohydrate accounts for the main composition of *E. prolifera.* Recent studies on *E. prolifera* were mainly focused on its polysaccharides in the aspect of structure feature and bioactive characterization. According to the research by Qi, Mao, Gao, and Chen (2012), the polysaccharides from *E. prolifera* (PE) consisted of xylose, galactose, arabinose, rhamnose and glucose, having the linkages of (1→4)-linked β-L-arabinopyranose residues with partially sulfate groups at the C-3 position. Based on its unique structure, many researches on its bioactivities are reported subsequently. Jiao et al. (2009) reported that PE has anti-tumor activity and that activity was exerted by enhancement of immune system instead of direct cytotoxicity. Kim, Lae Cho, Kamjanapratum, Shin, and You (2011) suggested that PE are strong immunostimulators because they significantly increased Con A-induced splenocyte proliferation and IFN-γ, IL-2 secretions. The investigation by Qi et al. (2012) demonstrated that PE could be a potential source of anticoagulant due to its activity to prolong thromboplastin time and thrombin time. Zhang et al. (2010) reported that PE possessed antioxidant activities which may be used as a possible supplement in food and pharmaceutical industries.

As the molecular weight of the PE ranges from 55 kDa to 511 kDa (Qi et al., 2012; Ray, 2006), the limited solubility and high viscosity restrict their further application. Therefore, transformation of PE to oligosaccharides of low viscosity and good digestion has attracted the interest of many researchers. Transformation by chemical hydrolysis has many drawbacks for oligosaccharides production, including low yields of oligosaccharides, high cost of separation, and environmental pollution (Wang, Liu, & Liang, 2012). Alternatively, enzymatic hydrolysis has become popular in recent years due to its advantages such as high efficiency, environmental

Abbreviations: PE, polysaccharides from *Enteromorpha prolifera*; DPE, degrdase for polysaccharides from *Enteromorpha prolifera.*

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compatibility and reproducibility (Liang, Hsieh, & Wang, 2012). Up to now, in the aspect of enzymatic application for algal polysaccharides degradase such as carrageenanase, alginatase, chitinases, and fucoidanase have been reported (Jouanneau, Boulenguer, Mazoyer, & Helbert, 2010; Li, Jiang, Guan, & Wang, 2011; Wang et al., 2012; Yu et al., 2013). However, there is scarcity in the report on efficient degradation of PE. Thus, selection of microbial enzymes of high specificity to degrade PE becomes a challenging and attractive task.

The objectives of this study were: (1) screening degradase for polysaccharides from *E. polifera* (DPE) producing bacterium and completing its identification, and (2) optimizing the conditions for degradases production. Although there are many literatures about the production and optimization of various enzymes to degrade marine-derived polysaccharides, to the best of our knowledge this is the first report on a newly isolated marine bacterium which produces enzymes to degrade PE specifically. This finding also provides an access to research in the structure of PE.

2. Materials and methods

E. prolifera was collected from the coasts of Qingdao, China. The PE was extracted with the improved method as described by Qi et al. (2012). The milled alga (80 g) was dipped into 60 volumes of tap water, homogenized and refluxed at 90 °C for 7 h. The supernatant was collected by centrifugation (4800 × *g*, 15 min) and concentrated, dialyzed in a cellulose membrane (molecular weight cut-off 3500) against flowing distilled water. The collection was precipitated by adding three volumes of 95% ethanol (v/v) and dried. The crude polysaccharides were dissolved in distilled water and used as the carbon source for the screening and isolation of DPE producing bacteria. All other reagents were of analytical grade and commercially available.

2.1. Screening and isolation of DPE producing bacteria

In our laboratory DPE producing bacteria were isolated from the surfaces of decayed *E. prolifera* and sediments samples collected from the coasts of Qingdao, China. The preliminary screening was carried out on a medium containing 5 g extracted crude PE and 10 g agar (Sigma, American) per 1000 ml mineral-salts medium (MM). The composition of MM was (g/L): K_2HPO_4 1.5, $NaNO_3$ 1.0, NaCl 15, and $MgSO_4$ 0.5. The initial pH of the medium was neutral. Colonies that formed clear zone on the screening plate were selected and purified further by the same plating method. Then all purified colonies were flask-cultured and enumerated in the medium containing 5 g PE as the sole carbon source per 1000 ml MM. Flasks were incubated on a rotary shaker (170 rpm min^{-1}; ASRS-124, Accurate Scientific Instrument, India) at 28 °C for 48 h. The liquid culture was centrifuged at 4000 × *g* for 15 min. The supernatant was used as the crude extracellular DPE. Finally, the bacteria exhibiting higher polysaccharidelytic activity was chosen and named strain A321.

2.2. Morphological, physiological and biochemical characterizations of the isolated bacteria

Temperature range for bacterial growth was determined to be from 4 °C to 40 °C. Range of pH for bacterial growth was between 5.0 and 11.0. Besides the Gram staining test under photomicroscopy, morphological trait of the isolated strain was observed under a scanning electron microscope. Phenotypic analysis and various biochemical tests were carried out by following what was described in Bergey's Manual of Systematic Bacteriology (second edition; 2004). Each experiment was carried out in triplicate.

Along with these assays, molecular identification of the microorganism was also performed by extracting, amplifying and sequencing 16S rDNA of the isolate. The genomic DNA for 16S rDNA sequencing analysis was extracted by using a DNA extraction kit according to the manufacturer's instructions (Sigma, American). The amplification of the 16S rDNA was conducted by using 5′-AGAGTTTGATCMTGCTCAG-3′ and 5′-ACGGCTACCTTGTTACGACTT-3′ as the primers in a thermal cycle (9700, ABI, USA) under the following conditions: 2 min at 94 °C, 30 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C, and one final step of 10 min at 72 °C. The amplified PCR products containing the 16S rDNA were sequenced by Sangon Biotech Corp., Ltd. (Shanghai, China). A comparison of nucleotide sequences was performed using the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) at the National Center for Biotechnology Information (NCBI, USA). Sequences were aligned using the program CLUSTAL.W. and a phylogenetic tree was made using the MEGA 5.05 program.

2.3. Enzyme assay

Degradase activity was determined as release of reducing sugar from the enzymatic hydrolysis of PE by using the DNS method under the assay conditions (hydrolysis at pH 5.5 and 35 °C) (Fenice, Selbmann, Zucconi, & Onofri, 1997). One unit (U) of degradase activity was defined as the amount of enzyme required to produce 1 μmol reducing sugar (glucose equivalent) in 1 min under the aforementioned conditions.

2.4. Optimization procedure

2.4.1. Effect of physical parameters on DPE production

One loopful of culture from the preliminary medium slant was transferred aseptically to shake flask medium and incubated for 24 h at 170 rpm at 28 °C. This was used as the seed. The seed was inoculated into the MM supplemented with PE (0.5%, w/v) and incubated on a rotary shaker with 170 rpm at different temperatures such as 24, 28, 32, and 37 °C. The effect of pH value (5.0–9.0) on the production of DPE by strain A321 was also studied. The degradase activity was assayed by the methods described earlier.

2.4.2. Effect of carbon and nitrogen sources on DPE production

The A321 was grown in the MM supplemented with different carbon sources (0.5%, w/v) including glucose, sucrose, starch, PE, and corn meal. The effect of organic nitrogen sources (0.2%, w/v) including peptone, yeast extraction, soybean meal, beef extract and inorganic nitrogen sources (0.1%, w/v) including NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, urea on DPE production was also studied.

2.4.3. Optimization by response surface methodology

Response surface methodology (RSM) was used to optimize the screening variables for enhanced DPE production using a central composite design (CCD). The significant variables utilized were PE, $NaNO_3$ and NaCl, each at five coded levels (−1.682, −1, 0, +1, +1.682) as shown in Table 1. The response values (Y) in each trial were the average of the duplicate samples.

2.4.4. Growth curve and changes in degradase activity during fermentation process

One ml seed culture was inoculated into a fresh 100 ml Erlenmeyer flask containing 20 ml optimized medium. The fermentation process was conducted under the optimized culture condition. Bacterial growth at regular periods was measured spectrophotometrically by monitoring the optical density at 600 nm and degradase activity was assayed simultaneously via procedure described earlier. Fitting of the growth curve and DPE production rate were plotted with the software Origin 8.0.

Table 1
Central composite design matrix for the experimental design and predicted response for degradase activity.

Run	Coded level			Degradase activity (U/ml)	
	X_1 (PE)	X_2 ($NaNO_3$)	X_3 (NaCl)	Actual value	Predicted value
1	0 (6 g/L)	0 (2.5 g/L)	0 (22 g/L)	0.53	0.55
2	0	0	0	0.55	0.55
3	−1 (3 g/L)	1 (4 g/L)	1 (34 g/L)	0.41	0.43
4	−1	1	−1 (10 g/L)	0.39	0.38
5	−1	−1 (1 g/L)	−1	0.36	0.39
6	1.68 (11.05 g/L)	0	0	0.56	0.59
7	0	0	−1.68 (0.2 g/L)	0.61	0.61
8	−1.68 (0.95 g/L)	0	1	0.23	0.20
9	1 (9 g/L)	−1	−1	0.62	0.59
10	1	−1	1	0.47	0.46
11	0	0	0	0.54	0.55
12	0	0	0	0.55	0.55
13	1	1	1	0.74	0.70
14	0	1.68 (5.02 g/L)	0	0.62	0.63
15	0	0	0	0.57	0.55
16	1	1	−1	0.71	0.71
17	0	0	1.68 (42.2 g/L)	0.55	0.55
18	0	−1.68 (0 g/L)	0	0.42	0.43
19	−1	−1	1	0.34	0.33
20	0	0	0	0.55	0.55

PE: polysaccharides from *Enteromorpha prolifera*.

2.5. Enzymatic hydrolysis

The crude PE was hydrolyzed by the DPE (120 U/g PE), pectinase (120 U/g PE), and cellulase (30 FPU/g) in stoppered Erlenmeyer flasks. The hydrolysis was performed in 0.1 M phosphate buffer (pH 7.0 for DPE, pH 5.0 for pectinase, and pH 4.0 for cellulase) with 0.9% (w/v) substrate at 35 °C. The duration of the enzymatic reactions was 9 h, and the content of reducing sugar was monitored every 2 h. The percent reducing sugar was calculated as

$$\%\,\text{reducing sugar} = \left(\frac{\text{mg of reducing sugars released}}{\text{mg of PE substrate}}\right) \times 100$$

3. Results and discussion

3.1. Isolation and screening of DPE producers

The unique habitats in coastal environment provide microbes with opportunities to develop novel capabilities for survival and for the production of metabolites not found in terrestrial environments (Bernan, Greenstein, & Maiese, 1997). Decayed *E. prolifera* samples were selected in this study to isolate DPE producing bacteria. With enrichment culture technique, 62 different bacteria were able to form clear zone on the screening plates. The colonies of the bacteria were cream, yellow, red, and white, respectively. Shake flask medium was used to select the bacteria that could produce enzymes to significantly degrade PE. Among the final candidates, strain A321 was selected for further experimentation because it exhibited the highest and steadily polysaccharidelytic activity. The colonies of the A321 were convex, round, moist and dense with smooth surfaces on the screening plates. The diameter of the colonies of 24 h culture was about 2–3 mm. The liquid culture of strain A321 appeared yellow in a shaking flask.

3.2. Morphological, physiological and biochemical characterizations of strain A321

Strain A321 was able to grow in the temperature range of 10–40 °C and the optimum temperature was 28 °C (data not shown). The optimum pH for strain A321 was 7.0 and the strain could grow in the pH range of 4–10. Strain A321 was found to be Gram negative bacteria. They are rod-shaped with the size of 0.5–0.6 μm × 1.9–2.2 μm. Detailed physiological characteristics of strain A321 are shown in Table 2. The starch hydrolysis was positive, as well as those for α-glucose, ammonium salt, nitrate reduction. As for the other tests, including the V.P. test, the M.R. test and indole production, results were negative. Strain A321 could not utilize D-Glucose, D-mannitose, D-mannitol, L-arbaitol, cellobiose, sucrose, and D-maltose. These data indicated that strain A321 belonged to genus *Alteromonas*.

3.3. 16S rDNA sequence determination

PCR products of 16S rDNA from strain A321 (1413 bp) were sequenced. Physiological study on the strain A321 was performed using the BIOLOG system (Biology, Inc., USA). Alignment of this sequence with sequences in the NCBI database indicated that 16S rDNA of strain A321 was significantly similar to that of *Alteromonas* species, such as strain BG20 (accession no.: EU714266.1 98%), strain YSN412 (accession no.: JN622161.1 95%), strain X3 (accession no.: GU975828.1 94%). Therefore, based on 16S rDNA sequence, strain A321 belonged to genus *Alteromonas*.

The phylogenetic tree was constructed according to the 16S rDNA sequence (Fig. 1). Since its sequence was similar to most *Alteromonas* sp. result of phylogenetic analysis was consistent with those of the phenotypic tests. Therefore, strain A321 was named *Alteromonas* sp. A321. It was deposited in China Center for type culture collection (CCTCC no: M2012132).

Table 2
Biophysiological-biochemical properties of strain A321.

Characteristic	Properties	Characteristic	Properties
Starch hydrolysis	+	α-Glucose	+
H_2S	–	D-Glucose	–
Gelatin liquefaction	–	D-Mannose	–
Spore	–	D-Mannitol	–
Utilization of citrate	–	L-Arbaitol	–
Indol test	–	Cellubiose	–
V.P. test	–	Sucrose	–
M.R. test	–	D-Maltose	–
Ammonium salt	+		
Nitrate reduction	+		

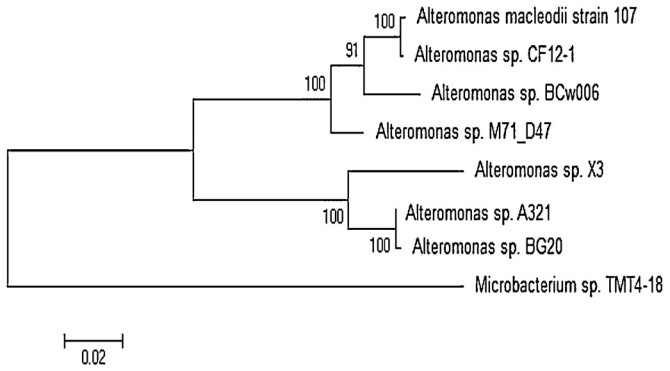

Fig. 1. Location of bacterium A321 in the phylogenetic tree based on the comparison of 16S rRNA gene sequence. The phylogenetic tree was generated using the maximum likelihood method.

3.4. Optimization procedure

3.4.1. Effect of physical parameters on DPE production

Results of the test on optimizing the incubation temperature for DPE production from *Alteromonas* sp. A321 revealed that the production gradually increased from 24 to 32 °C (Fig. 2A) and the maximal DPE production occurred at 32 °C. Increase in temperature beyond 32 °C had an adverse effect on the DPE production. Although the physiological changes induced by higher temperatures are not completely understood, it has been suggested that at high temperatures microorganisms may synthesize only a reduced number of proteins essential for growth and other vital physiological processes (Gawande & Kamat, 1999).

The pH of the medium is one critical environmental parameter affecting the cell growth, enzyme production and the transport of various components across the cell membrane (Kapoor, Nair, & Kuhda, 2008). *Alteromonas* sp. A321 produced maximum DPE at initial medium pH 7.3 (Fig. 2B). The decrease in the initial pH of the medium from 7.3 to 4.0 showed a significant decrease in the production of DPE. A loss of more than 60% of DPE production was observed at medium pH of 4.0. Increasing the initial pH of the medium from 7.3 to 9.0 showed a slight decrease in the production of DPE. The results implied that DPE production by *Alteromonas* sp. A321 was less susceptible to the alkaline condition than the acidic condition.

3.4.2. Effect of carbon and nitrogen sources on DPE production

Production of extracellular enzyme depends heavily on the composition of the medium (Deswal, Pal Khasa, & Kuhad, 2011). The influence of carbon sources on DPE production by *Alteromonas* sp. A321 was depicted in Fig. 2C. Among the tested carbon sources, PE was found to induce the maximum amount of DPE production. Although strain A321 could utilize other carbon sources for cell growth (date not show), DPE production would remain at the low level. The production of DPE decreased to about 20% of the maximum (i.e., PE), respectively, when other saccharides were used as the sole carbon source. The result indicated that PE was the most effective carbon source for DPE production by strain A321. It also implied that polysaccharides in the screening medium is usual necessary to induce high production of the enzymes to degrade the characteristic polysaccharides. Similar phenomenon has been reported in researches to obtain the highest production of α-agarase, chitinase, κ-carrageenase, and alginase (An et al., 2008; Gohel, Chaudhary, Vyas, & Chhatpar, 2006; Hassairi, Ben Amar, Nonus, & Gupta, 2001; Khambhaty, Mody, Jha, & Gohel, 2007).

In microorganisms, nitrogen sources (both organic and inorganic forms) are metabolized, primarily resulting in the production of amino acids, nucleic acids, proteins, and cell wall components (Reddy, Wee, Yun, & Ryu, 2008). Thus the effect of commonly used nitrogen sources on DPE production by A321 was studied (Fig. 2D). Both organic and inorganic nitrogen sources had positive effects on DPE production compared to control. In the decreasing influential order: $NaNO_3$ > beef extract > peptone > yeast extract > $(NH_4)_2SO_4$ > soybean meal > NH_4NO_3 > NH_4Cl > urea. It suggests among various nitrogen sources $NaNO_3$ induced maximum DPE production. Consequently, $NaNO_3$ is the best nitrogen source for DPE production in our study. Preference for nitrogen source varies with microorganisms. The highest κ-carrageenase

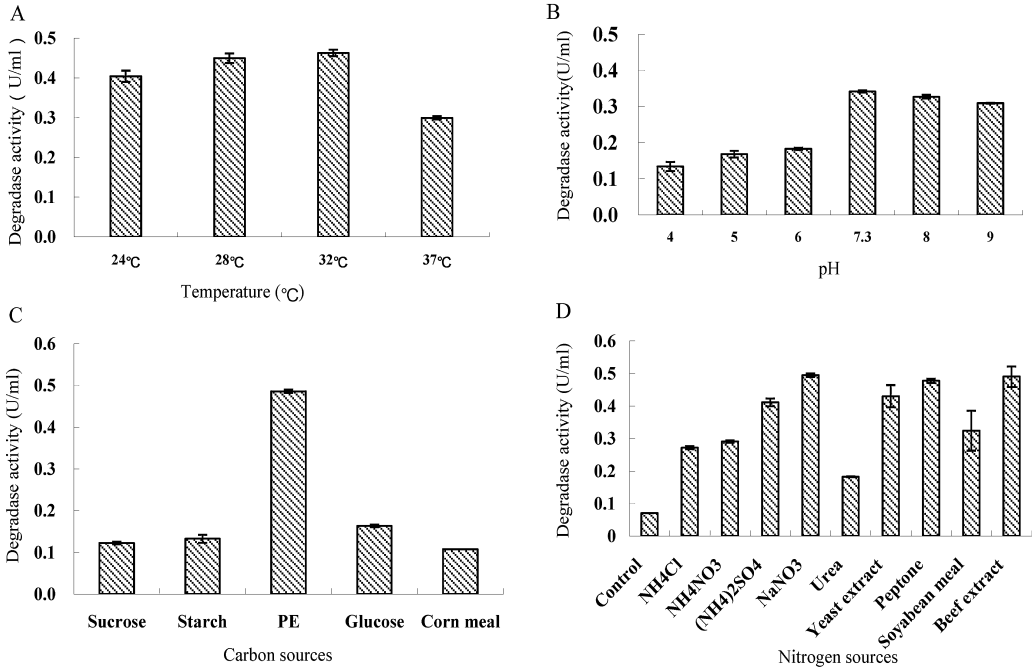

Fig. 2. Effect of culture condition and culture medium on DPE production. (A) The effect of temperature on DPE production. (B) The effect of pH on DPE production. (C) The effect of carbon sources on DPE production. (D) The effect of nitrogen sources on DPE production.

Table 3
ANOVA for response surface quadratic model for DPE production.

Factors	Statistics			
	Sum of squares	Mean square	*F*-Value	Prob > *F*
Model	0.30	0.033	47.37	<0.0001
X_1-PE	0.19	0.19	265.02	<0.0001
X_2-$NaNO_3$	0.046	0.046	65.32	<0.0001
X_3-NaCl	4.34E−0.03	4.34E−0.03	6.20	0.0320
X_1X_2	8.58E−0.03	8.58E−0.03	12.26	0.0057
X_1X_3	2.11E−003	2.11E−003	3.02	0.1130
X_2X_3	6.73E−003	6.73E−003	9.61	0.0112
X_1^2	0.041	0.041	58.46	<0.0001
X_2^2	7.99E−004	7.99E−004	1.14	0.3104
X_3^2	2.15E−003	2.15E−003	3.08	0.1099
Lack of fit	6.11E−003	6.11E−003	6.89	0.0269

PE: polysaccharides from *Enteromorpha prolifera.*

production was obtained when yeast extract was used as the nitrogen source by *Pseudomonas elongata* (Khambhaty et al., 2007), whereas peptone was the optimal nitrogen source for nattokinase production by *Bacillus subtilis* (Deepak et al., 2008).

3.4.3. Optimization of culture conditions for DPE production by CCD

Based on the above mentioned results, PE, $NaNO_3$, and NaCl were chosen as the key factors for maximizing the production of DPE. The optimal level of the key factors and the effects of their interactions on DPE production were further explored by the CCD of RSM. Based on preliminary experiments (data not shown), the levels of media components were set to produce the maximum yields of DPE (Table 1).

The design matrix and the corresponding results of RSM experiments to determine the effects of three independent variables (PE, $NaNO_3$, and NaCl) are shown in Table 1 along with the mean predicted values. The analysis of variance (ANOVA) was conducted to test the significance of the fit of the second-order polynomial equation for the experimental data (Table 3). The model terms, X_1, X_2, X_3, X_1X_2, X_2X_3, X_1^2 were significant ($p < 0.05$). The linear effects of PE and quadratic term PE square ($p < 0.001$) were determined to be more significant than the effects of the other variables. These results indicate that the concentration of carbon source bears a direct relationship to DPE production. The interactions between PE and $NaNO_3$, $NaNO_3$ and NaCl were significant, as shown by the low *p*-value (<0.05) for the interactive terms. The model *F*-value was 47.37, which implied the model is significant. There is only a 0.010% chance that a "Model *F*-value" could occur due to noise.

The responses of the CCD were fitted with a second-order polynomial equation

$$Y = +0.55 + 0.12X_1 + 0.058X_2 - 0.018X_3 + 0.032X_1X_2 - 0.016X_1X_3 + 0.029X_2X_3 - 0.053X_1^2 - 7.498E - 003X_2^2 + 0.012X_3^2 \quad (1)$$

where *Y* is the degradase activity; X_1, X_2 and X_3 are the code values of PE, $NaNO_3$ and NaCl, respectively.

For a good statistical model, the R^2 value should be in the range of 0–1.0, and the closer the value is to 1.0, the stronger the model is established and the closer it predicts the response (Reddy et al., 2008). The regression equation (Eq. (1)) obtained from the ANOVA showed that the R^2 was 0.9771. The R^2 value of this model indicates a good agreement between experimental and predicted values, and implies that mathematical model is reliable for computing DPE production in the present study. On the other hand, the adjusted and predicted R^2 are 0.9565 and 0.8311, respectively. The high degree of similarity between the predicted and experimental values reflects the accuracy and applicability of RSM to optimize the process for enzymes production.

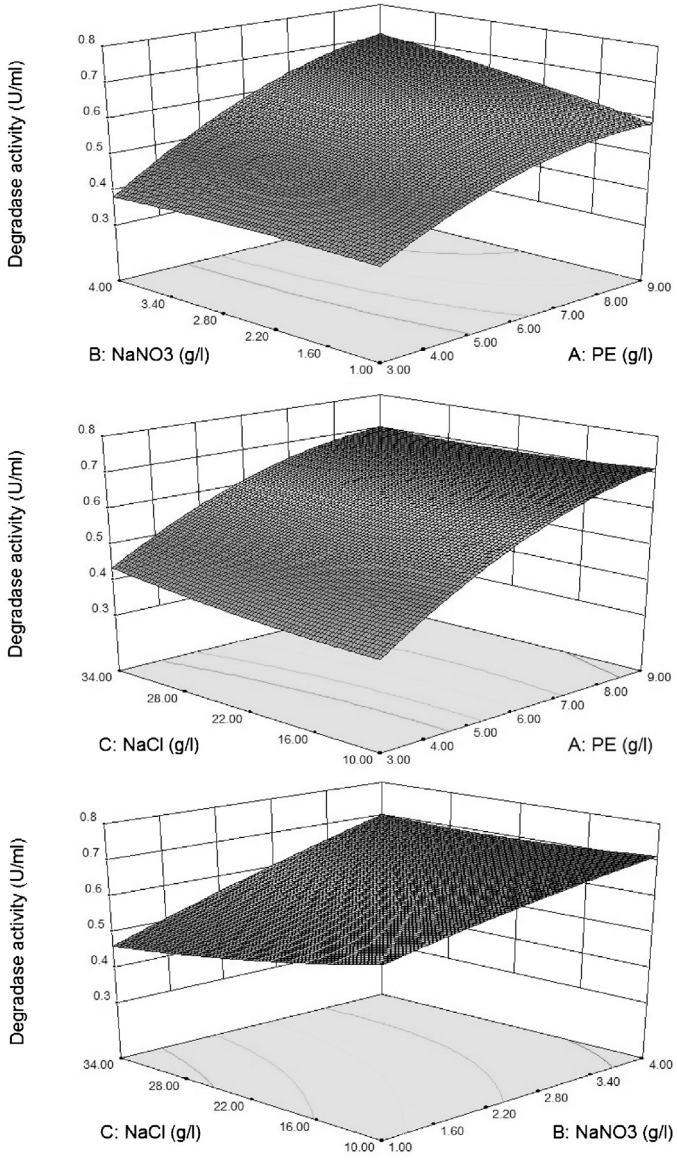

Fig. 3. Response surface plots of degradase production by *Alteromonas* sp. A321. (A) Interactive effect of PE and $NaNO_3$ on degradase production at fixed NaCl of 2.2%. (B) Interactive effect of NaCl and PE on degradase production at fixed $NaNO_3$ concentration of 0.25%. (C) Interactive effect of NaCl and $NaNO_3$ on degradase production at fixed PE concentration of 0.6%.

Response surface plots and contour plots are shown in Fig. 3A–C. The contour plots are not perfectly elliptical and are rather horizontal. Reddy et al. (2008) explained the similar phenomenon they observed by indicating that there may be less interaction occurring among the independent variables corresponding to the response surfaces. As shown in Fig. 3A, a linear increase in DPE production was observed when PE and $NaNO_3$ concentration were increased. Result of Fig. 3B shows the increase of DPE production is significant with an increase in the amount of PE to the optimum level. On the contrary, the effect of NaCl concentration (not statistically significant as reported by the model) on DPE production was not evident. A similar profile was observed in Fig. 3C with respect to $NaNO_3$ and NaCl concentration. Corresponding to the maximum DPE production of 0.709 U/ml, the optimal levels of the three variables determined by Design Expert 8.0 were PE 0.9%, $NaNO_3$ 0.4%, and NaCl 1.0%, respectively. Conversely, lower concentration of PE and

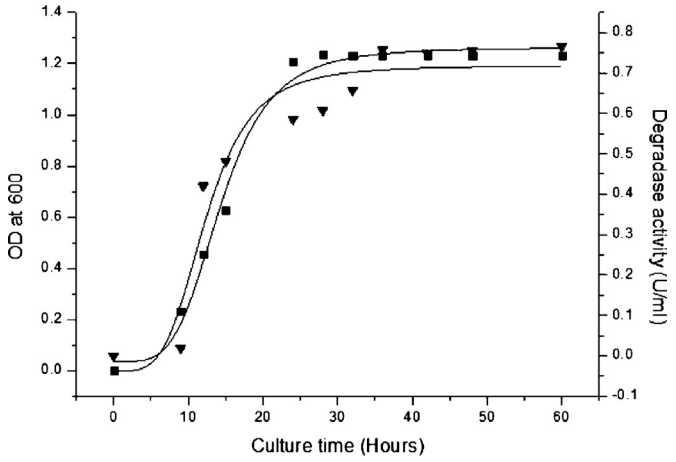

Fig. 4. Typical growth profile and production of degradase by *Alteromonas* sp. A321. Cell mass (■) and degradase activity (▼).

$NaNO_3$ could not fully support the cell growth, and DPE production was consequently lower than the optimum production, i.e., DPE production with the activity of 0.709 U/ml. Based on the optimum condition, verification experiment was carried out in triplicate, and the degradase activity reached 0.744 U/ml, which was as about two times higher than 0.391 U/ml obtained without optimization. The good correlation between predicted and experimental results confirmed the validation of the response model.

3.5. Growth curve and changes in degradase activity during fermentation process

Time course of the cell growth and DPE production from *Alteromonas* sp. A321 in the optimized medium were determined (Fig. 4). Strain A321 was in the lag phase in the first 6 h, in which the organism started synthesizing the necessary proteins, co-enzymes and vitamins needed for their growth. The exponential phase occurred between hour 6 and hour 20, and production of DPE increased simultaneously. For other enzyme producing bacteria the similar phenomenon were reported in the case of cis-epoxysuccinic acid hydrolase from *Bordetella* sp. strain 1–3 and in the case of organic solvent-stable protease from *Bacillus sphaericus* (Li et al., 2008; Liu, Fang, Lv, Wang, & Chen, 2010). Production of DPE reached the plateau at 36 h when the bacterial culture reached the stationary phase. Any further increase in incubation time did not increase the enzyme production. The curve fitting of bacterial growth and DPE production were conducted with the software Origin 8.0, which is for the application of data analysis, publication-quality graphing, and programming. The nonlinear curve fitting equation was $y = A_2 + (A_1 - A_2)/(1 + (x/x_0)\hat{}p) \times A_1$. About the growth curve, the Adj. R^2, A_1, A_2, x_0, p was 0.98, 0.21, 1.28, 15.88, and 5.29, respectively. In case of the curve of DPE production, the Adj. R^2, A_1, A_2, x_0, p was 0.93, −82513.89, 91.04, 0.25, and 1.91, respectively. Obviously, strain A321 is able to produce DPE efficiently and specifically in a shorter fermentation period. Streamlining the bacteria strain for industrial application needs additional investigation.

3.6. Enzymatic hydrolysis

The results of hydrolysis efficiency with different enzymes are shown in Fig. 5. It was observed that DPE showed superior specific activities for PE. Hydrolysis of PE was rapid and the % reducing sugar reached 44.11 during the first hour. The reducing sugar increased with low rate from 3 h to 7 h. Percent reducing sugar could reach 63.53% at the 7th. Further increase in hydrolysis time shows no effect on the production of reducing sugar. On the other hand, pectinase and cellulase hydrolyzed PE at a very low level. In the aspect of PE degradation, Li et al. (2013) reported a method of microwave-assistance acid hydrolysis. The yield of oligosaccharides obtained under the condition of 1 M HCl at 70 °C with microwave irradiation 600 W was 23.78%. Compared to the aforementioned method, the enzymatic hydrolysis could be milder and more efficient for the production of oligosaccharides.

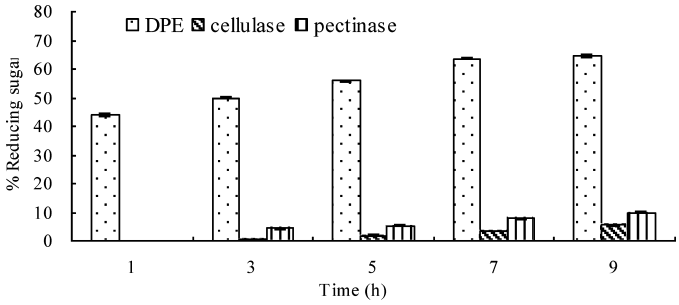

Fig. 5. The effect of different kinds of enzyme on % reducing sugar.

At present, no study has been reported to research enzymes of high specificity to degrade PE. This study provides the necessary information for recognizing this type of enzyme. It offers a new method to improve the application of *E. prolifera.* With its high efficiency to break down PE, we can use economic medium composition and optimize culture condition to further propel the application of DPE in oligosaccharides production. Further studies, including the structures of enzymatic hydrolysis production, protein purification, electrophoretic analysis, and enzyme characteristics, are underway in order to have a better knowledge for DPE application.

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