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Research article

Fermentation optimization and enzyme characterization of a new ι-Carrageenase from *Pseudoalteromonas carrageenovora* ASY5

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ABSTRACT

Background: A new ι-carrageenase-producing strain was screened from mangroves and authenticated as *Pseudoalteromonas carrageenovora* ASY5 in our laboratory. The potential application of this new strain was evaluated.

Results: Medium compositions and culturing conditions in shaking flask fermentation were firstly optimized by single-factor experiment. ι-Carrageenase activity increased from 0.34 U/mL to 1.08 U/mL after test optimization. Optimal fermentation conditions were 20°C, pH 7.0, incubation time of 40 h, 15 g/L NaCl, 1.5% (*w*/*v*) yeast extract as nitrogen source, and 0.9% (w/v) ι-carrageenan as carbon source. Then, the crude ι-carrageenase was characterized. The optimum temperature and pH of the ι-carrageenase were 40°C and 8.0, respectively. The enzymatic activity at 35–40°C for 45 min retained more than 40% of the maximum activity. Meanwhile, The ι-carrageenase was inhibited by the addition of 1 mmol/L Cd^{2+} and Fe^{3+} but increased by the addition of 1 mmol/L Ag^{+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, and Al^{3+}. The structure of oligosaccharides derived from ι-carrageenan was detected using electrospray ionization mass spectrometry (ESI-MS). The ι-carrageenase degraded ι-carrageenan, yielding disaccharides and tetrasaccharides as main products.

Conclusions: The discovery and study of new ι-carrageenases are beneficial not only for the production of ι-carrageenan oligosaccharides but also for the further utilization in industrial production.

1. Introduction

Carrageenans are sulfated linear polysaccharides of D-galactose extracted from various marine red algae (Rhodophyceae). These polysaccharides have repeating disaccharide sequences of alternating 3-linked β-D-galactopyranose and 4-linked α-D-galactopyranose or 3,6-anhydro-a-D-galactopyranose [1]. In these galactans, the β-linked galactose units are in the D configuration (G unit). However, whereas the α-linked galactose units are in the L configuration in agars (L unit), they are in the D configuration in carrageenans (D unit). Carrageenans are further classified based on the position and number of sulfate esters (S) and by the occurrence of 3,6-anhydro-bridges in the α-linked residues (DA unit) found in gelling carrageenans. For example, κ- (DA-G4S), ι- (DA2S-G4S) and λ- (D2S6S-G2S) carrageenans are distinguished by the presence of one, two or three ester sulfate groups per repeating disaccharide unit respectively [2]. ι-Carrageenan has an ester sulfate content of approximately 28–30% and a 3,6-anhydro-a-D-galactopyranose content of approximately 25–30%. κ-Carrageenan has an ester sulfate content of approximately 25–30% and a 3,6-anhydro-a-D-galactopyranose content of approximately 28–35%. Meanwhile λ-carrageenan has the highest ester sulfate content of approximately 32–39%, but no 3,6-anhydro-a-D-galactopyranose [3].

Carrageenans are degraded by κ-carrageenase (EC 3.2.1.83), ι-carrageenase (EC 3.2.1.157), and λ-carrageenase (EC 3.2.1.162) [4]. The κ-carrageenases have been widely studied, but ι- and λ-carrageenases are rarely reported. ι-Carrageenase is classified into family 82 of glycoside hydrolases, which can cleave the internal β-1,4 linkages of ι-carrageenans, yielding a series of homologous, even-numbered sulfated oligosaccharides [5]. These sulfated oligosaccharides exhibit various biological and physiological activities, such as anticoagulation [6], anti-inflammation [7], anti-thrombosis [8], antitumor activity [9], and viral inactivation [10]. In addition, they are also useful tools for the structural analysis of cell walls and isolation of protoplasts from red algae [11]. Therefore, degraded carrageenans have drawn considerable interest.

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Current degradation methods, such as acid hydrolysis, are highly rigorous such that labile and valuable native constituents are damaged during extraction. Specific enzyme degradation can retain the native structure of carrageenans without the risk of modification and is safer and more controllable than other degradation methods [12]. However, the application of ι-carrageenases also presents several problems. For example, ι-carrageenases Cgi A from *Zobellia galactanivorans* [13] and *Alteromonas fortis* [14] can effectively hydrolyze ι-carrageenan, but their main hydrolysates, namely, neo-ι-carratetraose and neo-ι-carrahexaose, are difficult to separate. A high ratio of neo-ι-carratetraose is produced by ι-carrageenases from *Microbulbifer thermotolerans*, while ι-carrageenases exhibit little activity in the absence of NaCl, thus increasing the expenditure on the repairment of reactor corrosion and oligosaccharide desalting [4]. Furthermore, developing fermentation processes for ι-carrageenase has not been extensively investigated, although some kinetic data on the production of this enzyme have been previously published. Therefore, superior producer strains have been explored to improve the yield and quality of ι-carrageenase fermentation to prepare these strains on a commercial scale.

Our preliminary experiments showed that the supernatant of marine bacterium *Pseudoalteromonas carrageenovora* ASY5 can degrade κ- and ι-carrageenans. In the present work, we obtained maximum ι-carrageenase activity by optimizing the medium components and culture conditions using a classical method in which one independent variable was changed while all other variables were fixed at certain level. The ι-carrageenase was obtained under these conditions and then characterized. This enzyme, which could degrade ι-carrageenan, yielding ι-carrageenan disaccharides and tetrasaccharides as the main products, can be used in industrial applications. This study is the first report on the optimization of medium components and culture conditions for ι-carrageenase production.

2. Materials and methods

2.1. Materials

ι-Carrageenan was purchased from Greenfresh Foodstuff Co., Ltd. All other chemical reagents were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd.

2.2. Bacteria

P. carrageenovora ASY5 (collection number CICC 23819 at the China Center of Industrial Collection) was isolated from the mangrove forest in Xiamen, Fujian province, China and preserved at the laboratory.

2.3. Culture medium and growth conditions

The seed culture medium (50 mL, pH 7.3) in 250-mL shake flasks consisted of beef paste (10 g/L), tryptone (10 g/L), NaCl (28.13 g/L), KCl (0.77 g/L), $CaCl_2$ (1.6 g/L), $MgCl_2$ (4.8 g/L), $NaHCO_3$ (0.11 g/L), and $MgSO_4$ (3.5 g/L). The original medium to produce ι-carrageenase (50 mL) in 250-mL shake flasks was composed of carrageenan (5 g/L), tryptone (5 g/L), NaCl (20 g/L), $CaCl_2$ (0.2 g/L), Na_2HPO_4 (3.82 g/L), and NaH_2PO_4 (1.32 g/L).

The seed culture medium was inoculated with overnight culture (2%, *v*/*v*, inocula) and incubated at 25°C at 180 rpm. Afterward, the fermentation medium (50 mL) in 250-mL shake flasks was inoculated with 2% seed broth and incubated at 18°C and 180 rpm. After cultivation for 40 h, the culture broth was centrifuged at 8000 × g for 30 min at 4°C to collect the supernatant. The supernatant was the crude enzyme and preserved at −20°C for further assays.

Cell growth and enzymatic activity in the culture broth were monitored to investigate the effects of NaCl, ι-carrageenan concentrations, carbon and nitrogen sources (1.5% nitrogen content, *w*/*v*), and fermentation temperature, duration, and initial pH. Cell growth was estimated by optical density at 660 nm (OD_{660}).

3. ι-Carrageenase activity assay

ι-Carrageenase activity was measured by using the 3,5-dinitrosalicylic acid (DNS) assay. The enzymatic hydrolysis reaction was conducted in 50 mM sodium phosphate buffer (pH 7.0) containing 600 μL 0.5% (*w*/*v*) ι-carrageenan and 400 μL ι-carrageenase at 40°C for 40 min. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as D-galactose per minute under the assay conditions. The enzyme that had been heated at 100°C for 10 min prior to the reaction was used as the control.

3.1. Determination of enzyme properties

The effect of acidity on enzymatic activity was determined under the standard assay condition in various buffers (50 mmol/L), namely, Na_2HPO_4-citric acid (pH 4.0–8.0), sodium phosphate buffer (pH 8.0–9.0), and glycine-NaOH (pH 9.0–10.5). The acidity stability of ι-carrageenase was measured under the standard assay condition after the enzyme was incubated in media with different pH levels ranging from pH 4.0 to pH 10.5 at 4°C for 75 min. The optimal temperature of the enzymatic activity was determined by measuring the activity at various temperatures (30–60°C). The thermostability of the enzyme was determined by preincubating the enzyme at different temperatures ranging from 35°C to 50°C for 75 min. The enzyme assay was performed in the presence of 1 mM of various cation ions, inhibitors, or detergents to determine their effects on ι-carrageenase activity. The substrate specificity of ι-carrageenase was determined using κ-carrageenan, ι-carrageenan, agar, alginate, carboxymethyl cellulose, and fucoidin as substrates. Relative activity was calculated as a percentage of maximum ι-carrageenase activity.

3.2. Analysis of the hydrolysis products

The enzyme solution (100 mL) was incubated with 150 mL of 0.5% (*w*/*v*) ι-carrageenan sodium in 50 mM sodium phosphate buffer (pH 7.0) at 40°C for 24 h. The enzyme hydrolysates were first dissolved in 400 mL ethanol for 12 h and centrifuged at 10,000 × g for 30 min. The supernatant was harvested as the hydrolysis product. Subsequently, ethanol was removed by vacuum evaporation, and the degradation product powder was prepared by freeze-drying. The molecular mass distribution of the products was determined by using electrospray ionization mass spectrometry (ESI-MS, Waters, USA).

3.3. Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4. Results and discussion

4.1. Optimization of medium compositions

4.1.1. Effects of carbon sources on cell growth and ι-carrageenase production

Previous results indicated that an inducer is necessary to initiate and maintain the enzyme synthesis rate [15,16]. In particular, Beltagy et al. showed that major and minor repeat units (dimeric units) produced from the natural polysaccharide by carrageenase can act as inducers for ι-, κ-, and λ-carrageenases [15]. These units are highly beneficial because of their cheap cost and abundance [17]. Interestingly, in this study, various simple sugars (α-lactose, glucose, sucrose, and maltose)

or major repeat units (sodium alginate and κ-carrageenan) could not induce the production of ι-carrageenase from *P. carrageenovora* in a medium (Fig. 1A). However, the growth of bacteria increased in the medium supplemented with κ-carrageenan. This phenomenon may have been caused by the rapid utilization of the polysaccharide by the bacterium, resulting in increased cell mass by vigorous growth. Therefore, ι-carrageenan was chosen as the best and sole carbon source in the follow-up experiments.

4.1.2. Effects of ι-carrageenan concentration on cell growth and ι-carrageenase production

P. carrageenovora ASY5 produced little amount of ι-carrageenase without ι-carrageenan in the medium. However, the quantity of enzyme production produced by this bacterium usually increased with ι-carrageenan concentration, suggesting that ι-carrageenan can prompt ι-carrageenase synthesis (Fig. 1B). ι-carrageenan concentration was limited to lower than 9 g/L in this experiment because of the high viscosity of the culture broth and state of gelation in the culture medium. Zhou et al. reported that WZUC10 produced little κ-carrageenase without κ-carrageenan in the medium, and the optimal carrageenan concentration was 3 g/L [18]. Dyrset et al. also revealed that the initial concentration of κ-carrageenan in the fermentation broth is limited to approximately 2.5 g/L because of its low solubility and high broth viscosity [19].

4.1.3. Effects of nitrogen sources on cell growth and ι-carrageenase production

Nitrogen source provides substances for the synthesis of protein, nucleic acid, and nitrogen metabolites. In this study, different nitrogen sources were added to the fermentation medium to investigate strain growth and enzyme production. Organic nitrogen sources (yeast extract, bacteriological peptone, and tryptone) were more effective ($P < 0.05$) for strain growth and enzyme synthesis than inorganic ones (sodium nitrate, ammonium nitrate, ammonium chloride, and

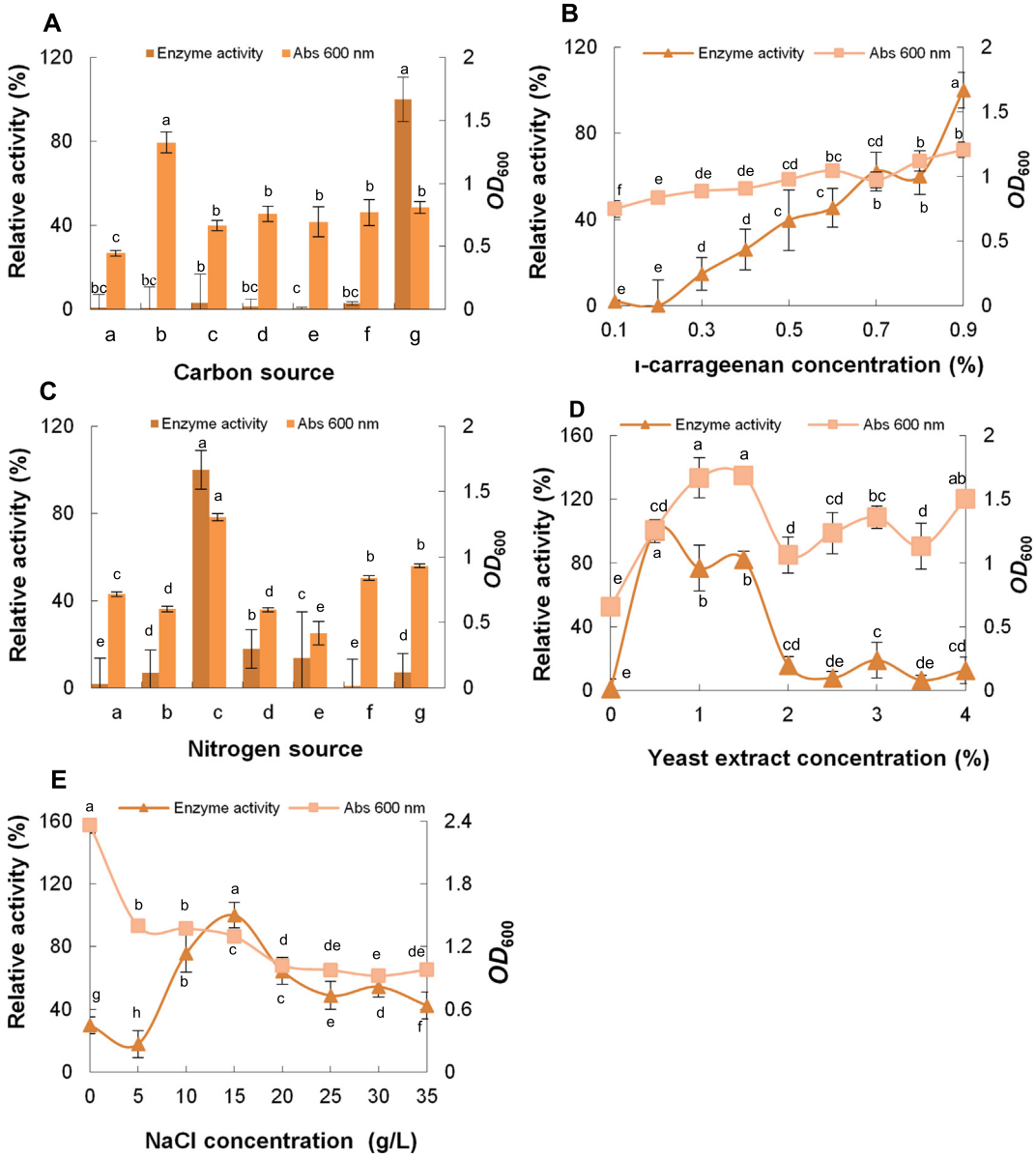

Fig. 1. The effect of medium composition on *Pseudoalteromonas carrageenovora* ASY5 cell growth and ι-carrageenase production during fermentation. (A) Effects of different carbon sources on cell growth and ι-carrageenase production: a, α-lactose; b, κ-carrageenan; c, maltose; d, glucose; e, sodium alginate; f, sucrose; and g, ι-carrageenan. (B) Effect of ι-carrageenan concentration on cell growth and enzyme production. Effects of different parameters on cell growth and enzyme production: (C) nitrogen (a, sodium nitrate; b, ammonium nitrate; c, yeast extract; d, ammonium chloride; e, diammonium phosphate; f, bacteriological peptone; and g, tryptone); (D) yeast extract and (E) NaCl concentration. Different lowercase letters on the bar denote significant differences ($P < 0.05$). Bar represent the standard deviations (n = 3).

diammonium phosphate) (Fig. 1C). Among these organic sources, yeast extract resulted in the highest ($P < 0.05$) enzyme production and cell growth. Several inorganic nitrogen sources, including ammonium nitrate, ammonium chloride, and diammonium phosphate, had negative effects ($P < 0.05$) on ι-carrageenase production. These results indicated that nitrogen source was essential for enzyme production, and the optimal nitrogen source for ι-carrageenase production was yeast extract.

4.1.4. Effects of yeast extract concentration on cell growth and ι-carrageenase production

The maximum enzyme concentrations in fermentations, in which yeast extract was selected as an independent research objective at different concentrations, are shown in Fig. 1D. Low yeast extract concentration limited enzyme production and had a maximum value at approximately 5 g/L ($P < 0.05$). Further increase in yeast extract resulted in reduced maximum carrageenase concentration ($P < 0.05$). These observations may indicate an optimum ι-carrageenase utilization rate determined by the cell concentration and duration of the ι-carrageenan metabolizing phase. Dyrset et al. reported that strain 9 reached maximum activity at a low yeast extract concentration of 0.5 g/L, while NCIMB 302 attained its maximum activity at 8 g/L of yeast extract [19].

4.1.5. Effects of NaCl concentration on cell growth and ι-carrageenase production

P. carrageenovora ASY5 needed NaCl to grow well. The effect of NaCl concentration on cell growth was examined at a range of 0–35 g/L. Fig. 1E shows that the strain did not grow well without or at low concentrations of NaCl, whereas growth was inhibited at high NaCl concentrations ($P < 0.05$). Slight change in cell growth was observed at 20–35 g/L, compared with κ-carrageenase from a marine

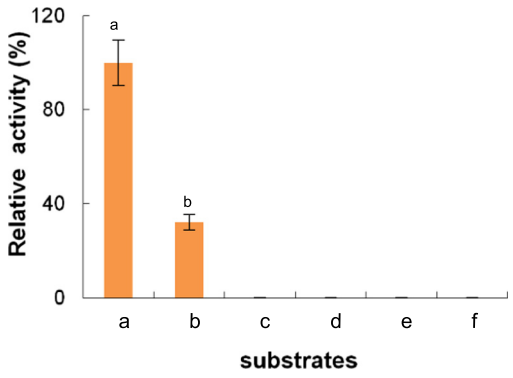

Fig. 3. Substrate specificity of carrageenase from *P. carrageenovora* ASY5. a, κ-carrageenan; b, ι-carrageenan; c, fucoidin; d, agar; e, carboxymethyl cellulose; and f, sodium alginate. Different lowercase letters on the bar denote significant differences ($P < 0.05$). Bar represent the standard deviations (n = 3).

Cytophage-like bacterium, which achieved the maximum cell growth at 10 g/L of NaCl and attained little change in cell growth at 10–60 g/L [18].

4.2. Optimization of incubation conditions with single-factor experiment

4.2.1. Effect of incubation time on cell growth and ι-carrageenase production

Fig. 2A shows that the biomass increased with incubation time. However, the enzyme activity increased at first and then decreased with prolonged incubation period. Fig. 2A also shows that the maximum ι-carrageenase activity was obtained in the middle of the growth phase (approximately 40 h) for fermentations with optical cell concentration of $OD_{600} = 0.8$. This result indicates that the increase in enzyme production was not due to a higher cell concentration or a prolonged production period but to an increase in the specific enzyme

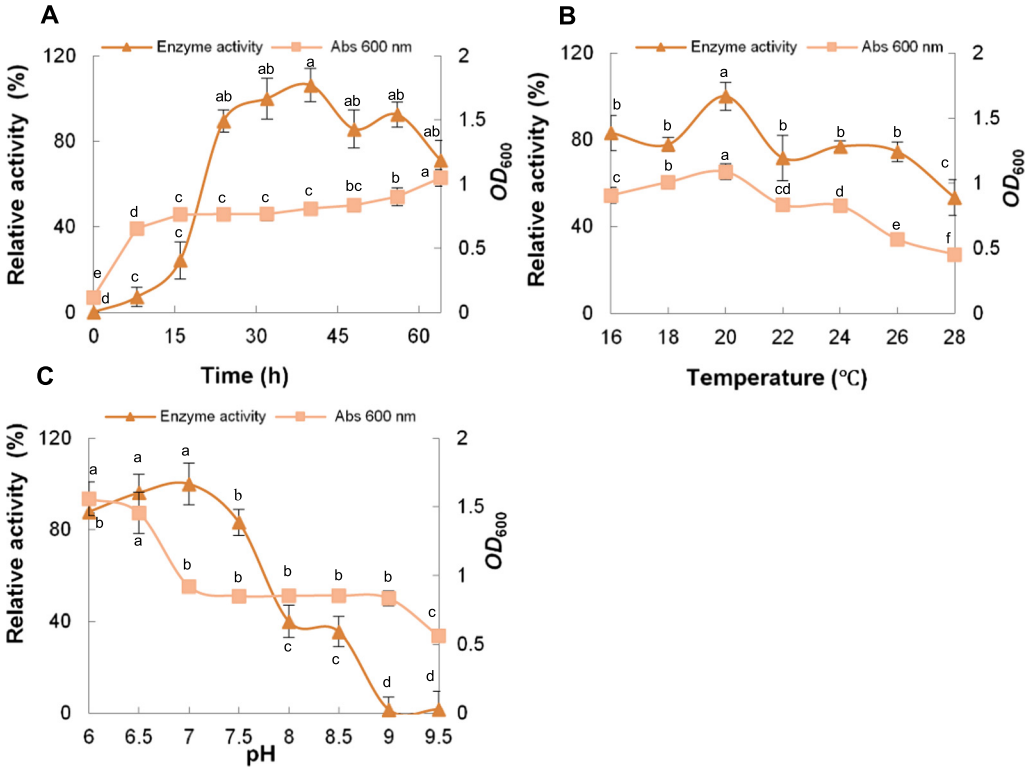

Fig. 2. The effects of incubation conditions on *P. carrageenovora* ASY5 cell growth and ι-carrageenase production. Effects of (A) incubation time, (B) incubation temperature, and (C) original pH on cell growth and ι-carrageenase production. Different lowercase letters on the bar denote significant differences ($P < 0.05$). Bar represent the standard deviations (n = 3).

Table 1
Different carrageenase activities released during the incubation of various carrageenans with *Pseudoalteromonas carrageenovora* ASY5. The bacterial strain was grown in the presence and absence of ι-carrageenan or κ-carrageenan. Carrageenase activity is expressed in units per milliliter (U/mL).

	Enzymatic activity (U/mL)		
	Induction	Kappa	Iota
Supernatant	Kappa	2.94	0
	Iota	2.17	0.84
	None	0.19	0

production rate. Interestingly, various microbes require different incubation times for maximum carrageenase production. For example, bacterium 1 [20] may consume 12 h while 8 days will be consumed in ALAB-001 [21] and *Bacillus* sp. SYR4 may require 14 days [22].

4.2.2. Effect of temperature on cell growth and ι-carrageenase production

Optimal temperature and pH correspond with the optimal conditions for the growth of the organism. *P. carrageenovora* ASY5 grew better and produced more ι-carrageenase at incubation temperature of 20°C (Fig. 2B). Incubation temperature higher than 20°C resulted in lower yield ($P < 0.05$) compared with those at lower temperatures. Similar to other metabolic enzymes [23,24,25], optimum carrageenase production in bacteria is performed at the neutral temperature, except for *Bacillus* sp. Lc50-1 [26] which requires 55°C for production.

4.2.3. Effect of original pH on cell growth and ι-carrageenase production

The production of carrageenases was also greatly influenced by optimum pH, and various microbes required different initial pH levels for maximum carrageenase production (Fig. 2C). *P. carrageenovora* ASY5 showed maximum carrageenase production activity at pH 7.0 ($P < 0.05$), while biomass decreased with increase in pH level. Similar results were obtained for ι-carrageenase production from *Cellulophaga* sp. QY3 [23] and κ-carrageenase from *P. carrageenovora* HLX250 [27].

Single-factor experiment analysis was performed to optimize fermentation composition to clarify the level of ι-carrageenase production. Based on the results described above, the optimal medium was composed of 15 g/L NaCl, 0.5% (*w*/*v*) yeast extract as nitrogen source, and 0.9% (w/v) ι-carrageenan as additional carbon source. The optimal culture conditions were temperature at 20°C, pH 7.0, and incubation time of 40 h. Under these conditions, ι-carrageenase activity reached 1.08 U/mL, which was 3.18 times higher ($P < 0.05$) than that of basic culture medium.

The development of fermentation processes to produce ι-carrageenase is rarely investigated, contrary to κ-carrageenases which have been extensively studied. Youssef et al. optimized parameters using response surface methodology and obtained *Cellulosimicrobium cellulans* κ-carrageenase activity that was 2.3 times higher than that obtained from the basal medium [28]. Similarly, Dyrset et al. showed the role of casamino acid in the fed-batch fermentation of *Pseudomonas carrageenovora* NUMB 302 and significantly achieved enhanced enzymatic activity by 2.6 times [19]. Furthermore, 32-fold increase in κ-carrageenase production compared with initial value was achieved in *Pseudomonas elongate* using statistical optimization method [11].

4.3. Properties of ι-carrageenase

4.3.1. Substrate specificity of carrageenase

Agars and carrageenans are 1,3-α-1,4-β-galactans from the cell walls of red algae, substituted by zero (agarose), one (κ-), and two (ι-carrageenan) sulfate groups per disaccharidic monomer. After the hydrolysis of agarose, κ-carrageenan, ι-carrageenan, sucrose, CMC-Na, and alginate using ι-carrageenase, κ- and ι-carrageenans could be effectively hydrolyzed by the enzyme (Fig. 3). By contrast, agarose, fucoidin, alginate, and cellulose were not hydrolyzed by the obtained enzyme. This phenomenon implies that the supernatant of marine bacterium *P. carrageenovora* ASY5 may be composed of two enzymes, namely, ι- and κ-carrageenases, or an enzyme that can simultaneously degrade ι- and κ-carrageenases. Ma et al. also reported that the supernatant of marine bacterium *Cellulophaga* sp. QY3 can degrade both ι- and κ-carrageenans, while recombinant CgiB_Ce from *Cellulophaga* sp. QY3 could degrade ι-carrageenan but not κ-carrageenan, λ-carrageenan, or agarose [23]. The last three compounds have backbones similar to that of ι-carrageenan. In addition, Liu et al. revealed that purified enzyme from *Pseudoalteromonas porphyrae* could not only actively convert κ-carrageenan into tetrasaccharides but could also effectively convert λ-carrageenan [29].

P. carrageenovora ASY5 was grown in the presence of different carrageenans, in the assumption that this process would help the strain induce enzymes involved in carrageenan biodegradation. Culture supernatant was assayed on κ- and ι-carrageenans to screen for carrageenase activity (Table 1). When κ-and ι-carrageenans were present in the culture medium, the κ-carrageenase activity was detected in the supernatant (2.94 and 0.19 U/mL under induction with κ-carrageenan and no inducer, respectively), but no ι-carrageenase activity was detected in the supernatant. By contrast, when ι-carrageenan was used as the inducer in the

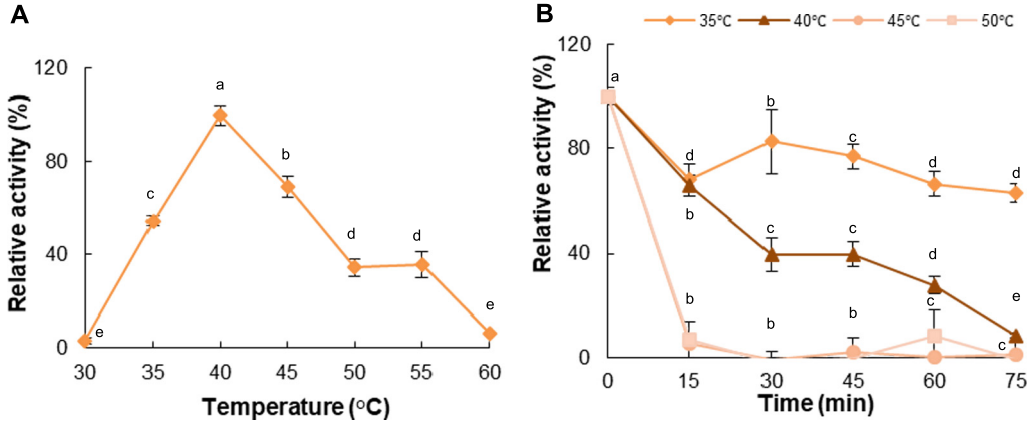

Fig. 4. The effect of temperature on *P. carrageenovora* ASY5 ι-carrageenase activity. (A) Effect of temperature on enzymatic activity of *P. carrageenovora* ASY5. ι-Carrageenase was measured at temperatures between 30°C and 60°C at pH 7.0 for 40 min to determine the optimum temperature. (B) Thermostability of *P. carrageenovora* ASY5. The enzyme was incubated at different temperatures (35°C–50°C) for 75 min to determine the thermal stability. Residual activity was determined by the standard assay method and calculated as a percentage of untreated ι-carrageenase activity. Different lowercase letters on the bar denote significant differences ($P < 0.05$). Bar represent the standard deviations (n = 3).

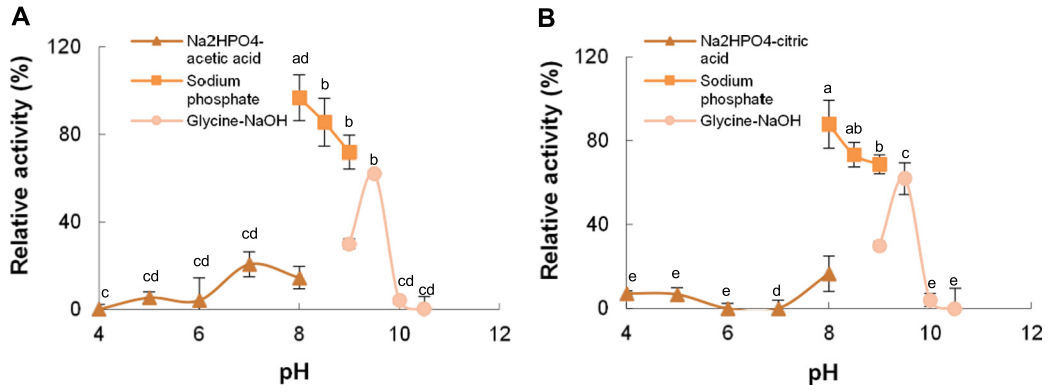

Fig. 5. The effect of pH on *P. carrageenovora* ASY5 ι-carrageenase activity. (A) Optimal pH of ι-carrageenase. ι-Carrageenan was dissolved in the following buffers under standard assay conditions:▲ 50 mmol/L Na_2HPO_4-citric acid (pH 4.0–8.0); ■ 50 mmol/L sodium phosphate buffer (pH 8.0–9.0); ● 50 mmol/L glycine-NaOH (pH 9.0–10.5). (B) The pH stability of ι-carrageenase. The enzyme was pre-incubated at 40°C over a range of pH levels (4.0–10.5) for 75 min. The residual activity of ι-carrageenase was measured with the standard method similar to that in Fig. 4. Different lowercase letters on the bar denote significant differences ($P < 0.05$). Bar represent the standard deviations (n = 3).

culture medium, the κ- and ι-carrageenase activities were measured in the supernatant. This result indicated that the supernatant of *P. carrageenovora* ASY5 was composed of two enzymes, namely, ι- and κ-carrageenases, with ι-carrageenan as the inducer.

4.3.2. Effect of temperature on ι-carrageenase activity

The ι-carrageenase activity was measured as a function of temperature from 30°C to 60°C, and the highest activity was measured at 40°C (Fig. 4A). Thermostability is an important and useful criterion for the industrial application of ι-carrageenase. Therefore, the thermostability of the enzyme was investigated by pre-incubating the enzyme in the same buffer as described in Section 2 for 75 min, and its remaining activity was determined. Fig. 4B shows that the ι-carrageenase was stable at 35°C, and residual ι-carrageenase activity still remained at 60% of the control after treatment for 75 min, indicating that the enzyme was stable up to 35°C. Fig. 4B also revealed that the enzyme was inactivated rapidly at temperatures higher than 40°C and was almost inactivated at 45°C or 50°C within 15 min ($P < 0.05$). Thus, the ι-carrageenase showed an apparent considerable thermo-sensibility. The ι-carrageenase Cgi A_Ce from the marine *Cellulophaga* sp. QY3 retained 80% of its activity after 1 h at 50°C and was completely inactivated after 1 h at 60°C. Moreover, the optimal temperature of the enzyme was 50°C [23]. The optimal temperature for the enzymatic activity of ι-carrageenase Cgi A produced by *M. thermotolerans* JAMB-A94T is 50°C [4]. The ι-carrageenase from a marine bacterium showed an optimum temperature of 40°C, and the enzyme was not active at 60°C, and 30% of maximal activity was expressed at 20°C [20]. The recombinant ι-carrageenase Cgi B_Ce from *Cellulophaga* sp. QY3 had the optimal temperature of 45°C and retained 70% of the original activity after incubation at the temperatures below 40°C for 1 h [30]. These results suggested that ι-carrageenase produced by *P. carrageenovora* ASY5 completely differed from that produced by other bacteria.

4.3.3. Effect of pH on ι-carrageenase activity

The ι-carrageenase activity was measured at various pH levels in buffers with the same ionic concentrations. The results (Fig. 5A) showed maximum activity at pH 8.0. The pH stability was tested by pre-incubating the enzyme for 75 min in appropriate buffers with the same ionic concentrations at different pH levels ranging from 4.0 to 10.5 at 4°C. The results in Fig. 5B show that the activity profile of the enzyme was stable from pH 8.0 to pH 9.5. For example, more than 60.0% of the residual activity was retained after treatment from pH 8.0 to pH 9.5 and 4°C for 75 min. These results suggest that the enzyme was very stable in the pH range of 8.0–9.5. In particular, 87% of the residual activity was retained after treatment at pH 8.0 and 4°C for 75 min. The optimal pH of ι-carrageenase Cgi A_Ce from *Cellulophaga* sp. QY3 was 7.0, and more than 70% of the original activity was retained after treating the enzyme at pH 5.0–10.6 and 4°C for 24 h [23]. The maximum activity of ι-carrageenase Cgi A produced by *M. thermotolerans* JAMB-A94T was observed at pH 7.5, and the enzyme was stable in a range of pH 7–10, retaining more than 80% of the original activity [4]. The optimal pH of ι-carrageenase from a marine bacterium exhibited an optimum pH of 8.0. When enzyme activity was assayed at 25°C, a shift in the optimum to 7.0 was observed [20]. However, the optimal pH of recombinant ι-carrageenase Cgi B_Ce from *Cellulophaga* sp. QY3 was 6.5 in 50 mM phosphate buffer, and the enzyme was stable in the pH range of 6.0–9.0 [30]. These results indicate that ι-carrageenase from *P. carrageenovora* ASY5 differed from that from other bacteria.

4.3.4. Effect of metal ions on ι-carrageenase activity

The ι-carrageenase activity was measured in the presence of various chemicals under standard assay conditions (Table 2). Cations Na^+ and K^+ significantly increased the activity of ι-carrageenase, with highest activity ($P < 0.05$) in 500 mM NaCl or 10 mM KCl. The ι-carrageenase activity was active in the absence of NaCl, but was remarkably enhanced by NaCl (100–700 mmol/L) ($P < 0.05$). The optimum NaCl concentration for ι-carrageenase was 700 mM, which was higher than that of ι-carrageenases from *Cellulophaga* sp. QY3 (500 mM) [23,30],

Table 2
Effects of metal ions on ι-carrageenase activity.

Metal ions	Concentration (mmol/L)	Relative activity (%)	Metal ions	Concentration (mmol/L)	Relative activity (%)
None	–	$100.0 \pm 1.5^{b,*}$	None	–	100.0 ± 1.5^{bc}
Na^+	100	73.4 ± 7.0^{cd}	K^+	1	126.6 ± 6.6^{a}
	300	84.0 ± 10.2^{bc}		3	119.4 ± 0.9^{ab}
	500	141.4 ± 1.8^{a}		5	134.6 ± 12.4^{a}
	700	150.4 ± 7.1^{a}		7	127.2 ± 1.7^{a}
	900	67.8 ± 4.8^{cd}		9	85.8 ± 8.4^{c}
	1000	53.8 ± 4.9^{de}		10	27.6 ± 2.0^{d}
	2000	41.2 ± 3.6^{e}			
None	–	100.0 ± 1.5^{ef}			
Li^+	1	103.2 ± 4.0^{ef}	Mn^{2+}	1	136.6 ± 3.1^{bc}
Ag^+	1	119.8 ± 7.0^{cde}	Zn^{2+}	1	143.8 ± 4.5^{b}
Ba^{2+}	1	211.4 ± 7.1^{a}	Fe^{2+}	1	129.8 ± 11.6^{bcd}
Cd^{2+}	1	94.6 ± 8.0^{f}	Fe^{3+}	1	72.4 ± 6.7^{g}
Ca^{2+}	1	148.6 ± 3.2^{b}	Al^{3+}	1	110.2 ± 3.0^{def}
Mg^{2+}	1	104.6 ± 6.8^{ef}			
Co^{2+}	1	207.8 ± 6.8^{a}			

Mean ± S.D. (n = 3).
* Different lowercase superscripts indicate the significant differences ($P < 0.05$).

Table 3
Effects of inhibitors and detergents on enzymatic activity.*

Inhibitors and detergents	Concentration (%)	Relative activity (%)	Inhibitors and detergents	Concentration (mmol/L)	Relative activity (%)
None	–	$100 \pm 2.5^{b,*}$	CTAB	1	119.6 ± 3.2^{ab}
Tween-20	1	70.2 ± 0.5^{ab}	PMSF	1	52.5 ± 2.8^{c}
Tween-80	1	115.8 ± 7.5^{b}	β-ME	1	109.7 ± 9.5^{ab}
Triton X-100	1	123.6 ± 8.7^{ab}	EDTA	1	0.2 ± 2.3^{d}
SDS	1	0 ± 2.2^{d}	DTT	1	180.2 ± 7.7^{a}

Mean ± S.D. (n = 3).
* Different lowercase superscripts indicate the significant differences ($P < 0.05$).

λ-carrageenase from *Bacillus* sp. (2 mM) [26], and κ-carrageenases from *Pseudoalteromonas* sp. QY203 (300 mM) [31] and *Shewanella* sp. Kz7 (200 mM) [32]. The presence of NaCl also showed inhibitory effect on the activity of κ-carrageenases from *P. porphyrae* LL1 and *Tamlana* sp. HC4 [29,33]. In addition, metal ions Cd^{2+} and Fe^{3+} had little effect on the enzymatic activity, suggesting that these ions altered the enzyme conformation. Other tested monovalent, divalent, and trivalent metal ions, such as Li^{+}, Ag^{+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, and Al^{3+}, showed a certain promoter action, whereas Ba^{2+} and Co^{2+} strongly accelerated the activity ($P < 0.05$). Similarly, Liu et al. [26] and Li et al. [29] also reported that κ-carrageenase activity was accelerated by Ba^{2+} and Co^{2+}, respectively. Interestingly, Ma et al. reported that ι-carrageenase activity was inhibited by a much wider range of ions, including Li^{+}, NH_4^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ba^{2+}, Fe^{3+}, and Al^{3+} [23]. These results indicate that several biochemical and physical properties of ι-carrageenase produced by *P. carrageenovora* ASY5 greatly differed from those of ι-, κ-, and λ-carrageenases produced by other marine bacteria.

4.3.5. Effect of inhibitors and detergents on ι-carrageenase activity

Table 3 depicts the effects observed in the presence of protein inhibitors and detergents. Among these effects, the acceleration with CTAB, DTT, and Triton X-100 was much pronounced and showed that the relative activity of ι-carrageenase increased by more than 20% ($P < 0.05$). The presence of the chelating agent EDTA negatively affected enzymatic activity ($P < 0.05$), demonstrating that the purified enzyme was a metalloenzyme [34]. The enzymatic activity was also strongly inhibited ($P < 0.05$) by PMSF, indicating that Ser residues were important for active sites of the enzyme. The results in Table 2 also show that SDS inhibited activity ($P < 0.05$) of the ι-carrageenase obtained in this study, similar to that of most κ-carrageenases previously reported [29]. However, EDTA did not inhibit the κ-carrageenase activities of *Vibrio* sp. CA-1004 [35] and *Cytophaga* sp.1 k-C783 [36] but strongly inhibited the activity of the enzyme from *Cellulophaga* sp. QY3 [30] and *Tamlana* sp. HC4 [33]. These results suggest that a metal ion was involved in the catalytic site of its ι- and κ-carrageenase. Similarly, SDS (1 mM)

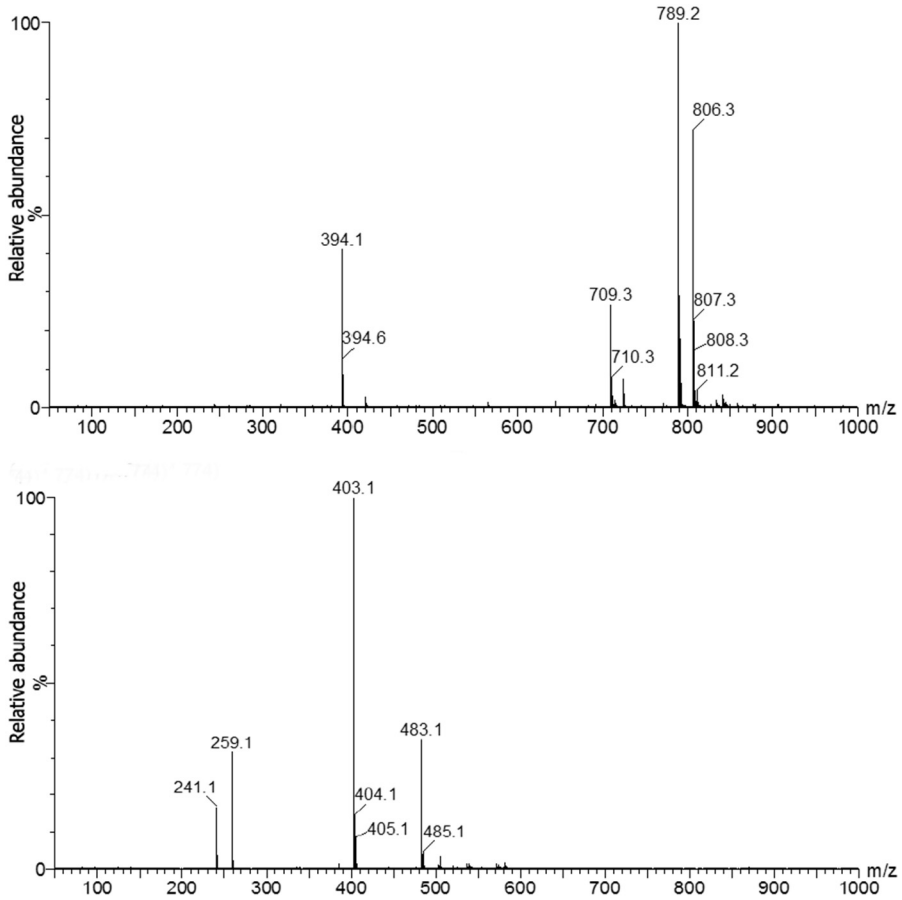

Fig. 6. ESI mass spectrum of the enzymatic products of ι-carrageenan.

had no effect on the activity of CgiA_Ce [23], and the activity of recombinant CgkX was also not inhibited by SDS [31].

4.4. Analysis of the hydrolysis products

The end degradation products of ι-carrageenan hydrolyzed by ι-carrageenase from *P. carrageenovora* ASY5 was analyzed using ESI-MS. Fig. 6 shows that the sample was a mixture of disaccharides and tetrasaccharides, For the disaccharide, the base peak occurred at m/z 403, which corresponds to the single-charged anion $[A\text{-}G4S]^-$. The peak at m/z 709 corresponds to a tetrasaccharide with only one sulfate group, that is, $[(A\text{-}G)(A\text{-}G4S)]^-$ or $[(A\text{-}G4S)(A\text{-}G)]^-$, while the base peak was found at m/z 789.2, corresponding to a tetrasaccharide with two sulfate groups, that is, $[(A\text{-}G4S)(A\text{-}G4S)]^-$ or $[(A\text{-}G)(A2S\text{-}G4S)]^-$. In addition, the mass spectrum exhibited major ions at m/z 241.0 and 483.0, corresponding to the molecular ions $[M\text{-}2H]^{2-}$ and $[M\text{-}H]^-$, respectively, of a ι-carrageenan disaccharide with a composition of $[A2S\text{-}G4S]^-$. The peaks found at m/z 394.1 in the mass spectrum corresponded to the double-charged ions of $[(A\text{-}G4S)]_2^{2-}$, which proved the presence of tetrasaccharide.

The ι-carrageenase CgiA_Ce from *Cellulophaga* sp. QY3 [23] also degraded ι-carrageenan into disaccharide and tetrasaccharide, while ι-carrageenase CgiB_Ce from *Cellulophaga* sp. QY3 [30] and ι-carrageenase A94Cgi from *M. thermotolerans* [4] degraded and hydrolyzed β-1,4-linkages of ι-carrageenan, yielding neo- ι-carratetraose as the main product. Sulfated oligosaccharides from marine algae have diverse biological and physiological activities, depending on structural parameters, such as carbohydrate structure, molecular mass, degree of sulfate esterification, and linking position of sulfo groups [37]. Therefore, degraded carrageenans, in particular carrabiose oligosaccharides, can be remarkably applied in the industry.

5. Conclusion

A new ι-carrageenase-producing strain *P. carrageenovora* ASY5 was studied in this paper. The nutritional requirement and fermentation conditions for *P. carrageenovora* ASY5 to produce ι-carrageenase were determined for the first time using classical optimization method. The important medium components identified by single-factor experiment were as follows: ι-carrageenan, 0.9 g/L; yeast extract, 5 g/L; and NaCl, 15 g/L. The fermentation conditions were temperature of 20°C, pH 7.0, and incubation time of 40 h. Under these conditions, the ι-carrageenase activity reached 1.08 U/mL, which was 3.18 times higher than that in basic culture medium. The optimal pH and temperature of the enzyme were pH 8.0 and 40°C, respectively. The enzyme was significantly stimulated by Co^{2+}, Ba^{2+}, and DTT, but inhibited by Cd^{2+}, Fe^{3+}, PMSF, SDS, and EDTA. The enzyme could actively degrade ι-carrageenan, yielding ι-carrageenan disaccharides and tetrasaccharides as the main products. The discovery and study of new ι-carrageenases are beneficial not only for the production of ι-carrageenan oligosaccharides but also for the further utilization in industrial production. Therefore, the ι-carrageenase produced by *P. carrageenovora* ASY5 needs further investigation for industrial applications.

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