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Gene Cloning and Characterization of an α-Amylase from *Alteromonas macleodii* B7 for *Enteromorpha* Polysaccharide Degradation

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Enteromorpha polysaccharides (EP) extracted from green algae have displayed a wide variety of biological activities. However, their high molecular weight leads to a high viscosity and low solubility, and therefore, greatly restrains their application. To solve this problem, bacteria from the surface of *Enteromorpha* were screened, and an *Alteromonas macleodii* strain B7 was found to be able to decrease the molecular weight of EP in culture media. Proteins harvested from the supernatant of the *A. macleodii* B7 culture were subjected to native gel electrophoresis, and a band corresponding to the *Enteromorpha* polysaccharide lyase (EPL) was detected by activity staining. The enzyme identity was subsequently confirmed by MALDI-TOF/TOF mass spectrometry as the putative α-amylase reported in *A. macleodii* ATCC 27126. The amylase gene (*amySTU*) from *A. macleodii* B7 was cloned into *Escherichia coli*, resulting in high-level expression of the recombinant enzyme with EP-degrading activity. AmySTU was found to be cold-adapted; however, its optimal enzyme activity was detected at 40ºC. The α-amylase was highly stable over a broad pH range (5.5–10) with the optimal pH at 7.5–8.0. The highest enzyme activity was detected when NaCl concentration was 2%, which dropped by 50% when the NaCl concentration was increased to 16%, showing an excellent nature of halotolerance. Furthermore, the amylase activity was not significantly affected by tested surfactants or the presence of some organic solvents. Therefore, the *A. macleodii* strain B7 and its α-amylase can be useful in lowering EP molecular weight and in starch processing.

Keywords: *Enteromorpha* polysaccharides, *Alteromonas macleodii*, identification, organic solvent tolerance, characterization, cold-adapted α-amylase

Introduction

The high-molecular-weight *Enteromorpha* polysaccharides (EP) extracted from green algae are highly sulfated polysaccharides. Studies indicate that EP have a variety of biological activities, such as anticoagulation [28], anti-allergy [30], anticancer [7], antitumor and immunomodulation [15], antioxidant [42], and other functionalities [14, 37]. In recent years, low-molecular-weight algal polysaccharides (LMWAP) have received widespread attention because of their higher biological activity, lower viscosity, and better solubility[6, 26, 39]. It is hypothesized that low-molecular-weight EP may also have a higher activity and better application.

LMWAP can be prepared from polysaccharides by enzymolysis or acidolysis. For acidolysis, it is difficult to control the LMWAP's molecular weight and their sulfated degree. However for enzymolysis, it is easy to control the LMWAP's molecular weight and one need not to worry about the removal of its side groups such as sulfuric acid groups.

The present study focused on screening bacterial strains possessing EP degradation activity. A cold-adapted α-amylase that can degrade EP was cloned and its enzymatic activity was characterized.

Materials and Methods

Chemicals and Reagents

Agar powder of bacteriological grade was purchased from BBI (Bio Basic, Markham, ON, Canada). Yeast extract and tryptone were bought from Oxoid (Basingstoke, UK). Unless otherwise stated, all chemicals used were of analytical grade or higher. Enzymes were purchased from TaKaRa (Dalian, China). Oligonucleotides were synthesized by BGI (Beijing, China).

Dry green alga *Enteromorpha* was purchased in Zhoushan, Zhejiang Province, China, in October 2010. The alga was washed with tap water and immediately dried in air. After being milled, the alga powder was then kept in plastic bags at room temperature.

Preparation of EP

The preparation of EP was followed according to the modified procedure reported by Jiao *et al*. [15]. First, the algal powder was extracted with 95% ethanol at 60°C for 2 h to remove pigments and micromolecular substances. Second, algal powder was suspended in 60 volumes of H_2O at room temperature for 1 h, and then homogenized at 90°C for 4 h with occasional stirring. After cooling to room temperature, the supernatant containing the EP solution was recovered by centrifugation at 7,000 ×g for 10 min. The EP solution was concentrated to a third of the initial volume by vacuum evaporation, precipitated by adding 3.75 volumes of 95% (v/v) ethanol, and then centrifuged at 7,000 ×g for 10 min. The recovered precipitation was washed sequentially with ethanol, acetone, and diethyl ether. The crude EP was harvested after air-drying, and then purified according to the TCA method [4] to remove proteins.

Isolation of EP-Degrading Bacterial Strains

The defined mineral salt medium supplemented with 0.3% of EP solution was used for enrichment and isolation of the EP-degrading strain. The inorganic salt ingredients (g/l) were as follows: NaCl, 30; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.2; Na_2HPO_4, 0.8; $MgSO_4$, 0.2; $FeCl_3$, 0.005; $CaCl_2$, 0.1; and $(NH_4)_6Mo_7O_{24}·4H_2O$, 0.0001. The initial pH of the culture medium was adjusted to 7.0 ± 0.1 with 0.1 M NaOH solution.

EP-degrading microorganisms were isolated from fresh *Enteromorpha* collected from Nan'ao Island (Shantou, Guangdong, China). The *Enteromorpha* was immediately taken to the laboratory and processed within 24 h. An aliquot of microorganisms collected from *Enteromorpha* was inoculated in enrichment medium for up to 4 weeks (the medium was replaced every 3 days). After enrichment, 1 ml of the culture was inoculated onto a 1.5% agar plate containing 0.3% EP as the sole carbon source. After 48 h of incubation, the colonies formed were transferred onto a fresh 1.5% agar plate containing 0.3% EP. This process was repeated 3–4 times until pure strains were acquired. The enzyme activity was measured again using EP as the sole carbon source and strain B7 with the highest EP-degrading ability was isolated.

16S rDNA Gene Sequence Analogy

PCR was used to amplify the 16S ribosomal DNA gene (rDNA) of the EP-degrading bacterium [13]. The primers were based on *E. coli*'s 16S rDNA (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1429R: 5'-GGTTACCTTGTTACGACTT-3'). An amplified DNA fragment was sequenced by BGI. The sequence obtained was analyzed with the alignment tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify closely matched species.

Determination of *Enteromorpha* Polysaccharide Lyase Activity

The activity of the enzyme towards starch was determined by measuring the release of the reducing sugar equivalent using the 3,5-dinitrosalicylic acid (DNS) method [23]. First, 100 µl of sample was added into 900 µl of phosphate buffer solution (PBS, pH 7.0, 20 mM) containing 0.5% substrate and incubated at 40°C for 10 min. The 1 ml reaction solution was then mixed with 2 ml of DNS reagent. The reaction was stopped by heating at 100°C for 5 min, and the mixture was then diluted to 10 ml with deionized water. Optical density was read at 540 nm, and values for reducing sugars were expressed as D-glucose equivalents. One unit of EPL activity was defined as the amount of enzyme that released 1 µg of reducing sugar (measured as D-glucose) from EP per minute under the above conditions.

Enzymatic degradation of EP was carried out in 20 mM Tris-HCl buffer containing 0.5% EP as the substrate at pH 7.5 and at 40°C for 10 min. The release of reducing sugar from EP was measured by using the Somogyi-Nelson method [24]. The EPL activity was determined by measuring the increase in absorbance at 755 nm. One unit of EPL activity was defined as the amount of enzyme that released 1 µg of reducing sugar (measured as D-rhamnose) from EP per minute under the above conditions.

Separation and Identification of EPL

Step 1: Preparation of EPL solution. The method for EPL preparation has been described [22]. Briefly, strain B7 was cultured for 48 h at 37°C. The supernatant was recovered by centrifugation at 8,000 ×g for 10 min at 4°C, and fractionated by using ammonium sulfate (40–50% saturation). The resulting precipitate was dissolved in 10 ml of 25 mM Tris-HCl buffer (pH 7.4) and then desalted by ultrafiltration against the same buffer. The dialysate was used as EPL solution for the native-polyacrylamide gel electrophoresis (native-PAGE) and the EPL activity assay.

Step 2: Native-PAGE and SDS-PAGE. The native-PAGE was performed on 12% (w/v) acrylamide gels at 4°C as described by Simpson [36]. Sodium dodecyl sulfate (SDS)-PAGE was carried out to estimate the protein molecular mass with a 4% stacking gel and a 12% separating gel as described by Laemmli [19]. Proteins were stained with Coomassie Brilliant Blue R-250.

Step 3: EPL Activity staining. The activity staining of EPL on native-PAGE was carried out according to Ghadi *et al*. [12]. After native-PAGE, the gel was soaked in 20 mM PBS buffer (pH 7.0) for 5 min. The gel was overlaid onto an agar plate containing 4% (w/v)

EP (in 20 mM PBS buffer, pH 7.0) on the top, and incubated at 37°C for 30 min. The gel was removed from the plate and then stained for proteins with Coomassie Brilliant Blue R-250. The agar plate was flooded with Lugol's iodine solution (1.0 g iodine crystals and 2.0 g KI dissolved in 300 ml distilled water) to visualize EPL activity.

Step 4: Identification of EPL. After activity staining, the EPL band in the gel was detected, which was then cut out and sent to BGI-Shenzhen for MALDI-TOF/TOF mass spectrometry analysis. The EPL was identified as an α-amylase and designated as AmySTU.

Gene Cloning

To express AmySTU in a heterologous system in *E. coli*, the gene was amplified with a 2× PCR master mix (Beyotime, Haimen, China). The primer sequences were as follows: forward primer, 5'-CCGGAATTCATGAAGAAACATGCGGCAG-3' (underlined letters: *Eco*RI cutting site); reverse primers, 5'-CCCAAGCTTTTC CATAAGCCACATTCG-3' (underlined letters: *Hin*dIII cutting site). The PCR was performed with a thermocycler programmed as 95°C for 5 min, 30 cycles of 94°C for 60 sec, 55°C for 60 sec, 72°C for 90 sec, and a final elongation of 72°C for 10 min. The amplification resulted in a 1,407 bp fragment. The fragment and vector pET-32a(+) were subjected to a double restriction digestion with *Eco*RI and *Hin*dIII. The ligation was used to transform competent *E. coli* DH5α. The plasmid with correct insert was sent to GBI-Shenzhen for sequencing. After identifying a positive clone, the recombinant vector was transformed into competent *E. coli* BL21. Positive clones were screened on solid Luria-Bertani (LB) medium containing 50 μg/ml ampicillin and agar (1.5%).

Heterologous Expression and Purification of the Recombinant α-Amylase

E. coli BL21 cells carrying the recombinant pET-32a(+)-amySTU plasmid were cultured overnight at 37°C in LB broth containing ampicillin. The overnight culture was inoculated into 1 L of fresh LB medium and incubated further at 37°C. Induction was done with 1 mM IPTG when A_{600} = 0.5–0.6 was reached. Afterwards, transformants were grown with constant shaking overnight at 25°C. Proteins were extracted from the supernatants with 40% saturation of ammonium sulfate. After centrifugation (10,000 ×*g*) for 30 min at 4°C, the precipitate was dissolved in 4 ml of 20 mM Tris-HCl buffer. Ultrafiltration was carried out to remove ammonium sulfate.

Recombinant AmySTU was purified with a 1.5 ml Ni agarose Fast Flow column (Henghui, Beijing, China). The column was first equilibrated with lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0). The resulting crude extract was then loaded onto the column, which was followed by three washes with washing buffer (50 mM NaH_2PO_4, 500 mM NaCl, 50 mM imidazole, pH 8.0). AmySTU was eluted with the elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0). The purity of the recombinant AmySTU was analyzed on a 12% SDS-PAGE gel [19].

The AmySTU concentration was measured according to the Bradford method [2].

Substrate Specificity for Recombinant AmySTU

The assay was conducted at 40°C for 10 min. The tested substrates were soluble starch, potato starch, β-cyclodextrin, carboxymethylcellulose, sodium alginate, carrageenan, agarose, agar, chitosan, and EP at the concentration of 1.0% (w/v) in Tris-HCl buffer (20 mM, pH 7.4).

Characterization of the Enzyme

Effects of temperature and pH. The effect of temperature on enzyme activity was carried out with a constant pH at 8.0 but at various temperatures ranging from 30°C to 70°C. The effect of pH on the activity was determined at a pH range of 5.5–10.0 with the following buffers: sodium acetate (pH 5.0–5.5), sodium phosphate (pH 6.0–7.5), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 9.5–10.0).

For measurement of pH stability, the enzyme was incubated at 30°C for 60 min in the above buffers at different pHs, and the residual activity was determined under enzyme assay conditions.

Effects of NaCl and $CaCl_2$. Enzyme activity was determined at NaCl concentrations between 0 and 25% (w/v), or $CaCl_2$ concentrations between 0 and 25 mM. The reaction without NaCl or $CaCl_2$ was considered as the control.

Effects of metal ions and chemical reagents. To determine the effect of metal ions on enzyme activity, the assay was performed in the presence of various metal ions at a final concentration of 10 mM. The activity of the enzyme without any additives was taken as 100%. The influence of EDTA, SDS, β-mercaptoethanol, and surfactants (10 mM) was studied by preincubating the enzyme with the compounds for 30 min at 25°C. Then, the remaining activity was determined using the standard enzyme assay. The activity determined in the absence of inhibitor was taken as 100%.

Organic solvent stability of the amylase. Purified AmySTU (1 ml) was incubated with 0.25 ml of organic solvent in a 2.5 ml microcentrifuge tube. The mixture was shaken (150 rpm) for 2 h at 25°C. Then, the remaining activity was measured under the standard enzyme assay conditions.

Results

Isolation and Identification of EP-Degrading Bacteria

Several EP-degrading bacterial strains were isolated by multiple rounds of screening for *Enteromorpha* bacteria capable of using EP as the sole carbon source. Strain B7, which had the highest EP-degradation ability than others, was selected for further study. The result from 16S rDNA sequencing showed that strain B7 has 99% similarity with *Alteromonas macleodii* str. 'Balearic Sea AD45' (GenBank Accession No. NR_074797) and *A. macleodii* str. 'Black Sea 11' (GenBank Accession No. CP003845). We therefore designated strain B7 as *A. macleodii* B7. To determine the
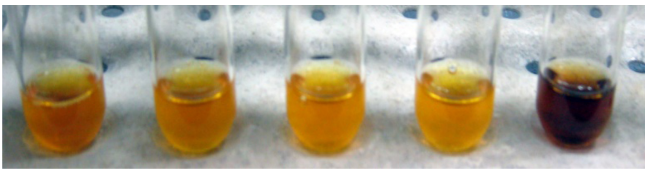
Fig. 1. The color change of *A. macleodii* B7 culture with iodine solution.

The picture was taken immediately after 0.2 ml of iodine solution was added into 1 ml of culture (left to right: 2, 3, 4, and 5 days, and control).

enzyme activity of B7 in the supernatant, 0.5% of crude EP powder was added in the culture containing strain B7 for up to 5 days. EP degradation could be detected by the color change of media when Lugol's iodine solution was added (Fig. 1). The color turned progressively lighter with longer incubation.

Separation and Identification of EPL

The method for the separation of EPL is described in the Materials and Methods section. The optimal concentration of ammonium sulfate for fractionation was 40%−50%. EPL was partially purified after ultrafiltration. The activity of EPL was visualized as a clear band after flooding the EP sheet with iodine solution (Fig. 2). MALDI-TOF/TOF mass spectrometry was performed for further identification of the separated EPL (Table 1), showing that EPL matched a putative α-amylase of *A. macleodii* ATCC 27126 (GenBank Accession No. ZP_04716377, gi:239995853). The partial sequence of the amylase and the seven matched peptides of EPL are shown in Fig. 3. We designated this amylase from *A. macleodii* B7 as AmySTU.
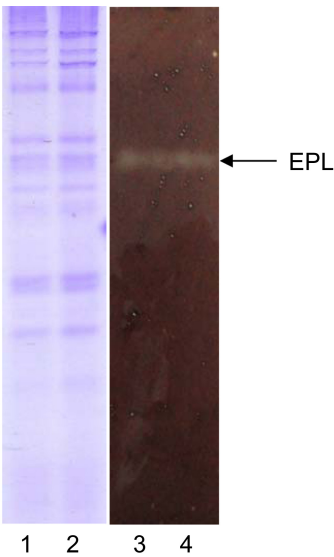

Fig. 2. Activity staining on native-PAGE of partially purified EPL.

Lanes 1 and 2: partially purified EPL. Lanes 3 and 4: clear bands shown on the EP sheet.

**Heterologous Expression and Purification of the Recombinant EPL**

The PCR primers were designed according to the CDS sequence of the putative α-amylase from *A. macleodii* ATCC 27126. A 1.4 kb sequence was amplified by PCR. The sequence of AmySTU showed high sequence homology to the putative α-amylase (*A. macleodii* ATCC 27126: 99%; *A. macleodii* str. English Channel 673: 99%; *A. macleodii* str. Black Sea 11: 99%; *A. macleodii* str. Balearic Sea AD45: 99%;

MKKHAAGVLI	GAALSTLSYQ	SYADVILHAF	NWTYDDVAAK	AQEIADLGYT
KVLVSPAYK<u>S</u>	<u>TGDQWWARYQ</u>	<u>PQDYR</u>VIDNP	LGDTADFQAM	VNALNAK<u>GVE</u>
<u>TYADIVFNHM</u>	<u>ANEAWKR</u>SDL	NYPGSEVLQQ	YSSNQGYYNG	ITLFGDVSSG
LFGGGDFHGT	YDQGGPKCIS	NYSDVGDVQY	NRLCGAAPDP	GLPDLDPNNW
VVAQQKAYLQ	ALKDMGVTGF	RVDAAK<u>HMTN</u>	<u>YHINAVFDSN</u>	<u>IK</u>NGVHVFGE
IITTGGAGSS	EYDNFLAPYL	SATGHSAYDF	PLFAQIRSAF	SFGGSMNALV
DPGAYGQALS	GDK<u>AITFSIT</u>	<u>HDIPLNDGFR</u>	<u>YQLLDPTDEH</u>	<u>LANAYVLGRD</u>
GGVPLLYSDN	NESGDNR<u>WVD</u>	<u>LYKR</u>DDIKGM	VKFHNGVQGS	GMQVVSHNDC
VIFFKRDHQG	VVGINKCADG	QDIWVDTSTD	NLWWYR<u>NYRD</u>	<u>TLSNDVQYIT</u>
<u>TQWHKFYVPG</u>	<u>R</u>SARMWLME			

Fig. 3. Partial amino acid sequence of the amylase and the seven matched peptides.

The sequences highlighted with bold red represent matched amino acid sequences of EPL by MALDI-TOF/TOF mass spectrometry.

Table 1. Database search result of the EPL by MALDI-TOF/TOF mass spectrometry.

Sample ID	Protein ID	Protein mass	Protein score	Significance score	Isoelectric point	Coverage rate	Description
B7-4	Gi | 239995853 | ref | ZP-047163.77 |	52152.1	124	81	4.89	25.37%	Alpha-amylase of *Alteromonas macleodii* ATCC 27126
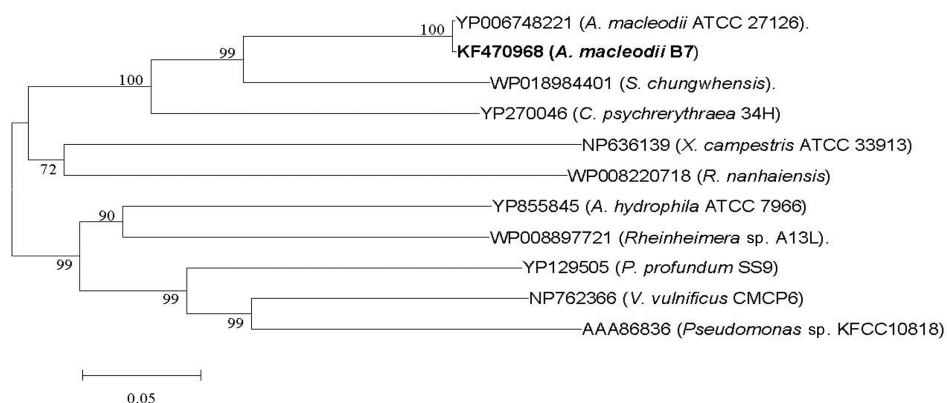
Fig. 4. Phylogenetic tree of AmySTU and other alpha-amlyases.

AmySTU is shown in bold face. The tree was constructed by the neighbor-joining method. Numbers indicate bootstrap values of 1,000 trials.

Aeromonas hydrophila: 57%). The homologies between AmySTU (GenBank Accession No. KF470968) and other well-known α-amylases are shown in Fig. 4.

To express AmySTU, the PCR product was introduced into pET-32a(+), which contains a hexahistidine N-terminal tag. The recombinant pET-32a(+)-His6-AmySTU was transformed into *E. coli* BL21 (DE3) cells. When the optical density of the transformed *E. coli* cells reached 0.6, cultures were induced with IPTG in a final concentration of 0.1 mM. After 12 h induction, AmySTU was detected in the supernatant fraction by 12% SDS-PAGE, revealing a Coomassie blue-stained band around 45 kDa (Fig. 5), which is 5 kDa less at than the predicted 50 kDa. The reason is currently under investigation.

Substrate Specificity

Purified AmySTU was hydrolytically active on a number of substrates. Apart from soluble starch, the enzyme also cleaved potato starch and β-cyclodextrin, whereas agarose, agar and EP were poor substrates. On the other hand, carboxymethylcellulose, sodium alginate, carrageenan, and chitosan were not cleaved by the enzyme (Table 2).

Characterization of the Amylase

For convenience, the characterization of amylase was carried out using soluble starch as the substrate.

Influence of temperature and pH on the activity and stability. The temperature profile of AmySTU activity is
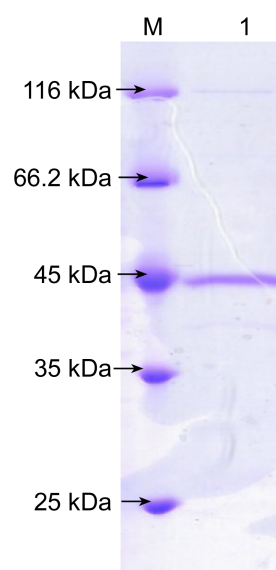
Fig. 5. SDS-PAGE of the purified recombinant AmySTU.

M: standard molecular mass markers; lane 1: purified AmySTU.

shown in Fig. 6. The enzyme displayed optimal activity at 40°C and retained 45% of its maximal activity at 60°C. AmySTU also showed 38% residual activity at 4°C. Up to now, the optimum temperatures for enzymatic activity of α-amylases that were normally used in industry were

Table 2. Substrate specificity of AmySTU.

Substrate	Enzyme activity (U/ml)
Soluble starch	142.5
Potato starch	178.4
β-Cyclodextrin	42.9
Agar	11.8
EP	5.0
Agarose	3.1
Carrageenan	ND
Sodium alginate	ND
Chitosan	ND
Carboxymethylcellulose	ND

ND, not detectable.

For the determination of substrate specificity, recombinant enzyme was incubated for 10 min in Tris-HCl buffer (20 mM, pH 7.5) at 40°C with different substrates (0.25% (w/v)).

ordinarily between 50°C and 55°C, which shows that the amylase excreted by strain B7 belongs to the cold-adapted enzyme [5, 10, 17].

The effect of pH on AmySTU activity and stability is shown in Fig. 7. The optimal activity of the enzyme was at pH 7.5–8.0. The enzyme was stable in a range of pH 5.5–9.5 after 60 min preincubation at 30°C. The enzyme retained about 65% of its original activity at pH 5.5–9.5.

Effects of NaCl and CaCl$_2$. To examine the effect of salt, AmySTU was incubated for 12 h at 30°C in various NaCl concentrations, and the residual activity was measured.

Fig. 7. Effect of pH on AmySTU activity (filled circles) and stability (open circles).

The relative activities were defined as the percentage of the maximum activity detected in the assay. For determining pH stability, the enzyme was incubated at different pH buffers for 60 min at 30°C, and the remaining activity was determined under standard assay conditions. Values are the mean ± SD of three independent experiments.

The enzyme showed maximum activity in 2% NaCl (Fig. 8). Results in Fig. 8 revealed that the enzyme was stable in NaCl concentrations of 0–20% and retained 58% of its activity in the presence of 16% NaCl.

For determining the effect of CaCl$_2$, AmySTU was incubated at 30°C in 20 mM Tris-HCl (pH 7.0) containing various concentrations of CaCl$_2$ for 12 h, and the remaining
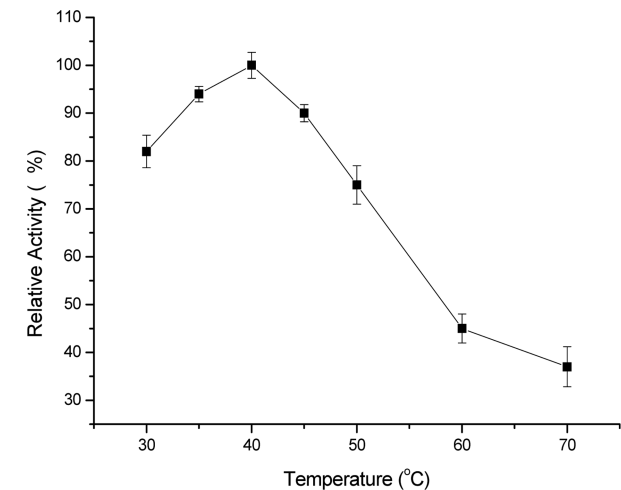

Fig. 6. Effect of temperature on AmySTU activity.

The relative activities were defined as the percentage of the maximum activity detected in the assay. Values are the mean ± SD of three independent experiments.
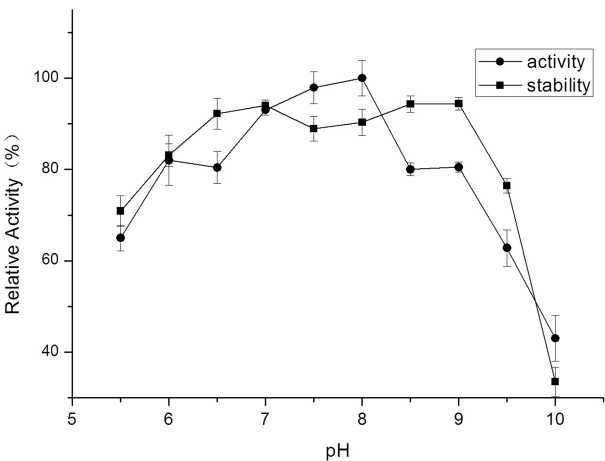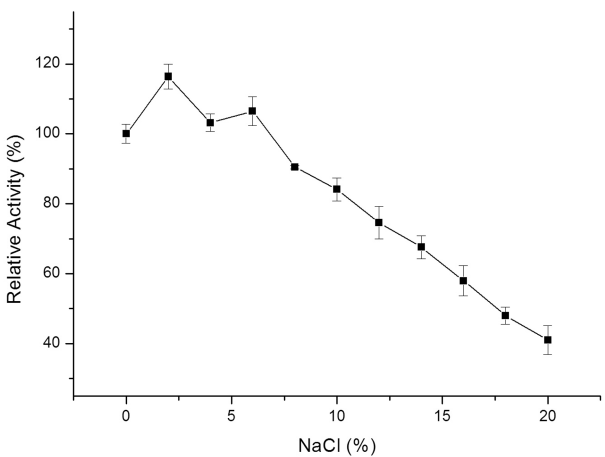

Fig. 8. Effect of NaCl concentrations on AmySTU activity.

The activity was measured in the reaction mixture containing various concentrations of NaCl. The relative activity was defined as the percentage to that of 0% NaCl detected in each assay. Values are the mean ± SD of three independent experiments.
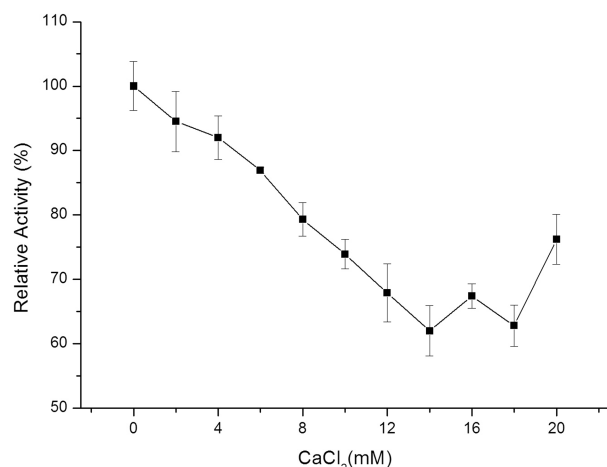
Fig. 9. Effect of $CaCl_2$ on the activity of AmySTU.
Values are the mean ± SD of three independent experiments.

activity was determined under standard assay conditions.

$CaCl_2$ was not required for the activity of the AmySTU against soluble starch. In the presence of increasing $CaCl_2$ concentrations, the activity of the enzyme was slightly inhibited. At 25 mM of $CaCl_2$, the enzyme retained around

60% of its activity (relative to the sample without $CaCl_2$) (Fig. 9).

Influence of different metal ions and chemical reagents on the amylase. The influence of various metal ions on the activity of AmySTU is presented in Table 3. None of the metal ions stimulated enzyme activity. In contrast, Co^{2+}, Cu^{2+}, Fe^{3+}, and SDS inhibited the enzyme. Other metal ions had no significant effects. Moreover, EDTA did not affect the amylase, indicating it is probably not a metalloenzyme [1].

AmySTU activity was studied in the presence of surfactants. The enzyme showed more than 95% activity in the presence of nonionic surfactants, such as Triton X-100, Tween 20, and Tween 80. However, 43% of the activity was lost in the presence of SDS.

Enzyme stability was also investigated. The enzyme activity decreased by 55%, 80%, 55%, 37%, and 76% after incubation in the presence of Cu^+, Ni^{2+}, Cu^{2+}, Co^{2+}, and EDTA, respectively, after 24 h incubation at 30°C. Surfactants and other metal ions had no big effects on the enzyme stability.

Effects of organic solvents. The effects of organic solvents on the stability of the amylase were determined. The

Table 3. Effects of various metal ions, surfactants, and organic solvents on the amylase activity.

Metal ion (10 mM)	Residual activity (%)		Organic solvent	Log Pow	Residual activity (%)	
	0 h	24 h			20%	50%
$MgCl_2$	94.6 ± 3.7	77.3 ± 7.9	None	-	100 ± 0.5	100 ± 1.5
$CuCl_2$	91.9 ± 2.7	36.7 ± 8.3	Dimethylformamide	-0.85	95.0 ± 0.4	81.8 ± 0.6
KCl	96.1 ± 3.3	91.7 ± 4.6	Methanol	-0.77	121.3 ± 1.1	123.3 ± 0.7
$BaCl_2$	92.6 ± 1.1	90.2 ± 3.6	Acetonitrile	-0.34	37.3 ± 0.8	6.87 ± 0.1
Ni Cl_2	88.1 ± 4.5	7.7 ± 4.5	Ethanol	-0.32	109.1 ± 0.9	109.8 ± 1.0
$CuSO_4$	59.4 ± 3.2	4.3 ± 5.4	Acetone	-0.24	104.7 ± 1.3	82.5 ± 0.7
$CoCl_2$	63.2 ± 4.9	26 ± 6.6	Formaldehyde	0	9.6 ± 0.3	18.0 ± 0.3
$AlCl_3$	94.5 ± 2.1	79.2 ± 5.4	Isopropyl alcohol	0.05	99.2 ± 1.0	96.0 ± 0.9
$Fe_2(SO_4)_3$	70.4 ± 1.7	68.2 ± 3.2	*n*-Butyl alcohol	0.88	100.2 ± 1.6	79.0 ± 0.6
$MnSO_4$	91.3 ± 1.4	83.9 ± 4.8	Isoamyl alcohol	1.28	118.3 ± 0.9	112.6 ± 1.9
β-Mercaptoethanol	102.5 ± 3.1	100.1 ± 2.5	Chloroform	1.97	68.6 ± 1.5	20.2 ± 0.8
Tween20	96.3 ± 2.4	102.9 ± 0.9	Toluene	2.73	90.4 ± 0.8	39.6 ± 3.0
Tween80	95.1 ± 1.7	91.1 ± 3.6	Dimethylbenzene	3.12	112.5 ± 0.9	17.3 ± 1.7
EDTA	85.9 ± 2.8	9.5 ± 2.5	*n*-Hexane	4.11	124.4 ± 1.3	135.8 ± 5
Triton X-100	102.2 ± 0.8	103.5 ± 1.1				
SDS	56.9 ± 2.3	28.4 ± 0.9				
DTT	94.2 ± 1.6	100.4 ± 4.3				

The enzyme was dialyzed against 20 mM tris-HCl buffer (pH 7.5). The activity of the enzyme was determined after preincubating with the metal ions at 25°C for 0 and 24 h respectively. The activity in the absence of any additives was taken as 100%. Values are the mean ± SD of three independent experiments.

amylase was incubated in the presence of 20% of various organic solvents at 25°C with shaking for 2 h, and the remaining activity was measured. The data in Table 3 showed that most of the organic solvents tested had no significant effect on enzyme activity, whereas some organic solvents stimulated amylolytic activity of the enzyme, with the highest activity detected in the presence of isoamyl alcohol (increased activity by 18%). However, the formaldehyde almost completely inhibited the activity, and only 9% relative activity was detectable.

Stability of the amylase in the presence of 50% of organic solvents was also tested. The amylase activity was not significantly influenced by 50% of DMF, methanol, ethanol, acetone, isopropyl alcohol, *n*-butyl alcohol, or isoamyl alcohol. The amylase was found to be dramatically inhibited by dimethylbenzene, as only 17% of its activity was retained when incubated in the presence of 50% of this organic solvent. In contrast, *n*-hexane had an activating effect on the purified enzyme, and maximum activity (135%) was observed in the presence of 50% *n*-hexane.

Discussion

The aim of this article was to obtain an EPL to degrade EP. All the references of EP are about the extract, structure, and biological activity. In this study, iodine solution was used to detect the EPL and an *A. macleodii* B7 was selected as the target bacterium.

For AmySTU, gene analysis indicated that this enzyme has a signal peptide of 23 amino acids. For convenience to purify, we removed the termination codon and retained the signal peptide when designing the primers. Thus, the production of translation has two his-tags: C-terminal his-tag and N-terminal his-tag. The N-terminal region including the His-tag is removed while secreted into media. The AmySTU can be purified by C-terminal his-tag. Western blot has verified that the protein AmySTU was really in the media.

The research of substrate specificity indicates that the enzyme is amylase and the optimum substrate is starch. Besides the above reasons, the reason we choose starch as the substrate to characterize the enzyme are as follows. First, the starch purchased is more pure than the EP, which we made ourselves. Thus, the enzyme activity for starch is more stable than for EP. Second, the enzyme activity for starch is much higher than EP. Thus, the error for enzyme activity is much smaller and the experimental data are more reliable. Third, starch and EP both are polysaccharides. The difference is that starch is composed of glucose whereas EP

are composed of rhamnose, xylose, glucose, and galactose [3, 31]. Chemical structural analysis of polysaccharides from Indian samples of *Enteromorpha compressa* (Ulvales, Chlorophyta) revealed a branched structure having 1,4- and 1,2,4-linked rhamnose 3-sulfate, 1,4-linked glucose, 1,3- and 1,6-linked galactose, 1,4- and terminally linked glucuronic acid and 1,4-linked xylose partially sulfated on O-2 [3]. The 1,4-linked rhamnose and 1,4-linked glucose may be the reason that the amylase can degrade the EP.

For the purified AmySTU, substrate specificity results showed that the AmySTU is an amylase that degrades EP, although the enzyme activity is low. α-Amylase from Sigma was also tested and it did hydrolyze the EP. The reason may be that EP are heteropolysaccharides and the enzyme can break some kind of chemical bonds. To our best knowledge, this is the first report of an α-amylase that can degrade the EP.

At present, the extensively studied cold-adapted enzymes are mainly produced by polar microorganisms, including *Alteromonas* [9], *Nocardiopsis* [41], *Janthinobacterium* [21], *Duganella* [21], and *Cytophaga* [25]. Some cold-adapted enzymes are from deep-sea microorganisms [27, 29]. Cold-adapted amylase from marine surface has not been reported.

The inhibition of amylase activity in the presence of Ca^{2+} has been reported in the case of metalloenzymes containing a metallic ion for catalytic activity [18, 32]. On the contrary, other reports also indicate no effect of Ca^{2+} on amylase activity [8, 34, 38].

Organic solvent-tolerant halophilic enzymes appear to be quite attractive for industrial applications such as bioremediation of carbohydrate-polluted salt marshes and industrial waste waters contaminated with organic solvents. However, reports about halophilic cold-adapted enzymes with organic solvent tolerance are scarce. As shown in Fig. 8 and Table 3, AmySTU showed excellent salt-tolerance and organic solvent-tolerance properties, such as obvious stimulation by some organic solvents (methanol, isoamylalcohol, and *n*-hexane). The organic solvent-tolerance behaviors might be due to the carry-over of nonpolar hydrophobic solvent, which provided an interface, thereby keeping the enzyme in an open conformation and resulting in the observed activation [40]. Li and Yu [20] found that the stability of amylase from *Thalassobacillus* sp. LY18 was dependent on the polarity of the solvents and increased only in the presence of water-insoluble solvents with higher log P_{ow} values. Shafiei *et al*. [35] also reported that hydrophilic solvents had no big significant effect on the activity of amylase from *Nesterenkonia* sp. strain F, and hydrophobic solvents increased the

activity of the amylase. However, in this article, no relationship between the stability against organic solvent and the polarity of the added organic solvent was found. The result was, however, consistent with previous observations by Fukushima *et al.* [11]; Karbalaei-Heidari *et al.* [16]; and Ruiz and Castro [33].

The enzyme is halotolerant and cold-adapted. Moroever, considering its high activity and stability in the presence of organic solvents, it could be potentially useful for practical applications in biotechnological processes with nonconventional media, such as cold washing.

Acknowledgments

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