J. Microbiol. Biotechnol. (2014), 24(1), 19–25
http://dx.doi.org/10.4014/jmb.1308.08055

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Purification and Characterization of Thermostable Agarase from *Bacillus* sp. BI-3, a Thermophilic Bacterium Isolated from Hot Spring

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Received: August 20, 2013
Revised: September 24, 2013
Accepted: September 25, 2013

First published online
September 25, 2013

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pISSN 1017-7825, eISSN 1738-8872

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An extracellular agarase was purified from *Bacillus* sp. BI-3, a thermophilic agar-degrading bacterium isolated from a hot spring in Indonesia. The purified agarase revealed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with an apparent molecular mass of 58 kDa. The optimum pH and temperature of the agarase were 6.4 and 70°C, respectively. The activity of the agarase was stable at high temperatures, and more than 50% activity was retained at 80°C for 15 min. Furthermore, the enzyme was stable in the pH range of 5.8–8.0, and more than 60% of the residual activity was retained. Significant activation of the agarase was observed in the presence of K^+, Na^+, Ca^{2+}, Mg^{2+}, and Sr^{2+}; on the other hand, Ba^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, and EDTA inhibited or inactivated the enzyme activity. The components of the hydrolytic product analyzed by thin-layer chromatography showed that the agarase mainly produced neoagarobiose. This study is the first to present evidence of agarolytic activity in aerobic thermophilic bacteria.

Keywords: Hot spring bacteria, thermostable agarase, purification, characterization

Introduction

Agar is a complex polysaccharide component of the cell walls of some red algae, and is composed of agarose and agaropectin. Agarose is a linear polymer of alternating units of 3-O-bound β-D-galactopyranosides and 4-O-bound 3,6-anhydro-α-galactopyranoses, whereas agaropectin has an intricate yet vague structure. There are only a few organisms capable of hydrolyzing agar, which allows agar to be widely used as a gelling agent for microbiological media [4].

Agarases are the hydrolytic enzymes that degrade agar into oligosaccharides, which have various chemical properties and biological activities. Based on the sites of hydrolysis, agarases are characterized as α-agarases and β-agarases. The α-agarases cleave the α-L-(1,3) linkages of agarose to produce oligosaccharides of the agarobiose series with 3,6-anhydro-L-galactopyranose at the reducing end, whereas the β-agarases cleave the β-D-(1,4) linkages of agarose to produce neoagarooligosaccharides with D-galactopyranoside residues at the reducing end [28]. Agarases can be used to prepare protoplasts and extract biological substances, such as unsaturated fatty acids, vitamins, carotenoids, betaine, among others, from algae [3].

Agarases are produced mainly by bacteria, including *Pseudomonas*, *Pseudoalteromonas*, *Vibrio*, *Streptomyces*, *Agarivorans*, *Alteromonas*, *Cytophaga*, *Bacillus*, and *Halomonas aquamarina* [6]. As agar is a polysaccharide produced by marine seaweeds, most of the agar-degrading bacteria are naturally inhabitants of the marine habitat, and only very few agar-degrading non-marine bacteria (freshwater, hot spring, soil, *etc.*) have been reported [7]. With rare exceptions, all the agarolytic prokaryotes are mesophiles, and only three strains of anaerobic thermophilic bacteria with an agarolytic activity are known [4]. All the known bacterial agarases are thermolabile enzymes, which are quickly inactivated at 60°C. Only the *Thermoanaerobacter wiegelii* B5 agarase is known to be active at a temperature range of 50–80°C

(optimum, 70°C) [4].

In the last decades, research on thermophilic microorganisms has progressed to a considerable degree owing to the thermostability of their enzymes, which have application in various fields of biotechnology [9,14]. Extracellular hydrolytic enzymes have been detected in numerous hyperthermophiles (both archaea and bacteria), with an optimum growth temperature exceeding 80°C [26], as well as in extreme and moderate thermophiles, both aerobic [13] and anaerobic [18], represented by bacteria with an optimum growth temperature of 55–75°C. In the present study, *Bacillus* sp. BI-3, a novel agar-degrading thermophilic aerobic bacterium, was isolated from a hot spring in Indonesia, and the characteristics of the thermostable agarase from this bacterium as well as its enzymatic products were investigated.

Materials and Methods

Isolation of Agarase-Producing Bacterial Strain

The agarase-producing thermophilic bacterial strain was isolated from green microbial mat collected from a hot spring on the coast of Kalianda Island, Indonesia (105°35′12′′; 5°44′46′′). The strain was inoculated on a medium containing 0.3% (w/v) peptone, 0.3% (w/v) yeast extract, 0.3% (w/v) NaCl, and 2.0% (w/v) agar, and incubated at 55°C. Positive colonies showing clear zones or pits were picked out from the selection plates containing agar as the sole source of energy and carbon.

Agarase Enzyme Assay

The specific activity of purified agarase was determined according to a modified method described by Ohta *et al*. [23]. An appropriately diluted enzyme solution was added to different substrates in sodium phosphate buffer (pH 6.5) and incubated at 70°C for 15 min. The activity was expressed as the initial rate of agar hydrolysis by measuring the release of reducing ends using the 3,5-dinitrosalicylic acid (DNS) procedure with D-galactose as the standard [20]. One unit of enzyme activity was defined as the amount of protein that catalyzes the production of 1 μmol of reducing sugar such as D-galactose per minute under assay conditions.

Preparation of Partially Purified Agarase Solution

To isolate agarase, the BI-3 strain was grown in 500 ml vials containing 350 ml of the above-mentioned medium supplemented with 0.3% agarose. The cells grown for 36 h were separated by centrifugation at 6,000 ×*g* for 10 min at 4°C. The enzyme in the supernatant was perturbed by the addition of solid ammonium sulfate at 75% saturation with slow stirring for 1 h. The precipitate formed was recovered by centrifugation at 6,000 ×*g* for 30 min at 4°C. The precipitate was dissolved in sodium phosphate buffer (pH 6.5) and dialyzed against 1,000 ml of the same buffer overnight with intermittent change of buffer every 4 h. The dialyzed sample was concentrated with polyethylene glycol 20,000 solution.

Separation, Purification, and Determination of Molecular Mass of the Agarase

The clear dialysate was applied onto a Q-Sepharose column (2.6 × 40 cm) equilibrated with sodium phosphate buffer (pH 6.5). The column was washed with 50 ml of the same buffer to remove unbound proteins. The enzyme was eluted in 1.5 ml fractions by a discontinuous gradient of NaCl (0–0.5 M) in the same buffer. The fractions with highest agarase activity were pooled and concentrated by using a 30 kDa Amicon Ultra-15 (MWCO 30 kDa; Millipore). Subsequently, the concentrate was loaded onto a Sephacryl S-200 column (Pharmacia, Uppsala, Sweden) equilibrated with sodium phosphate buffer (pH 6.5) and the proteins were eluted with the same buffer. The active fractions were analyzed for protein content by reading the absorbance at 280 nm and stored at 4°C until further use. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to estimate the protein molecular mass with a stacking gel (4% polyacrylamide) and a separating gel (10% polyacrylamide).

Effects of pH, Temperature, and Metal Ions

In each experiment, 1.0% agar solution and purified agarase were mixed and incubated at various times and temperatures as described earlier. The relative agarase activity was determined by the DNS method. The optimum pH of the enzyme was determined using the following buffers at 20 mM concentrations: NaAc-HAc buffer (pH 5.0–6.4), KH_2PO_4-NaOH buffer (pH 6.5–7.5), Tris-HCl buffer (7.5–10.0), and $NaCO_3$-$NaHCO_3$ buffer (9.0–10.0). The stability of the enzyme at various pH values was examined as follows: 10 ml of the enzyme was made to 100 ml using appropriate buffer solution and pre-incubated for 15 min at 70°C, and the residual activity was determined under standard assay conditions. The effect of temperature on the agarase activity was examined under standard assay conditions with varied temperatures (from 50°C to 85°C). The thermostability of the agarase was determined by assaying the residual activity of the enzyme at 70°C after pre-incubation for 15 min at the desired temperature. The effects of various ions or chelator ($CuSO_4$, KCl, $ZnSO_4$, $FeSO_4$, $BaCl_2$, NaCl, $SrCl_2$, $CoCl_2$, $MgSO_4$, $MnCl_2$, and $CaCl_2$) and EDTA on the catalytic activity of the agarase were determined by including the metal salts (2 mM) in the assay mixture and incubating at 70°C for 15 min. The control comprised the assay mixture without metal ion salts or chelator.

Identification of Enzyme-Hydrolyzed Products

Thin-layer chromatography (TLC) was used to identify the hydrolysis products of agar. Neoagarohexaose (NA6), neoagarotetraose (NA4), neoagarobiose (NA2), or neoagarohexaitol (NA6-OL) were purchased from Sigma. The reactions of purified agarase and agar or neoagarohexaitol were carried out as 100 ml reactions containing 10 ml of purified agarase and 90 ml of 1.0% agar at 70°C for 15, 30,

and 60 min, respectively. Subsequently, the reaction mixtures were applied to a silica gel 60 TLC plate (Merck, Germany). The TLC plates were developed using a solvent system consisting of *n*-butanol–acetic acid–water (2:1:1 (v/v)). After hydrolysis of the substrates, the resultant oligosaccharide spots were visualized by spraying naphtoresorcinol or aniline hydrogen phthalate on the plate, and then heating it on a hot plate.

Results and Discussion

Isolation and Characterization of Thermophilic Agarolytic Bacteria

Overall, 20 samples from hot springs located in Kalianda Island were inoculated onto plates containing solid medium with agar as the sole source of energy and carbon. After plating 10-fold dilutions on the plate and incubating the plate at 55°C, the sole colonies appeared on the surface, with clear zones or pits around the colonies. After replating these colonies onto semiliquid medium with 0.5% agarose, one strain of thermophilic bacteria, BI-3, was isolated. Analysis of the 16S rRNA sequences allowed us to ascribe this strain to the genus *Bacillus*, and we named it as *Bacillus* sp. BI-3. The pure strain is conserved in the Key Laboratory of Marine Bio-active Substances *SOA*.

To date, very few agar-degrading thermophilic bacteria from hot springs have been reported. Only Bannikova *et al*. [4] had described an anaerobic thermophilic bacterium displaying agarolytic activity, which was isolated from hot springs located in the Baikal region and Kamchatka and was found to belong to the genus *Thermoanaerobacter*. These results suggest that microorganisms displaying agarolytic activity are widespread. In previous studies, mesophilic agarolytic microorganisms were detected in marine habitats, where marine red algae could be the source of agar, acting as a substrate for them. However, agarolytic organisms had also been isolated from freshwater springs [1]. These data demonstrate that agarolytic prokaryotes can also inhabit terrestrial hot springs, where the polysaccharide of cyanobacteria, forming cyanobacterial mats [21], can act as the substrate for agarase.

Separation and Identification of Agarase BI-3

The agarase was purified by ammonium sulfate fractionation, followed by a combination of two chromatographic steps. The agarase was purified 47.78-folds with a specific activity of 373.62 U mg/l and a final yield of 10.68%. SDS-PAGE revealed the presence of a single protein band with agarase activity, whose apparent molecular mass was estimated to be 58 kDa (Fig. 1).

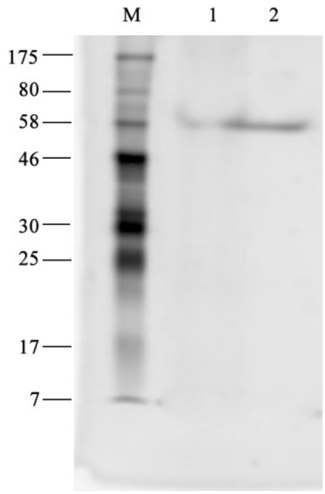

Fig. 1. Electrogram of SDS-PAGE of the purified agarase BI-3. M: Standard molecular weight markers; 1, 2: Agarase BI-3 of different concentrations.

The reported molecular mass of agarase varies from values as low as 12 kDa [11] in the case of *Bacillus megaterium* to as high as 210 kDa in the case of *Pseudomonas*-like bacteria [19]. The β-agarases are divided into three groups according to their sizes, and Group II includes β-agarases with a molecular mass of 50–80 kDa. The molecular mass of extracellular β-agarase secreted by the agar-liquefying bacterium *Alteromonas* sp. C-1 [16] has been found to be 52 kDa, that of AgaD from *Vibrio* sp. PO-303 has been noted to be 51 kDa [5], that of AgaA from *Agarivorans* sp. LQ48 has been observed to be 52 kDa [17], and that of AgaA from *Zobellia* sp. has been found to be 69 kDa [10]; all of these agarases belong to Group II β-agarases. In the present study, the molecular mass of agarase BI-3 was found to be 58 kDa, suggesting that the enzyme belongs to Group II β-agarases.

Effects of Temperature and pH on Agarase BI-3 Activity and Stability

Temperature and pH are considered to be decisive parameters for enzyme activity. The temperature optima of various agarases are higher than the gelling temperature of agar because compact bundles of gelled agar hinder enzyme action [10, 22, 27, 28]. Most of the agarases known to date are thermolabile enzymes incapable of displaying their activities at temperatures exceeding 50–60°C. It was observed that the activity of agarase BI-3 consistently increased from 55°C to 70°C with optimum activity at 70°C (Fig. 2A). However, an obvious decrease was observed when the agarase was incubated at temperatures above 80°C. The activity of agarase was stable at a high

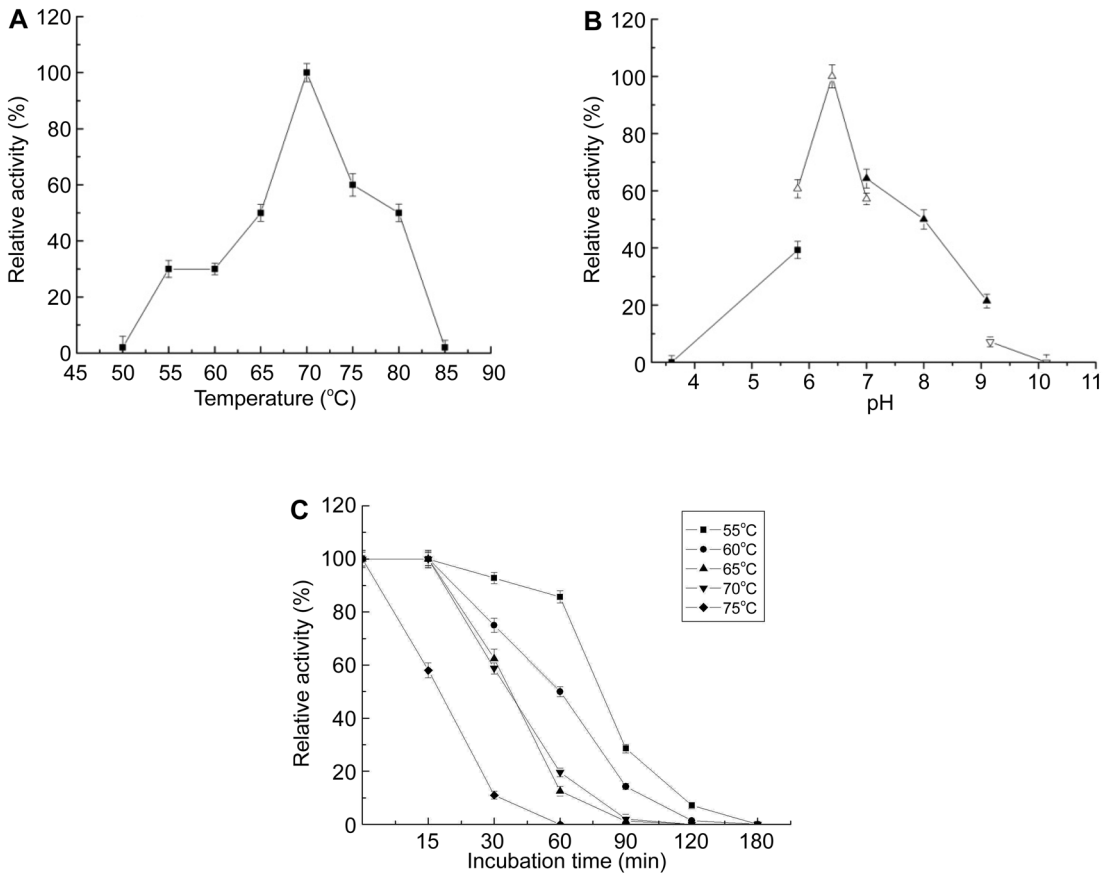

Fig. 2. Effects of temperature and pH on the specific activity of agarase BI-3.
(**A**) Effect of temperature on the specific activity of agarase BI-3; (**B**) Effect of pH on the specific activity of agarase BI-3; (**C**) Effect of different temperatures on the thermostability of agarase BI-3. —■—, NaAc-HAc buffer; —△—, KH_2PO_4-NaOH buffer; —▲—, Tris-HCl buffer; —▽—, $NaCO_3$-$NaHCO_3$ buffer.

temperature and retained more than 50% of its activity up to a temperature of 80°C (Fig. 2B). This study is the first to present evidence of agarolytic activity in aerobic thermophilic bacteria. The isolated agarase BI-3 displayed high optimum temperature and thermostability (Fig. 2C). The enzyme exhibited maximum agarase activity at pH 6.4 (Fig. 2B), whereas the optimum pH for many other agarases was noted to be 7.0 [10, 17, 24]. In previous studies, agarases active at alkaline pH had also been reported [7, 15]. Agarase BI-3 was stable in a wide pH range from 5.8 to 8.0, retaining more than 50% of its residual activity, similar to other agarases such as AgaY (6.0–8.0) [24].

Effects of Various Metal Ions and Reagent on Agarase BI-3 Activity

The effects of metal ions and other reagents on the agarase activity were investigated (Table 1). It has been reported that macroelements found in seawater, such as

Table 1. Effects of metal ions or chelators on agarase BI-3 activity.

Metal ions or chelators	Relative activity of agarase BI-3 (%)
Control	100
Mg^{2+}	125
Ca^{2+}	150
K^{+}	250
Na^{+}	375
Sr^{2+}	400
Ba^{2+}	50
Cu^{2+}	0
Zn^{2+}	0
Fe^{2+}	0
Co^{2+}	0
Mn^{2+}	0
EDTA	0

Na^+, K^+, Mg^{2+}, Ca^{2+}, and Sr^{2+}, activate some agarases [15, 29]; surprisingly, these metal ions could also obviously activate agarase BI-3 activity, although *Bacillus* sp. BI-3 was screened from freshwater. The reason for this observation may be the inhabitation of *Bacillus* sp. BI-3 in the hot spring located near the sea. However, similar to AgaY, the agarase activity was inhibited or inactivated by other metal ions such as Ba^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, and Fe^{2+}. Furthermore, although EDTA has been reported to be a poor inhibitor of AgaA34, it completely inactivated the activity of agarase BI-3. These results indicated that agarase BI-3 was a metal-ion-dependent enzyme and that some metal ions were essential for its activity.

Identification of Enzyme-Hydrolyzed Products

Neoagarobiose, along with some neoagarohexaose and neoagarotetraose, was the main product of agarose hydrolysis by agarase BI-3 (Fig. 3). The agarooligosaccharides, which were purchased from Sigma, were also run along with the agarase-BI-3-hydrolyzed products on TLC. None of the hydrolysis products produced by the purified agarase BI-3 matched with the R_f values of agarooligosaccharides (data not shown), whereas they matched with the R_f values of standard neoagarooligosaccharides. This clearly demonstrated that the agarase secreted by *Bacillus* sp. BI-3 is a β-agarase. Furthermore, agarase BI-3 was observed to be an endo-type glycosidase, and specifically cleaved only the β-1,4 linkages and not the β-1,3 linkages found in agarose. In previous studies, few bacteria capable of producing endo-type β-agarases that can hydrolyze agarose and neoagaro-oligosaccharides to yield neoagarobiose have been reported [2, 3, 12, 25]. Furthermore, only β-agarases from *Vibrio* sp. AP-2 [2], *Vibrio* sp. JT0107 [25], and *Vibrio* sp. PO-303 [3] have been observed to have the ability to release neoagarobiose from neoagarotetraose. The rest of the reported β-agarases have been noted to produce mixtures of neoagarobiose and neoagarotetraose as the final products [7].

The β-agarase BI-3 can hydrolyze neoagarohexaose to produce neoagarotetraose and neoagarobiose as described above. Since two β-1,4 linkages are present in neoagarohexaose, neoagarohexaitol was used as a substrate in order to clarify the cleavage site. The products of enzymic hydrolysis of the sugar alcohol were detected by spraying TLC plates with naphthoresorcinol (specific for compounds containing 3,6-anhydro-L-galactose) and aniline hydrogen phthalate (specific for reducing compounds). Two hydrolysis products were detected, as shown in Fig. 3B. The R_f value of one product corresponds to that of neoagarotetraose; the aniline hydrogen phthalate reagent indicated that the product was a reducing sugar, but not a sugar alcohol. The other product was not

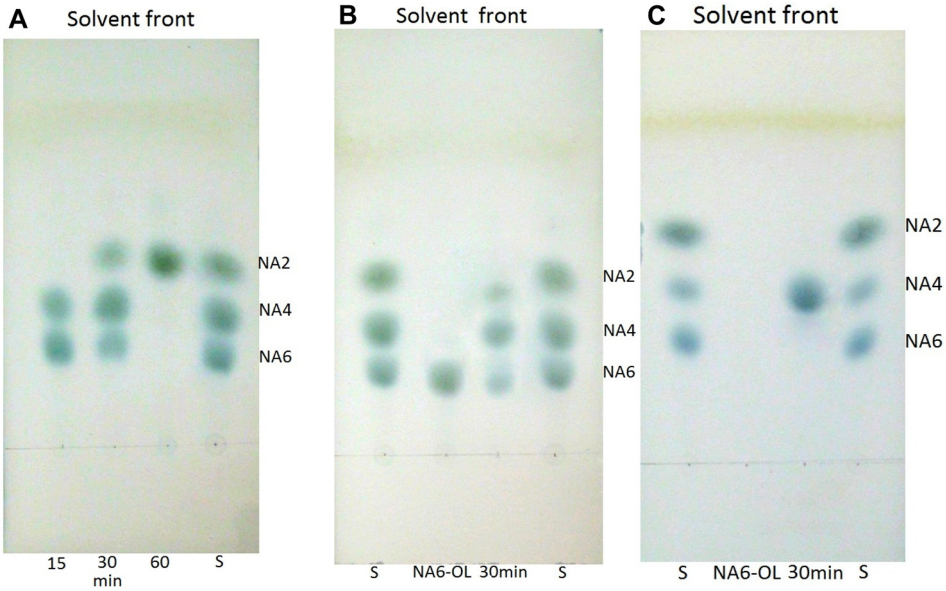

Fig. 3. Thin-layer chromatogram of the hydrolysis products of purified agarase BI-3 reaction.

(**A**) Thin-layer chromatogram of the hydrolysis products of agar by BI-3. S, standards (NA2, NA4, NA6); NA2, neoagarobiose; NA4, neoagarotetraose; NA6, neoagarohexaose. Numbers under the chromatogram indicate the reaction time. (**B**) and (**C**). Thin-layer chromatogram of the hydrolysis products of neoagarohexaitol by BI-3. The plates were sprayed with naphthoresorcinol (**B**) or aniline hydrogen phthalate (**C**). S, standards (NA2, NA4, NA6); NA2, neoagarobiose; NA4, neoagarotetraose; NA6, neoagarohexaose; NA6-OL, neoagarohexaitol.

neoagarobiose because its R_f value differed from that of the standard neoagarobiose sample, and the product was not a reducing sugar, as shown in Fig. 3C. From these observations, it was considered that the β-agarase BI-3 cleaves the β-1,4 linkage near a sugar alcohol in neoagarohexaitol. Thus the β-agarase BI-3 first cleaves the β-1,4 linkage near the reducing end of neoagarohexaose to produce neoagarotetraose and neoagarobiose, and then cleaves the β-1,4 linkage of neoagarotetraose to produce neoagarobiose.

In the present study, the β-agarase BI-3 reaction on agar, after prolonged incubation (60 min), produced neoagarobiose as the final product without other neoagarooligosaccharides. This unique character is useful for selective production of neoagarobiose from agar/agarose, which has both moisturizing and whitening effects on skin [12]. Moreover, the enzyme was noted to be active over a broad range of pH, with a high optimum temperature and thermostability. Thus, β-agarase BI-3 has greater potential for industrial production of neoagarobiose from agar/agarose at high temperature.

Acknowledgments

This study was financially supported by the Public Science and Technology Research Funds Project of Ocean (2011418027), Shandong Province Young and Middle-Aged Scientists Research Awards Fund (DS2010HZ001), and Basic Scientific Research Funds of FIO, SOA (AGY2011G17).

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