Heterologous expression of a newly screened β-agarase from Alteromonas sp. GNUM1 in Escherichia coli and its application for agarose degradation

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ARTICLE INFO

Article history:
Received 28 October 2013
Received in revised form 3 December 2013
Accepted 18 December 2013
Available online 4 January 2014

Keywords:
Alteromonas
β-Agarase AgaG1
Agarose Neoagarobiose Neoagarotetraose

ABSTRACT

The gene of agaG1 from Alteromonas sp. GNUM1 encoding a β-agarase (AgaG1) was heterologously expressed in E. coli BL21 (DE3). The recombinant strain was cultured at 37 °C and then AgaG1 was expressed at 25 °C and 0.5 mM IPTG. The optimum conditions for AgaG1 to hydrolyze agarose were pH 7.0 and 40 °C. The main products of agarose hydrolysis by AgaG1 were confirmed to be neoagarobiose and neoagarotetraose. A new agarose hydrolysis process using AgaG1 was developed, in which the reaction temperature was adjusted stepwise to avoid gelation problem with no chemical pretreatment step. The enzyme AgaG1 was found to be very effective and highly selective. When 10.0 g/L agarose was hydrolyzed, 98% of the agarose added was converted to 3.8 and 6.4 g/L of neoagarobiose and neoagarotetraose, respectively.

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1. Introduction

Bioenergy and chemicals production from biomass has been increasingly attracting public attention recently. Their production from marine algae such as red algae as carbon source has many advantages compared with those based on starch and lignocelluloses. Marine algal biomass can be cultivated and harvested easily in the sea without competing with food crops for lands as starch biomass does, potentially having unlimited cultivation space, and its use in quantity as raw material for bioenergy and chemicals would not cause forest denudation as the use of cellulosic biomass is expected to. Growing marine algae can fix a larger amount of CO2 per unit mass than starch crops and plants and thus their cultivation can contribute more positively in reducing global warming [1,2]. In addition, because marine algae do not compete with food crops, they can be a more ethical choice than starch biomass. Marine algae has a relatively low content of lignocelluloses and thus much easier to hydrolyze than cellulosic biomass.

The main component of cell wall of red algae represented by Gelidiales and Gracilariales is agar. Agar is composed of two similar but different types of polysaccharides, agarose and agaropectin. Agarose consists of a linear chain of alternating residues of 3-0-linked β-D-galactopyranose and 4-0-linked 3,6-anhydro-α-L-galactose [3]. Agaropectin has a similar linear structure to agarose, but it has side groups of sulfate esters, pyruvate acetal, and methyl ethers [3]. For the hydrolysis of agar or agarose, chemical or enzymatic treatment can be used. A chemical process using a strong acid is simple and efficient, but it generates together with galactose, the target sugar, toxic compounds such as 5-hydroxymethylfurfural and levulinic acid. Both of them are known to inhibit the growth of many microbial strains including yeast in ethanol fermentation [4,5]. In an enzymatic hydrolysis process on the contrary, galactose and 3,6-anhydro-α-L-galactose are selectively produced by the action of agarase(s) and agarobiose hydrolyase with no toxic compounds generation. There are two types of enzymatic methods for agar hydrolysis: β-agarolytic and α-agarolytic. The β-agarolytic method use β-agarase(s) (EC 3.2.1.81) which cleavages β-1,4 bonds in agarose to produce neoagarobiose, while the α-agarolytic method use α-agarase(s) (EC 3.2.1.158) which cleavages α-1,3 bonds to produce agarobiose [6]. However, there have been only few studies on the α-agarolytic method since α-agarase is very rare in nature. For this reason, the β-agarolytic method has been the first choice for enzymatic hydrolysis of agar [7]. Agaropectin can be also hydrolyzed by agarase(s), but less completely than agarose due to the structural difference between the two.
In our previous work, an agar degrading bacterial strain of Alteromonas sp. GNUM1 was isolated from the surface of Sargassum serratifolium, a macroalgae collected from the East Sea of Korea [8], and a β-agarase gene (agaG1, Gene accession number KF574012) was screened and characterized [9]. In this study, the newly identified β-agarase (AgaG1) was heterologously expressed in E. coli BL21. The extracellular portion of β-agarase production was successfully used for the hydrolysis of agarose to produce neogarabiose and neogarotetraose through stepwise temperature adjustment.

2. Materials and methods

2.1. Strains and plasmids

The genomic DNA of Alteromonas sp. GNUM1 was used for the original source of the β-agarase gene, agaG1. E. coli DH5α [F- endA1 thi-1 recA1 relA1 gyrA96 deoR supE44 proAB lacU169 Δ(lacZΔM15)] was used as the host strain for the cloning and maintenance of plasmids. T&A cloning vector [p2728 bp, RBC Bioscience, Taiwan] was used for PCR product cloning. Plasmids of pET22b(+) [5493 bp, Takara, Japan] with T7 promoter and pMoPac1 [6016 bp, Takara, Japan] with has lac promoter were used as cloning and expression vector. E. coli BL21 (DE3) [F-ompT gal dcm lon hsdS(rB-R− mB−) λDE3 [lacIq (DE3)] was used as the host for the expression of agaG1 gene.

2.2. Media and growth conditions

E. coli was grown on the Luria–Bertani (LB) medium, for the selection of the pET22b(+) and pMoPac1 transformants, 100 mg/L of ampicillin and 35 mg/L of chloramphenicol was used, respectively. Basically, batch culture was performed in a baffled Erlenmeyer flask at 37 °C and 200 rpm.

2.3. Construction of recombinant E. coli strains producing β-agarase

The genomic DNA of Alteromonas sp. GNUM1 was used as PCR template. For extracellular agarase expression, the agaG1 gene with its original signal sequence was amplified by using the following primers, agaG1-F (CATATGAGATTCAgagGATG, Ndel site underlined) and agaG1-R (AGCTGATTAAACTTAGGTCAGATTG, HindIII site underlined) and Taq DNA polymerase (NIRN Biotech, Korea). The PCR product was separated by electrophoresis with 0.7% agarose gel and was purified by using a gel extraction kit (Dyne Bio, Korea). The amplicon was ligated into T&A cloning vector and the recombinant plasmid transformed into E. coli DH5α. The recombinant plasmid was obtained by using a mini plasmid preparation kit (NIRN Biotech, Korea) and analyzed by sequencing. The sequenced plasmid digested with Ndel and HindIII, then 0.95 kb DNA fragment ligated into 5.5 kb pET22b(+) and 6.0 kb pMoPac1 by using T4 DNA ligase (Takara, Japan) resulting the construction of 6.3 kb pET22b-agaG1 and 4.6 kb pMoPac1-agaG1 of agarase production vector (Fig. 1). The expression vectors were transformed to E. coli BL21 by heat shock, and transformants were selected by growing on LB plate, supplemented with 50 mg/L of ampicillin and 35 mg/L of chloramphenicol, respectively. The agaG1 expression was induced by IPTG and confirmed by staining with Lugol’s solution.

2.4. β-Agarase expression

The β-agarase was expressed in a two-stage flask culture. First, the cells were grown at 37 °C. When the culture OD600 reached 1.2, the temperature was shifted to a lower level for induction, hereafter induction temperature, and then IPTG was added to the cultures for expression. At 8 h after the induction, the culture broth was centrifuged, and the supernatant was used for the measurement of extracellular agarase activity. The centrifuged cell lysate obtained by using a cell homogenizer (Model 500, Fisher scientific, USA) by 30% of amplitude during 3 min, was used for the measurement of intracellular agarase activity.

2.5. Degradation of agarose and products identification

The enzyme reaction was performed at 40 °C, 100 rpm in 50 mM Tris–HCl buffer (pH 7.0) containing 0.3 U/mL of enzyme and 1.0% agarose for 16 h. For thin-layer chromatography (TLC) analysis of the reaction products, samples were applied to a Silica Gel 60 TLC plate, and developed with 0.1-butanol–acetic acid–water solution (2:1:1, v/v/v). The developed reaction products were visualized by spraying 10% (v/v) sulfuric acid in ethanol and heating at 90 °C. For quantification, the reaction products were separated and purified to a pure form by using a recycling preparative high-performance liquid chromatography (HPLC) (LC-9104, Japan Analytical Industry Co., Ltd, Japan) with JAI GEL 252W and 251W columns (Japan Analytical Industry Co., Ltd., Japan). Each of the purified samples was identified by TLC agar and mass spectrometry (MS) (micrOTOF-Q II, Bruker Daltonik, Germany) and HPLC with a Asahipak NH2P-50 4E (250 mm × 4.6 mm, Shodex, Japan) column and ELSD detector (Sedex 75, Sedere, France) under conditions as previously described [10].

2.6. Analysis

Cell growth was monitored by measuring the optical density at 600 nm (OD600) and the agarase activity was measured by the dinitrosalicylic acid (DNS) method. Twenty five μL of the sample was mixed with 975 μL of 50 mM Tris–HCl buffer (pH 7.0) containing 0.2% agarose. After the incubation at 40 °C for 10 min, 1 mL of DNS reagent solution was mixed and heated in boiling water for 10 min until the color changed, and the absorbance at 540 nm (A540) was measured. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar (d-galactose) per minute under the given conditions. The neogarabiose and neogarotetraose, the main products from agarase degradation were quantified by using HPLC under the same conditions as described in Section 2. The agarase conversion to neogarabiose and neogarotetraose was estimated from the amount of these two compounds produced by the reaction by using conversion factors as follows.

The amount of agarose converted to neogarabiose, Neogarabiose produced (g) = Agarose converted (g) × fng
where fng = 1.06 (the conversion factor for agarose to neogarabiose; 324/306).

The amount of agarose converted to neogarotetraose, Neogarotetraose produced (g) = Agarose converted (g) × fg4
where fg4 = 1.03 (the conversion factor for agarose to neogarotetraose; 630/612).

Fig. 1. Scheme of pET22b-agaG1 and pMoPac1-agaG1.
3. Results and discussion

3.1. Construction of expression systems for the production of AgaG1 in E. coli

For heterologous expression of the AgaG1 in E. coli, the agarase-coding gene (agaG1) amplified from the genome of Alteromonas sp. GNUM1 was ligated into pET22b(+) and pMoPac1, respectively. The recombinant plasmids were transformed into E. coli BL21 (DE3), and β-agarase expression was induced by IPTG and the degradation of agar by the expressed agarase was confirmed by using Lugol’s solution on agar plate.

3.2. Selection of expression system and induction conditions for AgaG1 expression in E. coli

To identify effects of induction temperature on β-agarase expression level, the temperature was changed to 18 °C, 25 °C, 30 °C or 37 °C (control) when the culture OD₆₀₀ reached 1.2 at 37 °C, and then 1 mM of IPTG was added for induction. In the case of the pET22b-agaG1 transformant, cell growth increased by increasing the induction temperature (Fig. 2a). Both of the intracellular agarase activity and the total agarase activity showed the highest values of 4.4 and 5.4 U/ml, respectively when the induction temperature was 18 °C (Fig. 2b). They decreased as the induction temperature was increased. The extracellular activity showed no significant changes at about 1.0 U/ml in the range of 18–30 °C. In the case of pMoPac1-agaG1 transformant, cell growth was seriously inhibited by agarase expression when the induction temperature was higher than 25 °C (Fig. 2c). The highest intracellular and total agarase activities of 5.9 and 6.7 U/ml respectively were obtained at 18 °C of induction temperature, whereas the highest extracellular agarase activity of 2.3 U/ml was obtained at 25 °C (Fig. 2d). In this study, the extracellular portion was to be used for the subsequent step of agarase degradation because of its easiness of preparation, and thus the pMoPac1-agaG1 transformant and an induction temperature of 25 °C were selected for agarase production in the subsequent experiments.

For investigation of the effects of IPTG concentration on cell growth and agarase expression in the culture of the pMoPac1-agaG1 transformant, 0, 0.1, 0.2, 0.5, or 1.0 mM of IPTG was added at 25 °C of induction temperature. As shown in Fig. 3, cell growth was significantly retarded after induction when the IPTG concentration was 0.5 or 1.0 mM. The highest extracellular agarase activity of 3.7 U/ml was obtained at 0.5 mM of IPTG concentration.

3.3. Effects of temperature and pH on agarase activity

In the previous study, the AgaG1 was found to be a typical endoacting β-agarase and to have the optimum pH and temperature of pH 7.0 and 40 °C, respectively [9]. As a process of confirmation, the optimum pH and temperature for the β-agarase in the crude enzyme solution produced by fermentation were identified.

![Fig. 2](image-url). Effects of induction temperature on growth and AgaG1 expression at 1.0 mM IPTG. (a) Cell growth of E. coli BL21/pET22b-agaG1; (b) β-agarase activity of E. coli BL21/pET22b-agaG1; (c) cell growth of E. coli BL21/pMoPac1-agaG1; (d) β-agarase activity of E. coli BL21/pMoPac1-agaG1.
Firstly, 50 mM buffer solutions with various pHs ranging from 4.0 to 10.0 were used at for 40 °C. Citrate phosphate buffers were used for pH 4.0, 5.0 and 6.0, and MOPS buffers were used for pH 6.0 and 7.0. Tris–HCl buffers were used for pH 7.0, 8.0 and 9.0, and glycine–NaOH buffers were used for pH 9.0 and 10.0. Reactions were carried out for 10 min. The relative activity in Fig. 4a was defined as the percentage of the maximum activity. The pH-stability was assessed after a pre-incubation at various pHs (4 to 10) for 30 min. As shown in Fig. 4, the optimum pH for agarase activity was confirmed to be pH 7.0. At pH 6, 97% activity was retained. However, the relative activity was about 57% at pH 8.0 and pH 5.0. No significant level of activity was observed at pH 4.0 and pH 10.0 (Fig. 4a). Over 97% of relative activity was retained after the pre-incubation at pH 6, 7, 8 or 9, while about 80% at pH 5 or 10. AgaG1 was completely deactivated after the pre-incubation at pH 4 (Fig. 4a). The effects of temperature on the activity and stability of AgaG1 were investigated in the range from 20 to 50 °C at pH 7.0. The thermal stability was assessed after a pre-incubation at various temperatures (25–60 °C) in 50 mM Tris–HCl buffer (pH 7.0) for 30 min. As shown in Fig. 4b, the optimum temperature for agarase activity was found to be 40 °C, and over 90% of relative activity was retained at the temperature in the vicinity of 40 °C, that is, 37 °C or 45 °C. The enzyme was observed to completely lose its activity over 55 °C. The enzyme showed a good thermal stability up to 45 °C.

3.4. Identification of agarase degradation products

The products of agarase degradation by AgaG1 were identified preliminary by TLC and then by HPLC. Due to the lack of standard compounds, d-galactose, 4-β-galactobiase, and 3α,4β, 3α-galactotetraose were used as standard compounds for TLC. In the beginning of the reaction, Spots 2, 3 and 4 were observed as major ones (Fig. 5a). After 2 h, Spot 1 began to be observed. After 16 h, only two major spots of Spots 1 and 2 were detected. Spots 1 and spot 2 were observed at similar positions with d(+)-galactose and 4-β-galactobiase, respectively. In the HPLC analysis, peaks were observed at 5.2, 6.4, 8.2, 10.8, 14.5, and 19.5 min. However, after 16 h, only two peaks at 5.2 and 6.4 min were observed (Fig. 5b). The peaks at 5.2 and 6.4 min corresponded to Spots 1 and 2, respectively. To identify the final products, fractions corresponding to both peaks above mentioned were taken and purified by using recycling prep HPLC (Fig. 6). The purified samples were analyzed by TLC and HPLC again, and by MS. As shown in Fig. 6, Spot 1 was revealed to have a m/z ratio of 347 (M+Na)⁺, which was applicable to neoagarobiase, and Spot 2 was identified as neogarotetraose [m/z = 653 (M+Na)⁺]. It was expected that neoagarobiase and neoagarotetraose would have been observed at similar positions to 4-β-galactobiase and 3α,4β,3α-galactotetraose, respectively in TLC analysis. Instead, however, neoagarobiase and neoagarotetraose showed similar.

![Fig. 3. Effects of IPTG concentration on growth and AgaG1 expression by E. coli BL21/pMoPac1-agaG1 with induction at 25 °C: (a) cell growth curve and (b) extracellular β-agarase activity.](image)

![Fig. 4. Effects of pH and temperature on AgaG1 activity: (a) pH-dependency at 40 °C (filled circle) and pH-stability (empty circle); (b) temperature-dependency at pH 7.0 (filled circle) and thermostability (empty circle).](image)
positions to \(\alpha\)-galactose and 4-\(\beta\)-galactobiose, respectively. In the second round of HPLC analysis, neoagarobiose was detected at 5.2 min, and neoagarotetraose was detected at 6.4 min (Fig. 6c) confirming the reproducibility of the HPLC analysis.

To identify the final substrate of AgaG1 used, it was tested for the hydrolysis of neoagarotetraose and neoagarohexaose prepared from agarose by using another \(\beta\)-agarase of DagA [11]. Only neoagarohexaose was degraded to release neoagarobiase and neoagarotetraose, while neoagarotetraose was not degraded into neoagarobiase (Fig. 7). This clearly showed that AgaG1 produced neoagarobiase and neoagarotetraose from agarose or neoagaro-oligosaccharide longer than hexamer.

It is known that there are four glycoside hydrolase (GH) families in \(\beta\)-agarase classified based on similarities of amino acid sequence: GH16, GH50, GH86, and GH118. According to a protein BLAST at the NCBI (http://www.ncbi.nlm.nih.gov), AgaG1 belongs to GH16 family [9]. GH16 family agarases have endolytic activity and thus they can degrade agarose rapidly, but cannot hydrolyze neoagarotetraose to neoagarobiase as AgaG1 does [7]. Most of GH16 family agarases produce neoagarotetraose and neoagarohexaose as final products from agarose (Table 1). Only few of them including AgaG1 have a capability of degrading neoagarohexaose, being a very rare GH16 family agarase.

3.5. Production of neoagarobiase and neoagarotetraose from agarose degradation

Considering that the gelling temperature of agarose is 43–45 °C, it would be ideal if the optimum temperature is higher than 45 °C.

![Fig. 5. Analysis of agarose hydrolysis products: (a) thin-layer chromatography; G: \(\alpha\)-galactose; G2: 4-\(\beta\)-galactobiose; G4: 3\(\alpha\),4\(\beta\),3\(\alpha\)-galactotetraose; (b) high-performance liquid chromatography.]

![Fig. 6. Analysis of products purified by recycling prep-high-performance liquid chromatography: (a) mass spectrometry for purified Spot 1; (b) mass spectrometry for purified Spot 2; (c) TLC for purified products, G: \(\alpha\)-galactose; G2: 4-\(\beta\)-galactobiose; G4: 3\(\alpha\),4\(\beta\),3\(\alpha\)-galactotetraose; P: Hydrolysate before purification; (d) high-performance liquid chromatography for purified spots 1 and 2.]
Unfortunately, however, the optimum temperature for AgaG1 used in this study is 40 °C. Kim proposed an agarose pretreatment with acetic acid to partially degrade it with the purpose of avoiding gelation problem [12]. However, such an additional step of chemical pretreatment could be effective in avoiding gelation problem, but it would make the whole process time-consuming and costly. The use of acid causes an additional material cost and would require a neutralization step. A neutralization step causes a further material cost for the alkali used. Often, neutralization generates a significant amount of salts, which can inhibit microbial activity and thus should be removed before fermentation.

In this study, agarose degradation by AgaG1 was started at 46 °C to avoid gelation problem with no separate pretreatment step. The dosage of enzyme was 0.5 U/ml. After 10 min of reaction at 46 °C, the temperature was reduced to 40 °C, the optimum point at which the main part of the process operated. During the operation at 46 °C, the enzyme activity might have been somewhat lower than the maximum value, but was expected to be high enough to partially degraded agarose to a level not to cause gelation. As mentioned earlier, AgaG1 was an endo-type agarase, which was known to randomly degraded agarose and thus rapidly lower the viscosity of agarose solution. Actually, there was no problem of gelation during the rest of the process at 40 °C. Only the time profiles of neoagarobiose, neoagarotetraose and neoagarohexaose are presented in Fig. 8 since little amount of oligomers larger than hexamer was observed to exist in the reaction mixture even in the beginning, for example, at 2 h of reaction. Neoagarobiose and neoagarotetraose concentrations increased monotonically with reaction time, while neoagarohexaose concentration showed a maximum at 2 h then decreased. The reaction was terminated at about 8 h, and the final concentrations of neoagarobiose and neoagarotetraose were 3.8 g/L (0.012 M) and 6.4 g/L (0.010 M), respectively.

In this study, the conversion of agarose could not be calculated because the residual amount of agarose could not be measured. However, it could be concluded that almost all the agarose added was hydrolyzed. The concentration of neoagarobiase produced (3.8 g/L) was equivalent to 3.6 g/L agarose and the concentration of neoagarotetraose (6.4 g/L) to 6.2 g/L agarose. This implied that 9.8 g/L agarose out of 10 g/L had been converted to neoagarobiase and neoagarotetraose.

Neoagar-oligosaccharides have a potentially high economic value because of their biological activities. They can be used as active ingredients in the healthcare, food, pharmaceutical, and cosmetic industries. Neoagar-oligosaccharides produced by using β-agarase DagA have shown to have high anti-cancer, anti-obesity and anti-diabetes effects (unpublished data). Neoagarobiase and neoagarotetraose are known to have high anti-oxidative properties [13]. More specifically, neoagarobiase was reported as a rare

![Fig. 7. Degradation of neoagarohexaose and neoagarotetraose: (a) HPLC analysis of neoagarohexaose degradation products and (b) HPLC analysis of neoagarotetraose degradation products.](image)

![Fig. 8. Production of neoagarobiase and neoagarotetraose by AgaG1 at pH 7.0 and 40 °C.](image)

### Table 1

Products from agarose hydrolysis by bacterial glycosidic hydrolase (GH) 16 family agarases.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Protein</th>
<th>Accession no.</th>
<th>Main product</th>
<th>Reference</th>
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<tr>
<td>Alteromonas sp. GNUM1</td>
<td>β-agarase AgaG1</td>
<td>KF574012</td>
<td>NA2, NA4</td>
<td>This study</td>
</tr>
<tr>
<td>Streptomyces coelicolor A3(2)</td>
<td>β-agarase DagA</td>
<td>CAB61795</td>
<td>NA4, NA6</td>
<td>[11]</td>
</tr>
<tr>
<td>Flammulina velutipes strain YT</td>
<td>β-agarase AgaYT</td>
<td>AEX80424</td>
<td>NA4, NA6</td>
<td>[21]</td>
</tr>
<tr>
<td>Pseudoalteromonas sp. AG4</td>
<td>β-agarase AgaP</td>
<td>ADD80418</td>
<td>NA4, NA6</td>
<td>[22]</td>
</tr>
<tr>
<td>Agarivorans sp. IQ48</td>
<td>β-agarase AgaA</td>
<td>ACM50513</td>
<td>NA4, NA6</td>
<td>[23]</td>
</tr>
<tr>
<td>Pseudoalteromonas sp. CY24</td>
<td>β-agarase AgaA</td>
<td>AAN39119</td>
<td>NA4, NA6</td>
<td>[24]</td>
</tr>
<tr>
<td>Vibrio sp. strain PO-303</td>
<td>β-agarase AgaA</td>
<td>BAF52129</td>
<td>NA4, NA6</td>
<td>[25]</td>
</tr>
<tr>
<td>Saccharophages degradans 2–40</td>
<td>β-agarase AgaB</td>
<td>BAF34350</td>
<td>NA2, NA4</td>
<td>[26]</td>
</tr>
<tr>
<td>Zobelia galactanivorans</td>
<td>β-agarase AgaA</td>
<td>AAT67062</td>
<td>NA4</td>
<td>[27]</td>
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<tr>
<td>β-agarase AgaB</td>
<td>AAF21820</td>
<td>NA4, NA6</td>
<td>[28]</td>
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<td>β-agarase AgaD</td>
<td>CBM41186</td>
<td>NA4</td>
<td>[29]</td>
<td></td>
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</table>

NA2: neoagarobiase; NA4: neoagarotetraose; NA6: neoagarohexaose.
compound with both skin moisturizing and whitening effects on melanoma cells [14].

Although the final products are neogalactobiose and neoagarotetraose in this study, often agarose is needed to be completely hydrolyzed to a mono-sugar level to produce d-galactose and 3,6-anhydro-L-galactose. This can be accomplished in two steps: (1) agarose degradation by agarase(s) to neogalactobiose and then (2) neoagarobiose degradation to by α-neogalactobiose hydrolase (NABH) [15]. An agarase belonging to the GH50 family, encoded in the Sco3487 gene was newly screened from Streptomyces coelicolor A3(2) in our group, whose final product was neoagarobiose. However, this agarase with an exo-lytic activity was found to be very slow in hydrolysing agarose [16]. The first step to produce neogalactobiose from agarose is expected to be very effective if a mixture of Sco3487 agarase and AgaG1 agarase since AgaG1 is fast in liberating neoagarotetraose, which is the final substrate of Sco3487 agarase.

D-Galactose and 3,6-anhydro-L-galactose produced from the hydrolysis of neogalactobiose by NABH have a number of applications. The crude galactose solution can be used for the production of energy material such as ethanol by Saccharomyces cerevisiae [17], and butanol. Galactose in a pure form can be used as raw material for the synthesis of high-value chemicals such as d-tagatose, a low-calorie sweetener [18] and isopropyl β-D-1-thiogalactopyranoside [19]. It was reported that 3,6-anhydro-L-galactose exhibited skin whitening and anti-inflammatory activities [20]. It would be desirable if 3,6-anhydro-L-galactose in the crude galactose solution can be metabolized by microorganisms in terms of substrate utilization efficiency. Unfortunately, however, there have been reported no such cases. Only indirect evidences of its utilization by some microalgae-associated microorganisms have been observed in our group (data not given).

4. Conclusions

An enzymatic hydrolysis process to produce neogalactobiose and neoagarotetraose from agarose has been developed, which needs no pretreatment step to avoid agarose gelation problem. Agarose was hydrolyzed by a newly screened Alcaligenes sp. β-agarase cloned and expressed in E. coli. The enzyme was found to be very effective and highly selective. When 10.0 g/L agarose was hydrolyzed, 98% of the agarose added was converted to 3.8 and 6.4 g/L of neogalactobiose and neoagarotetraose, respectively.

Acknowledgements

This work was supported by the New & Renewable Energy Technology Development Program of the Korea Institute of Energy Technology Evaluation and Planning (KETEP) funded by the Korea government Ministry of Knowledge Economy (No. 200930109001B) and the Advanced Biomass R&D Center (ABC) of Global Frontier Project funded by the Ministry of Education, Science and Technology (ABC-2011-0031356).

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