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# High temperature and low acid pretreatment and agarase treatment of agarose for the production of sugar and ethanol from red seaweed biomass

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## HIGHLIGHTS

- Agarose was prehydrolyzed using acetic acid (1–5%, w/v) at 110–130 °C for 10–30 min.
- Prehydrolyzed agarose was converted to monomers by agarases (62.8% of theoretical maximum sugar yield).
- Prehydrolyzed agarose was converted to ethanol by SSF using agarases and yeast.

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## ABSTRACT

To obtain fermentable sugar from agarose, pretreatment of agarose by using acetic acid was conducted for short durations (10–30 min) at low acid concentrations (1–5% (w/v)) and high temperatures (110–130 °C). On testing the pretreated agarose by using an endo-β-agarase I (DagA), an exo-β-agarase II (Aga50D), and neoagarobiose hydrolase (NABH), we observed that the addition of the endo-type agarase did not increase the sugar yield. Use of the crude enzyme of *Vibrio* sp. EJY3 in combination with Aga50D and NABH including acetic acid pretreatment resulted in a 1.3-fold increase in the final reducing sugar yield (62.8% of theoretical maximum based on galactose and 3,6-anhydrogalactose in the initial agarose), compared to those obtained using Aga50D and NABH only after acetic acid pretreatment. The simultaneous saccharification and fermentation of pretreated agarose yielded ethanol of 37.1% theoretical maximum yield from galactose contained in the pretreated agarose.



## 1. Introduction

Marine algae are considered as the third-generation biomass and have been recognized as promising biomass (Brennan and Owende, 2010; Singh et al., 2011; Singh and Gu, 2010). Particularly, red algal biomass such as *Gelidium amansii* has been identified as a promising source for bioethanol production owing to its high carbohydrate content and the absence of lignin (John et al., 2011). The main component of red algae (*Rhodophyta*) is agarose, which comprises β-linked repeats of neoagarobiose subunits (Renn, 1997). Neoagarobiose is composed of D-galactose and 3,6-anhydro-L-galactose (AHG), in which each monomer is bonded to the other by an α-linkage (Lahaye and Rochas, 1991). In nature, agarases are responsible for the hydrolysis or depolymerization of agarose and are mainly produced by marine bacteria. The agarase system is classified into two groups such as α- and β-agarases according to their modes of action (Fu and Kim, 2010). Whereas α-agarases attack the α-linkages in agarose, β-agarases cleave the β-linkages (Flament et al., 2007). The β-agarase system, which is relatively better known than the α-agarase system, depolymerizes agarose into neoagarobiose by the action of β-agarases I and II (Morrice et al., 1983). The neoagarobiose is then cleaved by α-neoagarobiose hydrolase (NABH) into D-galactose and AHG (Ha et al., 2011; Lee et al., 2009).

Although, unlike lignocellulose, lignin is absent from algae (Bak et al., 2009; Ko et al., 2009; Martone et al., 2009), chemical pretreatment of biomass would still be required to enhance the enzymatic saccharification of the algal carbohydrates. In particular, in the case of agarose, its high degree of polymerization (DP) and tendency to form gels easily impede enzyme action, thereby interfering with the hydrolysis (Renn, 1997; Warren, 1996). Thus, to produce fermentable sugars from the red algae effectively, chemical pretreatment such as acid pretreatment is necessary for augmenting saccharification (Kim et al., 2012; Lahaye and Rochas, 1991; Yun et al., 2013).

Previously, we have reported a chemical liquefaction process involving a treatment with a high concentration (5.5–54.7%) acetic acid at a moderately high temperature of 80 °C for a long duration (1–5 h) to increase the sugar yield from the enzymatic hydrolysis

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of agarose (Kim et al., 2012; Yun et al., 2013). However, the practical implementation of the reaction conditions was difficult to adopt in the actual operation, especially due to the long duration and high acid concentration. Moreover, agarooligosaccharides of odd-numbered DPs were not hydrolyzed by the four enzymes used in (i.e., DagA, Aga16B, Aga50D, and NABH).

In the present study, we carried out further optimization of pretreatment using acetic acid to reduce acid concentration and pretreatment time and determination of the optimal combination of different types of agarases in order to increase the saccharification yield from agarose. In addition, we attempted to identify new types of agarases that could cleave agarotriose into monomeric sugars such as D-galactose and AHG towards enhancing the final sugar yield.

## 2. Methods

### 2.1. Production of recombinant agarases

The recombinant agarases such as Aga50D, DagA, and NABH were produced as described in a previous study (Kim et al., 2012). The genes for DagA (EMBL id: CAB61795), Aga50D (EMBL id: ADB81904), and NABH (EMBL id: ABD81917) were cloned and overexpressed in *Escherichia coli*. From 1 l culture of recombinant *E. coli*, more than 50, 30, and 5 mg of purified Aga50D, NABH, and DagA were obtained, respectively.

### 2.2. Preparation of crude enzyme of Vibrio sp. EJY3

*Vibrio* sp. EJY3 was cultured on minimal medium containing 23 g/l synthetic sea salt (Instant Ocean sea salt, Aquarium Systems, Mentor, OH), 50 mM Tris–HCl, 2 g/l agarose, 1 g/l yeast extract, and 0.5 g/l ammonium chloride at 30 °C for 4 h (Roh et al., 2012). After harvesting the culture, the cell pellet was sonicated and centrifuged. The resulting supernatant was then concentrated using an Amicon tube (Millipore, Billerica, MA) to obtain the crude enzyme. The protein in the crude enzyme was quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), and bovine serum albumin (Sigma–Aldrich) was used as the standard.

### 2.3. Pretreatment of agarose using acetic acid

Agarose was dissolved in different concentrations of acetic acid solutions, thus giving 7% (w/v) of the final agarose concentration. The agarose in the acetic acid solution was pretreated using a microwave digester (Milestone, Shelton, CT). The acid pretreatment was performed at 110 °C, 120 °C, and 130 °C with 1%, 3%, and 5% (w/v) concentrations of acetic acid for varied reaction times of 10, 20, and 30 min. After pretreatment, the agarose solution was neutralized to pH 7 with 1–5 M sodium hydroxide. Furthermore, the sample volume was adjusted to 28 ml using distilled water. Thus, the final agarose concentration in all the neutralized pretreated agarose was 5% (w/v).

### 2.4. Enzymatic hydrolysis of acetic acid-pretreated agarose

The neutralized pretreated agarose (5%, w/v) was subjected to the enzymatic hydrolysis by agarases in a 100 μl reaction mixture (pH 7.0 of Tris–HCl buffer) at 30 °C. The enzyme dosages used were 1.1 nmol (0.03 mg protein) of DagA (an endo-type β-agarase I; 0.07 U/mg protein) per g substrate, 2.4 nmol (0.03 mg protein) of Aga50D (an exo-type β-agarase II; 0.62 U/mg protein) per g substrate, and 2.4 nmol (0.06 mg protein) of NABH (2.56 U/mg protein) per g substrate. One unit of the enzymes was defined as the amount of enzymes required to release 1 μmol of reducing sugar from agarose per min at 30 °C in Tris–HCl buffer pH 7.0. The crude enzyme obtained from *Vibrio* sp. EJY3 was tested (Roh et al., 2012) for hydrolyzing oligoagarosaccharides with DP3, which could not be cleaved by Aga50D and NABH. The filtrate obtained from the pretreated agarose by Aga50D and NABH was incubated with 0.5 mg of the crude enzyme (100 mg protein/g substrate).

**Table 1**
Reducing sugar and hydroxymethyl furfural (HMF) yields from the acetic acid pretreatment of agarose.

| Pretreatment conditions | | Reducing sugar yield (%, w/w)[g] | HMF yield (%, w/w)[g] |
|---|---|---|---|
| Acetic acid 1% (w/v) | 120 °C 20 min | 9.5 ± 0.5 | 0.2 ± 0.1 |
| | 120 °C 30 min | 17.7 ± 3.6 | 0.2 ± 0.1 |
| | 130 °C 20 min[a] | 11.8 ± 2.1 | 0.1 ± 0.1 |
| | 130 °C 30 min | 19.4 ± 5.2 | 0.3 ± 0.2 |
| Acetic acid 3% (w/v) | 110 °C 20 min | 9.0 ± 0.3 | 0.1 ± 0.1 |
| | 110 °C 30 min[b] | 19.6 ± 3.2 | 0.7 ± 0.1 |
| | 120 °C 10 min | 9.6 ± 1.8 | 0.5 ± 0.3 |
| | 120 °C 20 min | 14.89 ± 1.7 | 0.5 ± 0.2 |
| | 120 °C 30 min | 19.6 ± 1.6 | 0.9 ± 0.1 |
| | 130 °C 10 min | 20.8 ± 4.9 | 0.6 ± 0.1 |
| | 130 °C 20 min | 22.1 ± 1.2 | 1.3 ± 0.2 |
| | 130 °C 30 min[c] | 30.6 ± 4.8 | 2.1 ± 0.8 |
| Acetic acid 5% (w/v) | 110 °C 20 min | 14.6 ± 2.4 | 0.5 ± 0.0 |
| | 110 °C 30 min | 16.9 ± 0.3 | 0.3 ± 0.2 |
| | 120 °C 10 min | 16.4 ± 0.8 | 0.5 ± 0.4 |
| | 120 °C 20 min | 19.4 ± 1.3 | 0.4 ± 0.1 |
| | 120 °C 30 min[d] | 23.6 ± 0.2 | 0.6 ± 0.1 |
| | 130 °C 10 min[e] | 26.1 ± 2.1 | 0.6 ± 0.1 |
| | 130 °C 20 min | 30.9 ± 2.5 | 1.2 ± 0.9 |
| | 130 °C 30 min[f] | 33.7 ± 0.1 | 2.3 ± 1.3 |

[a–f] Pretreated agarose samples selected for further enzymatic hydrolysis and SSF based on the following criteria.
[a] Low saccharification and low HMF yields.
[b] Medium saccharification and low HMF yields.
[d,e] Medium saccharification and medium HMF yields.
[c,f] High saccharification and high HMF yields.
[g] Reducing sugar (by the DNS method) and HMF yields were expressed as mean ± standard deviation on the basis of the maximum amount of galactose and 3,6-anhydro-L-galactose (AHG) from the initial agarose.