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Agarose hydrolysis by two-stage enzymatic process and bioethanol production from the hydrolysate

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ABSTRACT

A two-stage enzymatic process was developed for agarose hydrolysis without acid pretreatment. In the first stage, agarose was hydrolyzed to produce neoagarobiose using an optimized dosage of AgaG1 and DagB at pH 7.0 and 40 °C. Agarose gelation was avoided by momentarily elevating the reaction temperature for the first 10 min, instead of employing a pretreatment step with acid prior to the enzyme reaction. In the second stage, neoagarobiose was further hydrolyzed to produce galactose using neoagarobiose hydrolase (NABH) at pH 7.0 and 35 °C. The overall yield of galactose from agarose was 88% of the theoretical maximum. The crude galactose solution produced from agarose hydrolysis was used directly for ethanol production by yeast to demonstrate its potential. In overall, 20 g/l of agarose could be converted to 3.71 g/l of ethanol.

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1. Introduction

Ethanol can be produced by fermentation from various types of biomass including starch, lignocelluloses, and marine algae biomass. The bioethanol produced from marine algae has several advantages over that based on starch and lignocellulosic biomass. It does not compete with human foods, does not cause forest denudation, has a low content of lignocellulose, and can fix a larger amount of CO_2 per unit mass [1,2].

The main constituent of red algae, one of the marine algae biomass, is agarose. Agarose is a linear chain of alternating residues of 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactose [3]. Agarose can be hydrolyzed chemically or enzymatically. During chemical hydrolysis, galactose from the galactosyl residue is retained, while the anhydrogalactose formed is further converted to 5-hydrooxymethyl furfural (5-HMF) and then to levulinic acid (LA), both of which are known to be toxic to many microorganisms including yeast to lower fermentation efficiency [4,5]. Therefore, the resulting crude galactose solution could be used as the medium for ethanol production only after a pretreatment step, for example, by nanofiltration and electrodialysis for their removal [6]. In contrast, an enzymatic hydrolysis process does not produce 5-HMF and LA, and leaves 3,6-anhydro-L-galactose intact. The resulting crude galactose solution, containing no toxic compounds, can be used directly to produce ethanol by fermentation.

Enzymatic hydrolysis of agarose is known to include three steps, in principle: (1) agarose liquefaction by β-agarase I to neoagaro-oligosaccharides; (2) neoagaro-oligosaccharides degradation by β-agarase II to neoagaoriobiose; and (3) neoagarobiose decomposition by α-neoagarobiose hydrolase (NABH) to galactose and anhydrogalactose [7].

In a series of efforts by our research group to search for enzymes needed for agarose degradation, the first involved AgaG1, screened from *Alteromonas* sp. GNUM1. This enzyme is an endo-type β-agarase, which hydrolyzes agarose to neoagarobiose and neoagarotetroase. It was expressed in *Escherichia coli* BL21 [8–10]. The second enzyme, DagB, an exo-type β-agarase which hydrolyzes agarose and neoagarotetraose to neoagarobiose, was screened from *S. coelicolor* A3(2) and expressed in *S. lividans* TK24 [11]. Finally, a newly screened microbial strain of *Alcanivorax* sp. A28-3 (KCTC12788BP) was found to produce NABH, although its amino acid sequence is yet to be identified. When neoagarobiose was treated with the cell lysate of *Alcanivorax* sp. A28-3, galactose and anhydrogalactose were produced as the final products [12].

In this study, a two-stage process for the saccharification of agarose to galactose and anhydrogalactose was developed and optimized. In the first step, agarose was degraded to neoagarobiose using a mixture of AgaG1 and DagB, produced by a recombinant *E. coli* and a recombinant *S. lividans*, respectively. In the second step,

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neoagarobiose was decomposed to galactose and anhydrogalactose using the cell lysate of *Alcanivorax* sp. A28-3. The resulting galactose solution was used by *Saccharomyces cerevisiae* KL17 (KFCC11493P), a galactose-utilizing yeast strain newly screened by our group for ethanol production.

2. Materials and methods

2.1. Strains and media

E. coli BL21 and pMoPac1 were used as the host and plasmid for the expression of *agaG1* gene, respectively [10]. *E. coli* BL21/pMoPac1-AgaG1 was grown on the Luria-Bertani (LB) medium containing 35 mg/l of chloramphenicol.

S. lividans TK24, which was obtained from The John Innes Foundation (United Kingdom) and pUWL201PW [13] were used as the host and vector for the expression of *DagB*, respectively. The *S. lividans* TK24/pUWL201PW-DagB culture was performed in a R2YE medium with no sucrose, containing per liter: 0.25 g K_2SO_4, 10.12 g $MgCl_2{\cdot}6H_2O$, 10 g glucose, 0.1 g casamino acids, 5 g yeast extract, 10 ml of 0.5% K_2HPO_4, 80 ml of 3.68% $CaCl_2{\cdot}2H_2O$, 15 ml of 20% L-proline, 100 ml of 5.73% *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES; pH 7.2), and 2 ml of trace elements solution [14]. For stable maintenance of the plasmid, 50 mg/l of thiostrepton was used [11].

Alcanivorax sp. A28-3 (KCTC12788BP) was used for the production of NABH. The strain was cultured in an artificial sea water media (ASW-YP) containing per liter 6.1 g Trizma base (pH 7.2), 12.3 g $MgSO_4$, 0.74 g KCl, 0.13 g $(NH_4)_2HPO_4$, 17.5 g NaCl, 0.14 g $CaCl_2$, 0.2 g yeast extract, 3.0 g bacto peptone, and 2.0 g agar.

S. cerevisiae KL17 (KFCC11493P) was used for the production of ethanol, and was cultured in an YPG medium containing per liter: 10 g yeast extract, 20 g peptone, and 10 g galactose, or in an YPEH medium containing per liter: 10 g yeast extract and 20 g peptone, and actual enzyme hydrolysate of agarose.

2.2. Preparation of enzyme solutions

2.2.1. AgaG1

E. coli BL21/pMoPac1-AgaG1 culture was performed in baffled Erlenmeyer flask. Cells from the cell stock were used to inoculate 10 ml of LB medium for the seed culture. The culture was performed at 37 °C and 200 rpm. After overnight culture, 2% of the seed culture broth was inoculated into 200 ml of LB medium. When the culture OD_{600} reached 1.2, the temperature was decreased to 18 °C and then AgaG1 expression was induced using 1.0 mM of IPTG. The culture was performed for 8 h at 18 °C after induction. The harvested cells were disrupted by using a homogenizer (Model 500, Fisher scientific, USA) to recover AgaG1 inside the cells. Cell debris was removed by centrifugation for 20 min at 5000 *g*-force. The supernatant was used for the hydrolysis of agarose.

2.2.2. DagB

S. lividans TK24/pUWL201PW-DagB was grown in a baffled Erlenmeyer flask. Cells from the cell stock were used to inoculate 10 ml of R2YE medium for the seed culture. The culture was performed at 28 °C and 200 rpm. After 2 days of seed culture, 2% of the seed culture broth was inoculated into 200 ml of R2YE medium. After 2 days of culture, cells were removed by centrifugation for 20 min at 5000 *g*. The supernatant was used for the hydrolysis of neoagarotetraose.

2.2.3. NABH

Alcanivorax sp. A28-3 was cultivated in an Erlenmeyer flask. Cells from the cell stock were used to inoculate 10 ml of ASW-YP medium for the seed culture at 28 °C and 200 rpm. After 1 day of seed culture, 2% of the seed culture broth was inoculated into 200 ml of ASW-YP medium. After 2 days of culture, the harvested cells were disrupted by using a homogenizer (Model 500, Fisher scientific, USA) to recover NABH inside the cells. Cell debris was removed by centrifugation for 20 min at 5000 *g*. The supernatant was used for the hydrolysis of neoagarobiose.

2.3. Two-stage hydrolysis of agarose

In the first stage, 10 or 20 g/l of agarose (Lonza, Switzerland) was hydrolyzed to neoagarobiose by using a mixture of AgaG1 and DagB, both of which had the same optimum condition of pH 7.0, and 40 °C [10,11]. The enzyme reaction was performed pH 7.0, 40 °C, and 100 rpm for 12 h in an Erlenmeyer flask containing 100 ml of reaction mixture. Tris-HCl buffer at 50 mM was used. To avoid the problem of agarose gelation, the reaction was started at 46 °C. The temperature was lowered to 40 °C after 10 min, when gelation was no longer a problem. In the second stage, neoagarobiose produced in the first stage was treated with the NABH solution at pH 7.0, 35 °C, and 100 rpm for 12 h in a flask containing 100 ml of reaction mixture. Tris-HCl buffer was used.

2.4. Ethanol production

S. cerevisiae KL17 (KFCC11493P) was used. It had been newly screened from the soil by our group, and exhibited efficient uptake of galactose to produce ethanol [15]. Seed culture was carried out in 10 ml of the YPG medium. The main culture was inoculated with 2% seed culture broth. As required, 100 ml of YPG medium, YPEH medium, or YPGH (YPG media + 0.5% of 5-HMF) medium was used for the main culture. All the yeast cultures were carried out at 30 °C and 200 rpm.

2.5. Analysis

The agarase activity was measured by the dinitrosalicylic acid (DNS) method as previously described [10]. One unit of enzyme activity was defined for both AgaG1 and DagB as the amount of enzyme liberating 1 μmole of reducing sugar per minute, at pH 7.0 and 40 °C. One unit of NABH activity was defined as the amount of enzyme liberating 1 μmole of galactose per minute, at pH 7.0 and 35 °C. Neoagarobiose and neoagarotetraose were quantified by using a high-performance liquid chromatograph (HPLC) (Waters, USA), with an Asahipak NH2P-50 4E column (250 × 4.6 mm, Shodex, Japan) and an ELSD detector (Sedex 75, Sedere, France) under conditions described previously [16]. Galactose and ethanol were quantified by using an YSI 2700 Select Biochemistry Analyzer (YSI, USA). The agarose conversion to neoagarobiose and neoagarobiose conversion to galactose, were estimated from the amount of these two compounds produced by the reaction using the following conversion factors.

The amount of agarose converted to neoagarobiose:

$$\text{agaroseconverted(g)} = \text{neoagarobioseproduced(g)}/f_{\text{biose/agarose}} \quad (1)$$

where $f_{\text{biose/agarose}} = 1.06$ (=324/306).

The amount of neoagarobiose converted to galactose:

$$\text{neoagarobioseconverted(g)} = \text{galactoseproduced(g)}/f_{\text{galactose/biose}} \quad (2)$$

where $f_{\text{galactose/biose}} = 0.56$ (= 180/324).

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