Improvement of a Unified Saccharification and Fermentation System for Agar-bioethanol Production in Yeast

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Received: September 17, 2019 / Revised: October 23, 2019 / Accepted: October 29, 2019

We improved on a unified saccharification and fermentation (USF) system for the direct production of ethanol from agarose by increasing total agarase activity. The pGMF α -NGH plasmid harboring the *NABH558* gene encoding neoagarobiose hydrolase and the *AGAG1* and *AGAH71* genes encoding β -agarase was constructed and used to transform *Saccharomyces cerevisiae* 2805. *NABH558* gene transcription level was increased and total agarase activity was increased by 25 to 40% by placing the *NABH558* gene expression cassette upstream of the other gene expression cassettes. In the 2805/pGMF α -NGH transformant, three secretory agarases were produced that efficiently degraded agarose to galactose, 3,6-anhydro-L-galactose (AHG), neoagarobiose, and neoagarohexaose. During the united cultivation process, a maximum of 2.36 g/l ethanol from 10 g/l agarose was produced over 120 h.

Keywords: Unified enzymatic saccharification and fermentation (USF) system, β -agarase, neoagarobiose hydrolase, bioethanol, recombinant yeast

The utilization of renewable resources is essential to achieve sustainable fuel production to solve the problem of excessive production of greenhouse gases that cause global warming [1]. Biomass is a renewable energy source, and bioethanol has attracted attention as a biomass-derived fuel. Among the many types of biomass, seaweed (marine biomass) is considered to be a promising raw material for bioethanol production owing to several advantages, including higher carbohydrate level and low lignin and hemicellulose content [2]. Specifically, marine red-algal biomass has emerged as a promising alternative for bioethanol production [3]. The major constituent of red macroalgal biomass is the recalcitrant agar polysaccharide. Agar is composed of agarose as the

major component and agaropectin as the minor component. Agarose is the major cell wall component that consists of D-galactoses and 3,6-anhydro-L-galactoses (AHG) connected by alternating α -1,3 and β -1,4 linkages [4, 5]. In the practice of converting red algal biomass into bioenergy, the critical step in the agarose decomposition process is the release of fermentable monosugars (e.g. galactose). However, utilization of agarose as a biomass has been limited owing to a lack of established methods for pretreatment and an effective saccharification system.

Kim *et al.* reported a sugar platform equipped with acetic acid, multiple agarases, and neoagarobiose hydrolase, and the enzyme system converted 79.1% of agarose to monosugars [6]. They used mild conditions for chemical liquefaction of agarose, and 4.4 g/l ethanol concentration was produced from 30 g/l agarose. In a previous study, we developed a unified enzymatic saccharification and fermentation (USF) system for the direct production

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of bioethanol without an agarose pre-chemical liquefaction process [7]. Recombinant yeast harboring multiple agarase genes directly produced fermentable sugars from agarose and a maximum of 1.97 g/l ethanol from 10 g/l agarose. Agarases, which are classified into two groups, α -agarase and β -agarase, hydrolyze agarose into oligosaccharides and monosugars. α -Agarases cleave the α -1,3 linkages of agarose to produce agarooligosaccharides [8] while the β -agarases cleave the β -1,4 linkages of agarose to produce neoagarooligosaccharides [9]. The end product of the β -agarase action is converged to a neoagarobiose (L-3,6-anhydrogalactosyl- α -1,3-D-galactose) that is further hydrolyzed by the key enzyme neoagarobiose hydrolase (NABH) into D-galactose and AHG [10, 11]. α -Agarases, including NABH, are difficult to utilize due to their low activity and productivity compared with β -agarases, and optimization for production of the α -agarases is sometimes necessary [12, 13]. In this study, we describe the effect of increased total agarase production on an improved unified enzymatic saccharification and fermentation system for increased bioethanol production from red algae (agarose).

In the present study, *Saccharomyces cerevisiae* strain 2805 (*MATa pep4::HIS4 prb1-1.6R can1 GAL2 his3-200 ura3-52*) was used as the host for agarase gene expression. β -agarase, encoded by the *AGA71* [14] and *AGAG1* [15] genes, can degrade agarose to neoagarohexaose (NA6), neoagartetraose (NA4), and neoagarobiose (NA2). α -Neoagarobiose hydrolase encoded by the

NABH558 gene [16] mainly degrades neoagarobiose to D-galactose and AHG and can directly degrade NA4 and NA6 to D-galactose and AHG (Fig. 1A). Cultivation methods using YPD nutrient medium [1% yeast extract, 2% peptone, 1% dextrose] and synthetic complete (SC) medium have been described in a previous report [7]. YPDG (YPD containing 1% galactose) medium was used for galactose-inducible gene expression. The methods for yeast transformation, reverse transcription PCR (RT-PCR), thin layer chromatography (TLC), and HPLC were previously described by Lee *et al.* [7]. β -agarase and NABH enzyme activity were measured by respective assay methods and active staining analysis [13, 14].

In a previous study, we constructed the pGMF α -HGN plasmid carrying the *AGA71*, *AGAG1*, and *NABH558* gene expression cassettes (ECs) [7]. The order of the genes in the pGMF α -HGN plasmid was arranged according to the order of substrate degradation, including agarose. However, transcription level of the *NABH558* gene was reduced compared to the other β -agarase genes. Lee *et al.* [17] reported that the direction of transcription and the order of genes affected the production and activity of enzymes in a simultaneous expression system. Therefore, we constructed pGMF α -HGNN and pGMF α -NGH plasmids (Fig. 1B and Table 1) with the goal to increase total agarase production, especially NABH production. The pGMF α -HGNN plasmid was constructed by inserting an additional *NABH558* gene expression cassette (*GAL10p-MFas.s-*

Fig. 1. Scheme of agarose degradation by various agarases (A) and plasmids containing various agarase gene expression cassettes (ECs) (B). NA4 and NA6 indicate neoagartetraose and neoagarohexaose, respectively.

Table 1. Plasmids list used in this study.

Plasmids	Descriptions
pGMF α -AgaH71 [7]	<i>GAL10p-MFas.s-AGA71-GAL7t</i> , <i>Ura3</i> selective marker
pGMF α -GH	<i>AGAG1</i> EC-AGA71 EC, <i>Ura3</i> selective marker
pGMF α -HGN [7]	<i>AGA71</i> EC- <i>AGAG1</i> EC- <i>NABH558</i> EC, <i>Ura3</i> selective marker
pGMF α -HGNN	<i>AGA71</i> EC- <i>AGAG1</i> EC- <i>NABH</i> EC- <i>NABH558</i> EC, <i>Ura3</i> selective marker
pGMF α -NGH	<i>NABH558</i> EC- <i>AGAG1</i> EC- <i>AGA71</i> EC, <i>Ura3</i> selective marker

NABH558-Gal7t) into the pGMF α -HGN plasmid. To construct the pGMF α -NGH plasmid, the *AGAG1* gene expression cassette (*GAL10p-MFas.s-AGAG1-GAL7t*) was cloned into the pGMF α -AgaH71 [7] plasmid, resulting in the pGMF α -GH plasmid. Subsequently, the *NABH558* gene expression cassette was also cloned into the pGMF α -GH plasmid, resulting in the pGMF α -NGH plasmid. These plasmids utilize the *GAL10* promoter, which is a galactose-inducible promoter, and the *MFa* signal sequence (*MFas.s*) for recombinant agarase secretion. Overlapping cloning methods and universal primers used for plasmid construction were described by Lee *et al.* [7].

Each constructed plasmid was used to transform the *S. cerevisiae* 2805 strain (uracil auxotroph) and transformants were selected on SC-Ura plates. Agarase activity in these clones was analyzed by DNS assay and direct

Table 2. Comparison of cell growth, total agarase activity and plasmid stability in yeast transformants expressing various agarases.

Transformants	Dry cell weight (g/l)	Agarase activity (unit/ml)	Plasmid stability (%)
2805/pGMF α -AgaH71	6.78	0.91 $\pm$ 0.02	80
2805/pGMF α -GH	6.82	1.16 $\pm$ 0.01	73
2805/pGMF α -HGN	7.26	1.45 $\pm$ 0.04	78
2805/pGMF α -HGNN	6.51	1.17 $\pm$ 0.03	58
2805/pGMF α -NGH	6.30	1.56 $\pm$ 0.06	75

Each transformant was cultured in 50 ml YPDG (1% galactose) medium for galactose-inducible gene expression at 30 °C for 48 h.

active staining using iodine solution. As shown in Table 2, total agarase activity in the 2805/pGMF α -GH transformant, which harbors two agarase genes, was increased by 30% compared with the 2805/pGMF α -AgaH71 transformant. In the 2805/pGMF α -HGNN transformant, which harbors an additional *NABH558* gene expression cassette, total agarase activity was not increased compared to the 2805/pGMF α -HGN transformant. However, when the *NABH558* gene expression cassette was positioned upstream of the other gene expression cassettes, as in the 2805/pGMF α -NGH transformant, total agarase activity was increased by 25 to 40% (Fig. 2A). Plasmids were stably maintained in each transformant and most agarase activities were found in the extracellular medium (Fig. 2B) with secretory effi-

Fig. 2. Comparison of specific agarase activity in yeast transformants (A) and active staining of agarase expressed in transformants (B). Transformants were cultivated in YPDG medium for 48 h to facilitate the expression of β -agarase and NABH, and specific activity units were divided by dry cell weight (DCW). H, 2805/pGMF α -AgaH71 transformant; GH, 2805/pGMF α -GH transformant; HGN, 2805/pGMF α -HGN transformant; HGNN, 2805/pGMF α -HGNN transformant; and NGH, 2805/pGMF α -NGH transformant. The cells were incubated in YPDG medium and then stained with iodine solution.

Fig. 3. Analysis of *NABH558*, *AGAG1* and *AGAH71* gene transcription level using RT-PCR. Total RNA was isolated from 2805/pGMF α -HGN and 2805/pGMF α -NGH transformants and cDNA was synthesized from total RNA by RT-PCR. The *AGAH71*, *AGAG1*, *NABH558*, and *ACT1* (internal control) genes were amplified by PCR using each cDNA as template, and the products were visualized by agarose gel electrophoresis. The intensity of PCR products was measured using the Scion image program.

ciencies ranging from 82% to 93% (data not shown).

In the case of other coexpression systems, it has been observed that gene expression profiles differ as the direction of transcription differs [18, 19]. In this study, the three genes have the same direction of transcription and, therefore, the three genes were successfully transcribed (Fig. 3). Furthermore, *NABH558* gene transcription level in the 2805/pGMF α -NGH transformant was increased by arranging the *NABH558* gene expression cassette upstream of the other gene expression cassettes. We suspected that the binding of RNA polymerase to the *GAL10* promoter domain upstream of the *NABH558*, *AGAG1*, and *AGAH71* ORFs had been affected by the RNA polymerase from the proceeding gene, suppressing the expression level of the following gene. Thus, we inferred that the increased level of *NABH558* gene transcription affected the total agarase activity in the 2805/pGMF α -NGH transformant.

To produce ethanol from agarose, the 2805/pGMF α -HGN and 2805/pGMF α -NGH strains were cultured in YPG [YP+2% galactose] medium for the induction of agarase genes and, subsequently, YPA [YP+1% agarose] for saccharification and ethanol fermentation. After inducing agarase gene expression, cultivated cells were transferred to pre-warmed YPA medium. Cultivation was started at 40°C and the agarases were allowed to hydrolyze agarose to fermentable sugars for 48 h. Subsequently, ethanol fermentation was induced at 30°C for 72 h. In the TLC analysis of cultivation supernatants, agarose in the medium was degraded to galactose, 3,6-anhydro-L-galactose (AHG), neoagarobiose, and neoagrotetraose with neoagarohexaose still remaining (Fig. 4).

Galactose was not observed 72 h after fermentation, indicating that galactose was consumed in ethanol production (Fig. 4, lane 5 and 7). Neoagarobiose and neoagrotetraose were decreased in the 2805/pGMF α -NGH strain during the fermentation process compared to the 2805/pGMF α -HGN strain (Fig. 4, lane 7). The pattern of hydrolysis products was similar in HPLC analysis (data not shown). We inferred that increased NABH activity lead to the increase in neoagarobiose and neoagrotetraose degradation. Furthermore, the unified enzymatic saccharification and fermentation of the 2805/pGMF α -NGH strain resulted in a maximum 2.36 g/l ethanol concentration from 10 g/l agarose, indicating that

Fig. 4. TLC analysis of cultivation supernatants in the 2805/pGMF α -HGN and 2805/pGMF α -NGH strains. Lane 1, AHG; lane 2, NA2 and NA4; lane 3, galactose; lane 4 and 6, cultivation supernatant at 48 h after saccharification and lane 5 and 7, cultivation supernatant at 72 h after ethanol fermentation.

Fig. 5. Comparison of cell growth and ethanol concentration by enzymatic saccharification of agarose and ethanol fermentation in the 2805/pGMF α -HGN and 2805/pGMF α -NGH strains. Open circles and closed circles indicate the 2805/pGMF α -HGN and 2805/pGMF α -NGH strain, respectively.

ethanol productivity increased about 20% compared with the 2805/pGMF α -HGN strain (Fig. 5). Therefore, agarose was efficiently degraded by increasing agarase production, resulting in improved agar-bioethanol productivity. Although it is still difficult for this strain to convert AHG to ethanol, we expect to obtain high concentrations of ethanol from agarose by improving the agarose hydrolysis step to produce larger quantities of fermentable sugars.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2018R1D1A1B07047291).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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