

# Production of Ethanol from Agarose by Unified Enzymatic Saccharification and Fermentation in Recombinant Yeast

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The unified saccharification and fermentation (USF) system was developed for direct production of ethanol from agarose. This system contains an enzymatic saccharification process that uses three types of agarases and a fermentation process by recombinant yeast. The pGMF $\alpha$ -HGN plasmid harboring *AGAH71* and *AGAG1* genes encoding  $\beta$ -agarase and the *NABH558* gene encoding neoagarobiose hydrolase was constructed and transformed into the *Saccharomyces cerevisiae* 2805 strain. Three secretory agarases were produced by introducing an *S. cerevisiae* signal sequence, and they efficiently degraded agarose to galactose, 3,6-anhydro-L-galactose (AHG), neoagarobiose, and neoagarohexose. To directly produce ethanol from agarose, the *S. cerevisiae* 2805/pGMF $\alpha$ -HGN strain was cultivated into YP-containing agarose medium at 40°C for 48 h (for saccharification) and then 30°C for 72 h (for fermentation). During the united cultivation process for 120 h, a maximum of 1.97 g/l ethanol from 10 g/l agarose was produced. This is the first report on a single process containing enzymatic saccharification and fermentation for direct production of ethanol without chemical liquefaction (pretreatment) of agarose.

**Keywords:** Unified enzymatic saccharification and fermentation (USF) system,  $\beta$ -agarase, neoagarobiose hydrolase, bioethanol, recombinant yeast

## Introduction

Biomass is a renewable energy source, and bioethanol has attracted attention as a biomass-derived fuel. Among the many types of biomass, seaweeds (marine biomass) are considered to be a promising raw material for bioethanol production owing to several advantages, including high productivity per unit area, high concentration of carbohydrate, and low concentration or lack of lignin, which inhibits the activity of enzymes and microorganisms that degrade polysaccharides contained in the seaweeds [1]. Specifically, marine red-algal biomass has appeared as a promising alternative for bioethanol production [2].

The major constituent of red-algal biomass is a recalcitrant agar polysaccharide. Agar is a gelatinous substance derived from a polysaccharide; it accumulates in the cell walls of red algae and is composed of agarose and agarpectin. This

polymer consists of 3,6-anhydro-L-galactoses (AHG) and D-galactoses connected by alternating  $\alpha$ -1,3 and  $\beta$ -1,4 linkages [3, 4]. In the practice of converting red algal biomass into bioenergy or valuable biochemicals including neoagarooligosaccharides the decomposition process of agarose to give fermentable monosugars (*e.g.* galactose) is the critical step. The sugar degradation that converts biomass into fermentable monosugars can be achieved by chemical liquefaction and enzymatic saccharification of the insoluble polymer (lignocellulosic biomass) [5–8]. However, no standard processes for decomposing the agar polysaccharide, the major constituent of red algal biomass, have been established. The acid hydrolysis for chemical liquefaction of agarose requires extreme reaction conditions including low pH and high temperature, and the products need to be neutralized before subsequent fermentation. In addition to the reaction extent of the chemical, decomposition is hard to control, and

over-decomposition often releases fermentation inhibitors such as furfurals that give rise to problems during bioethanol production [9]. The process is also prone to causing environmental pollution [10]. Moreover, the enzymatic hydrolysis of agarose is preferable to acid hydrolysis owing to its lower adverse environmental impact and energy requirement for the bioethanol production process. Agarase is a glycosidic hydrolase that hydrolyzes agarose into several oligosaccharides and is classified into two groups,  $\alpha$ -agarase and  $\beta$ -agarase, depending on the mode of action.  $\alpha$ -Agarases cleave the  $\alpha$ -1,3 linkages of agarose to produce a series of agarooligosaccharides [11], while the  $\beta$ -agarases cleave the  $\beta$ -1,4 linkages of agarose to produce a series of neoagarooligosaccharides [12]. The end product of the  $\beta$ -agarase action is converged to a disaccharidic neoagarobiose (L-3,6-anhydrogalactosyl- $\alpha$ -1,3-D-galactose) that is further hydrolyzed by a key enzyme of neoagarobiose hydrolase (NABH) into two monosugars, D-galactose and AHG [13, 14].  $\alpha$ -Agarases including NABH are difficult to isolate due to their low activity and productivity compared with  $\beta$ -agarase, and optimization for production of the  $\alpha$ -agarases is sometimes necessary [15, 16]. Therefore,  $\alpha$ -agarases have not been widely used for industrial applications. 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 [17]. They used mild conditions (with a dilute acetic acid at 80°C for 1–6 h) for chemical liquefaction of agarose, and 4.4 g/l ethanol concentration was produced from 30 g/l agarose by simultaneous saccharification and fermentation (SSF). In this study, we describe construction of a recombinant yeast that directly produces fermentable sugars from agarose by introducing multiple agarase genes and direct production of bioethanol

from agarose hydrolysis by unified enzymatic saccharification and fermentation (USF) without a pre-chemical liquefaction process of agarose.

## Materials and Methods

### Strains and Gene Information

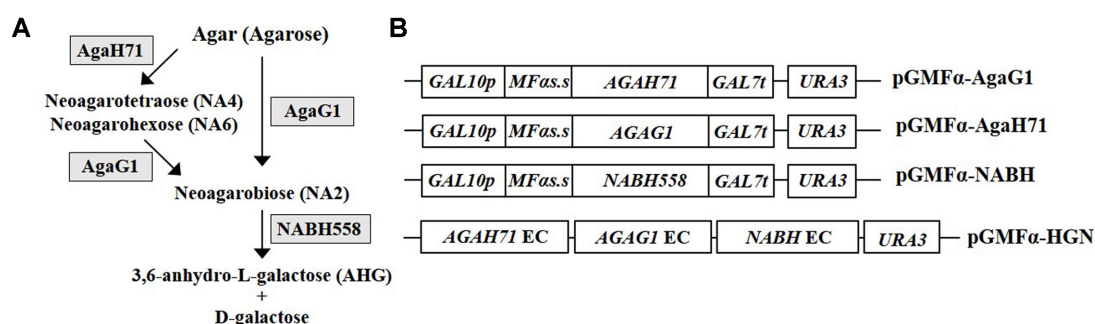
*Escherichia coli* DH5 $\alpha$  was used for the amplification of plasmids and subcloning of the agarase genes. *Saccharomyces cerevisiae* strains BY4742, 2805 and FY834 [18] were used as the host strains for expression of the agarase genes; these strains are uracil auxotrophs and are thermotolerant (Table 1). The *AGAH71* gene derived from *Pseudoalteromonas hodoensis* H7 [19], the *AGAG1* gene derived from *Alteromonas* sp. GNUM-2 [20], and the *NABH558* gene derived from *Gayadomonas joobiniege* G7 [21] were used as genes for production of agarases.  $\beta$ -Agarase encoded by the *AGAH71* gene can degrade agarose to neoagarotetraose (NA4) and neoagarohexaose (NA6), and  $\beta$ -agarase encoded by the *AGAG1* gene can degrade neoagarohexaose and neoagarotetraose to neoagarotetraose and neoagarobiose (NA2).  $\alpha$ -neoagarobiose hydrolase encoded by the *NABH558* gene can degrade neoagarobiose to D-galactose and 3,6-anhydro-L-galactose (Fig. 1A). The agarase genes were provided from pGEX-AgaG1, pGEX-AgaH71, and pET-NABH558 plasmids, respectively [19, 20], and the pGMF $\alpha$ -XYLP plasmid [22] was used as a vector for subcloning each agarase gene. pGMF $\alpha$ -XYLP contains a *GAL10* promoter, which is a galactose-induced promoter and signal sequence for efficient secretory production of agarases, and possesses the *URA3* gene as a selective marker (Table 1).

### Construction of Expression Plasmids

For the efficient expression of the *AGAH71*, *AGAG1*, and *NABH558* genes in *S. cerevisiae* strains, pGMF $\alpha$ -AgaH71, pGMF $\alpha$ -AgaG1, and pGMF $\alpha$ -NABH plasmids, respectively, were constructed. Each gene was ligated under the control of an inducible yeast *GAL10* promoter and fused to the *S. cerevisiae*

**Table 1.** Strains and plasmids used in this study.

Strains or plasmids	Genotypes or descriptions
<i>S. cerevisiae</i> BY4742	<i>MAT<math>\alpha</math> leu2<math>\Delta</math>0 ura3<math>\Delta</math>0 his3-<math>\Delta</math>1 lys2-<math>\Delta</math>0</i>
<i>S. cerevisiae</i> 2805	<i>MAT<math>\alpha</math> pep4::HIS4 prb1-1.6R can1 GAL2 his3-200 ura3-52</i>
<i>S. cerevisiae</i> FY834	<i>MAT<math>\alpha</math> ura3-52 his3-<math>\Delta</math>200 leu2-<math>\Delta</math>1 lys2-<math>\Delta</math>202 trp1-<math>\Delta</math>63</i>
pGEX-AgaH71	$\beta$ -agarase from <i>Pseudoalteromonas hodoensis</i> H7
pGEX-AgaG1	$\beta$ -agarase from <i>Alteromonas</i> sp. GNUM-1
pET-NABH558	Neoagarobiose hydrolase from <i>Gayadomonas joobiniege</i> G7
pGMF $\alpha$ -XYLP	<i>Gal10p-MF<math>\alpha</math></i> signal sequence- <i>Gal7t</i> , <i>Ura3</i> selective marker
pGMF $\alpha$ -AgaG1	<i>Gal10p-MF<math>\alpha</math>.s-AGAG1-Gal7t</i> , <i>Ura3</i> selective marker
pGMF $\alpha$ -AgaH71	<i>Gal10p-MF<math>\alpha</math>.s-AGAH71-Gal7t</i> , <i>Ura3</i> selective marker
pGMF $\alpha$ -NABH	<i>Gal10p-MF<math>\alpha</math>.s-NABH558-Gal7t</i> , <i>Ura3</i> selective marker
pGMF $\alpha$ -HGN	<i>AGAH71 EC-AGAG1 EC-NABH EC</i> , <i>Ura3</i> selective marker



**Fig. 1.** Scheme of agarose degradation by acting various agarases (A) and plasmids containing various agarase gene expression cassettes (EC) (B).

mating factor *a* signal sequence (*MFas.s*). To construct each plasmid, an In-Fusion HD Cloning Kit (Clontech Laboratories, Inc. Japan) designed for directional cloning was used. The pGMFα-AgaH71 (Fig. 1B) plasmid, which includes components controlling the inducible expression of the *AGAH71* gene, was constructed by inserting a PCR product containing the 824 bp *AGAH71* gene in-frame into the *Sall*-*Xba*I site of the pGMFα-XYLP plasmid. The PCR product containing the *AGAH71* gene with a flanking *Sall*-*Xba*I site was generated using the pGEX-AgaH71 plasmid as a template and AgaH71-F and AgaH71-R as forward and reverse primers, respectively. The oligonucleotides used are listed in Table 2. The PCR products containing the *AGAG1* gene (906 bp) and *NABH558* gene (1,080 bp) with a flanking *Sall*-*Xba*I site were generated using pGEX-AgaG1 and pET-NABH558 plasmids as templates and AgaH71-F/AgaH71-R and NABH-F/NABH-R as the primer set. The generated *AGAG1* and *NABH558* gene products were also ligated in-frame into the *Xba*I-*Sall* site of the pGMFα-XYLP plasmid, resulting in pGMFα-AgaG1 and pGMFα-NABH plasmids, respectively. Furthermore, we designed and constructed the pGMFα-HGN plasmid for simultaneous expression of the *AGAH71*, *AGAG1*, and *NABH558* genes. To construct the pGMFα-HGN plasmid, the pGMFα-NABH plasmid was used as the starting plasmid. The *AGAG1* gene expression cassette

(*GAL10p*-*MFas.s*-*AGAG1*-*GAL7t*) was amplified using the pGMFα-AgaG1 plasmid as a template, and the In-Gal10p-F/In-Gal7t-R primer set was cloned in-frame into the *Sac*I site of the pGMFα-NABH vector, resulting in the pGMFα-GN plasmid (9.3 kb). The *AGAH71* gene expression cassette was also amplified using the pGMFα-AgaH71 plasmid as a template using the same primer set, and the amplicon was cloned into the pGMFα-GN plasmid. Finally, the pGMFα-HGN plasmid (11.2 kb) including *AGAH71*, *AGAG1*, and *NABH558* gene expression cassette (E.C) was constructed (Fig. 1B). The order of the genes in the pGMFα-HGN plasmid was arranged according to the degradation order of enzymes for substrates including agarose, NA6, NA4, and NA2 (Fig. 1A).

#### Yeast Transformation, Media, and Culture Conditions

The constructed plasmids were transformed into *S. cerevisiae* BY4742, 2805, and FY834 strains using high-frequency transformation methods, and *Ura*<sup>+</sup> transformants were selected on synthetic complete (SC) medium [16]. To induce expression of agarase genes, yeast transformants were cultured in 10 ml (for test tube culture) or 50 ml (for baffled flask culture) YPDG [10 g yeast extract, 20 g peptone, 10 g dextrose, and 10 g galactose per liter] media for galactose-inducible gene expression at 30°C for 48 h. For

**Table 2.** Oligonucleotides list used in this study.

Oligonucleotides	Sequences (5'→3')
AgaH71-F	CGCTCTAGATAAGAGAGCAGCTGATTGGAG
AgaH71-R	CCGGTCGACTTACTGGGCTTTAT
AgaG1-F	GGCTCTAGATAAGAGAGCGATTCTTTCAT
AgaG1-R	CGCGTCGACTTATTGGACACTA
NABH-F	CCGTCTAGATAAGAGATCTGAAAAAAATT
NABH-R	CGCGTCGACTTAGTTGGAGTTCT
In-Gal10p-F	ACATGATTACGAATTAATTCGAGCTCGGTACCCGGGGATCC
In-Gal7t-R	ATGGATCCCCGGGTACCGAGAAACGACGGCCAGTGCCAAG
ACT1-F	ATCCAAGAGAGGTATCT
ACT1-R	CACACTTCATGATGGAG

production of ethanol from agarose, yeast transformants were precultured at 30°C in a 10 ml YPG [10 g yeast extract, 20 g peptone, and 20 g galactose per liter] medium. After a 24 h incubation, cultivated solution (C.S, cell+medium) or cultivated cells (C.C, only cell pellet) were transferred into 50 ml YPA [10 g yeast extract, 20 g peptone, and 10 g agarose per liter] medium. Cultivation processed at 40°C for 48 h (saccharification process) and then continued for 72 h after shifting to 30°C (fermentation process). Optical density (OD) of the yeast transformants was periodically measured using a spectrophotometer at 600 nm, and the reading was converted to dry cell weight (DCW) by the pre-determined conversion factor, 0.31 DCW (g/l)/OD.

#### Assay of Agarase Activity

The activity of agarase toward agarose was measured based on the release of the reducing sugar equivalent using the 3,5-dinitrosalicylic acid (DNS) method [16, 19, 23]. The enzyme solution was incubated at 40 or 45°C for 10 min in 20 mM Tris-HCl (pH 7.0) buffer containing 0.2% agarose. After adding the DNS solution, the enzyme mixture was boiled for 10 min and cooled, and spectrophotometric activity measurements were subsequently carried out at 540 nm. The amount of liberated reducing sugar was measured using galactose as a standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min under these assay conditions. Agarase activity was also assessed by direct staining with iodine solution (0.05 M I<sub>2</sub> in 0.12 M KI) in which a clear halo distinguished cells expressing agarase.

#### Reverse Transcription PCR (RT-PCR)

RT-PCR was performed to determine the gene transcription levels of *AGAH71*, *AGAG1*, and *NABH558* in the *S. cerevisiae* transformants. Total RNA of each strain was extracted according to the method of Chomczynski and Sacchi [25] with minor modifications, and 1 μg of total RNA was used to synthesize the cDNA using the HyperScript First-Strand Synthesis Kit (GeneAll) and an oligo dT as primer in a 20 μl reaction system. The cDNA synthesis reaction conditions were 10 min at 30°C, 60 min at 42°C and 15 min at 72°C. The synthesized cDNA was used as a template for PCR, and AgaH71-F/R, AgaG1-F/R, NABH-F/R, and ACT1-F/R primer sets were used to amplify the *AGAH71*, *AGAG1*, *NABH558*, and *ACT1* genes, respectively. *ACT1* was used as an internal control.

#### TLC Analysis of Hydrolysis Products

The hydrolyzed products of agarose by recombinant agarase were detected by thin layer chromatography (TLC) using silica gel 60 plates [24]. The reaction mixture was prepared with 0.3% agarose substrate in 20 mM Tris-HCl (pH 7.0) buffer and the agarase enzymes. The reaction was carried out at 40–45°C for 24 h. Aliquots of the reaction mixture (6 μl) were spotted onto silica gel 60 plates and doubly ascended in a solvent system of n-butanol/ethanol/water (5:3:2, v/v/v). The separated products

were visualized by spraying with 10% (w/v) sulfuric acid prepared in absolute ethanol and heating the plates to 110°C.

#### Ethanol Determination

Ethanol production from agarose was attempted using the *S. cerevisiae* 2805/pGMFα-HGN strain. The 2805/pGMFα-HGN strain was precultivated in YPG medium for 24 h and cultivated solution (C.S), and cultivated cells (C.C) were transferred into YPA (1% agarose) medium. The cells were incubated for 48 h at 40°C and then incubated for a further 72 h after shifting to 30°C. The concentration of ethanol was determined by HPLC (Agilent 1100 series, Agilent, Inc., USA) with a refractive index detector (RID). Aminex HPX-87H column (Biorad) was used with an oven temperature of 65°C and a flow rate of the mobile phase (5 mM sulfuric acid) of 0.6 ml/min.

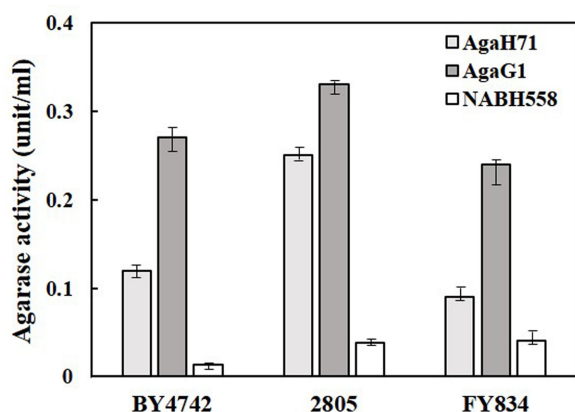
## Results and Discussion

#### Construction of Agarase Expression Plasmids

The agarase genes (*AGAH71*, *AGAG1*, and *NABH558*) were each amplified and cloned in an inducible expression vector (pGMFα-XYLP). The constructed expression plasmids had the *MFα* signal sequence for the secretory production of recombinant agarase genes, and the signal sequence was joined to the agarase genes (Fig. 1B). The expected junction between each signal sequence and agarase gene was confirmed using DNA sequencing of the pGMFα-AgaH71, pGMFα-AgaG1, and pGMFα-NABH plasmids. The sequence of the original target for the removal of the *MFα* propeptide is KREAEAEA, which is cleaved after the dibasic peptide K-R by the yeast endopeptidase *KEX2* [16, 26]. Thus, according to the design of the pGMFα-AgaH71, pGMFα-AgaG1, and pGMFα-NABH expression plasmid, the prosegment of *MFαs* is expected to be cleaved after the dibasic residues Lys-Arg by a single *KEX2* endopeptidase in *S. cerevisiae* [27], and the mature form of agarase was able to be secreted.

#### Optimal Host Strain for the Expression of Agarase Genes

To select suitable host strains for agarase expression, the pGMFα-AgaH71, pGMFα-AgaG1, and pGMFα-NABH plasmids were transformed into the *S. cerevisiae* BY4742, 2805, and FY834 strains, all of which have excellent ability to produce ethanol from galactose. Ten clones were randomly selected after incubation on an SC-Ura plate per each strain, and the agarase activity in these clones was analyzed by direct staining using iodine solution. The agar was degraded by the secreted recombinant agarase. Halos were evident around the *S. cerevisiae* transformants compared with the host strains, BY4742, 2805, and FY834, which did



**Fig. 2.** Comparison of agarase activity in each host strain harboring a pGMF $\alpha$ -AgaH71, pGMF $\alpha$ -AgaG1, and pGMF $\alpha$ -NABH plasmid, respectively.

not degrade the agar (data not shown). The clone exhibiting the highest level of agar degradation was chosen, and each strain harboring a pGMF $\alpha$ -AgaH71, pGMF $\alpha$ -AgaG1, or pGMF $\alpha$ -NABH plasmid was cultivated in 10 ml YPDG medium for 48 h. The enzymatic reactions of  $\beta$ -agarase and NABH were carried out at 45°C and 40°C, respectively. Agarase activity was compared by DNS assay. As shown in Fig. 2, when the *S. cerevisiae* 2805 strain was used as the host strain, agarase activities were improved 1.2-2.8 fold compared with other host strains used. Also,  $\beta$ -agarase activity commonly showed higher than NABH activity. Therefore, the *S. cerevisiae* 2805 strain was selected as the optimal host strain for expression of agarase genes.

### Secretory Production of Recombinant $\beta$ -Agarase and NABH

The signal sequence (MF $\alpha$ s.s) was joined to the *AGAH71*, *AGAG1*, and *NABH558* genes of each expression plasmid for the secretory production of two  $\beta$ -agarases and NABH, respectively. Activity and secretory efficiency of recombinant  $\beta$ -agarase and NABH were analyzed in *S. cerevisiae* 2805/

pGMF $\alpha$ -AgaH71, 2805/pGMF $\alpha$ -AgaG1, and 2805/pGMF $\alpha$ -NABH transformants. The clone exhibiting the highest expression level in test tube cultivation was chosen for the subsequent baffled-flask culture experiment. The ability of each transformant to produce secretory  $\beta$ -agarase and NABH together with their growth is shown in Table 3. As expected, most  $\beta$ -agarase and NABH activities were found in extracellular medium (supernatant) and secretory efficiencies were 87%-96%. Although NABH activity was still lower than  $\beta$ -agarase activity, recombinant NABH was efficiently secreted in the extracellular fraction (96%). To directly produce ethanol from agarose, enzymatic saccharification by the three cooperating genes was necessary. Therefore, the *AGAH71*, *AGAG1*, and *NABH558* gene expression cassettes were cloned into the pGMF $\alpha$ -HGN plasmid, and the plasmid was transformed into the *S. cerevisiae* 2805 strain.  $\beta$ -agarase and NABH were induced by cultivation into YPDG medium for 48 h in the *S. cerevisiae* 2805/pGMF $\alpha$ -HGN transformant. The total activity of agarase including  $\beta$ -agarase and NABH was 1.47 unit/ml, and 90% of total agarase was successfully secreted in supernatant (Table 3).

### Analysis of Agarase Expression Levels by RT-PCR

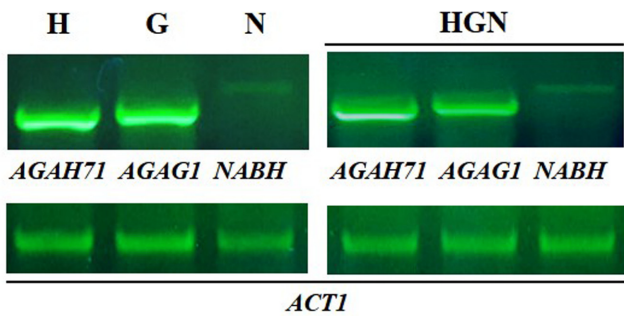
The genes encoding  $\beta$ -agarase from (marine) bacteria have been efficiently expressed in yeast such as *S. cerevisiae* and *Pichia pastoris* (methylotrophic yeast) [28, 29], but, commonly, the expression level of  $\alpha$ -agarase was low. The expression level of NABH was also low in the present study, despite the use of the same expression system. Therefore, we performed RT-PCR to examine the transcription level of each gene in each transformant. mRNA was prepared from the *S. cerevisiae* 2805/pGMF $\alpha$ -AgaH71, 2805/pGMF $\alpha$ -AgaG1, 2805/pGMF $\alpha$ -NABH, and 2805/pGMF $\alpha$ -HGN strains, and cDNA was subsequently synthesized. The cDNA was amplified using a specific primer set, and the transcription level was assessed. As shown in Fig. 3, the *AGAH71* and *AGAG1* genes were successfully amplified, and the trans-

**Table 3.** Comparison of cell growth and agarase activity in yeast transformants expressing various agarases.

Transformants	Dry cell weight (g/l)	Agarase activity (unit/ml)	
		$\beta$ -agarase activity	NABH activity
2805/pGMF $\alpha$ -AgaH71	6.11	0.87 (0.13)	-
2805/pGMF $\alpha$ -AgaG1	5.80	0.74 (0.08)	-
2805/pGMF $\alpha$ -NABH	5.79	-	0.12 (0.005)
2805/pGMF $\alpha$ -HGN	6.18	1.47 (0.17)	

The activity was indicated as extracellular enzyme activity and intracellular enzyme activity was indicated in parenthesis.





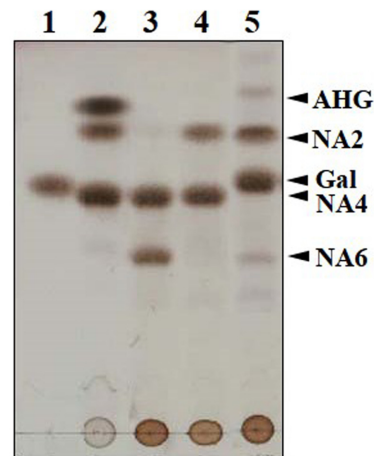
**Fig. 3.** Analysis of the transcription levels of agarase genes using RT-PCR.

The *AGAH71*, *AGAG1*, *NABH558*, and *ACT1* (internal control) genes were amplified by PCR using each cDNA as template. H; 2805/pGMF $\alpha$ -AgaH71 strain, G; 2805/pGMF $\alpha$ -AgaG1 strain, N; 2805/pGMF $\alpha$ -NABH strain and HGN; 2805/pGMF $\alpha$ -HGN strain.

cription level of the *AGAH71* gene was indistinguishable from that of the *AGAG1* gene. However, transcriptional level of the *NABH558* gene was lower than that of the other genes in the 2805/pGMF $\alpha$ -NABH and 2805/pGMF $\alpha$ -HGN strains. Thus, we inferred that the lower activity of NABH was a result of reduced transcription level. In bacterial and yeast, low activity and productivity of recombinant  $\alpha$ -agarase are caused by low transcription and translation levels [16]. The reason (mechanism) is not clear yet, but this can be overcome by promoter optimization, codon usage, and gene rearrangement.

#### TLC Chromatogram Analysis of Hydrolysis Products

Degradation action for agarose of agarase produced by each yeast transformant was analyzed using a TLC chromatogram (Fig. 4).  $\beta$ -Agarase produced in the 2805/pGMF $\alpha$ -AgaH71 strain could degrade agarose to neoagarotetraose and neoagarohexaose (Fig. 4, lane 3).  $\beta$ -Agarase produced in the 2805/pGMF $\alpha$ -AgaG1 strain could efficiently degrade agarose to neoagarotetraose and neoagarobiose (Fig. 4, lane 4). Neoagarobiose hydrolase produced in the 2805/pGMF $\alpha$ -NABH strain could not directly degrade agarose because it uses neoagarobiose as a substrate (data not shown). Therefore, simultaneous degradation of agarose was attempted by  $\beta$ -agarases and NABH produced in the 2805/pGMF $\alpha$ -HGN strain. Agarose was degraded to galactose, 3,6-anhydro-L-galactose (AHG), neoagarobiose, and some neoagarohexose (Fig. 4, lane 5). Although neoagarobiose was not completely degraded, most galactose as fermentable sugar was directly produced from agarose by enzymatic saccharification in the 2805/pGMF $\alpha$ -HGN strain.

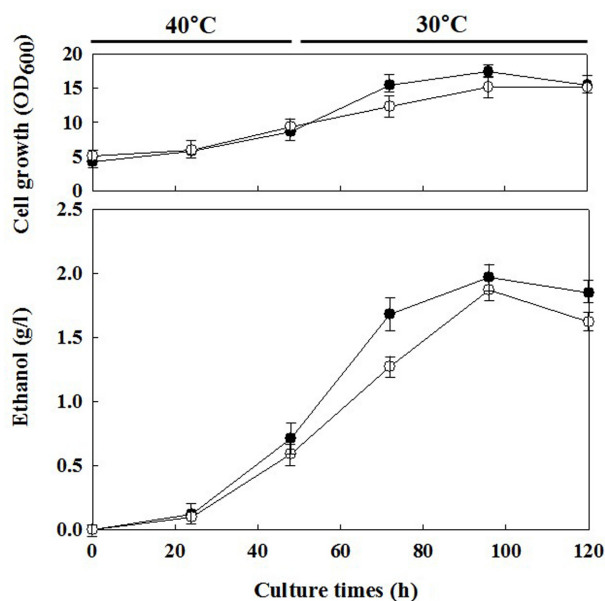


**Fig. 4.** TLC chromatograms of hydrolysis products of each agarase on agarose.

Lane 1; galactose, lane 2; AHG, NA2, NA4 mixture, lane 3; agarose+AgaH71, lane 4; agarose+AgaG1, lane 5; agarose+HGN (AgaH71-AgaG1-NABH).

#### Ethanol Production of Unified Saccharification and Fermentation

To produce ethanol from agarose, the 2805/pGMF $\alpha$ -HGN strain was cultured into YPG [YP+galactose] for induction of agarase genes and, subsequently, YPA [YP+agarose] for saccharification and ethanol fermentation. After inducing agarase gene expression, cultivated solution (C.S) or cultivated cells (C.C) of the 2805/pGMF $\alpha$ -HGN strain were transferred in pre-warmed YPA medium. Because agarose is easily polymerized (solidified) at a low temperature, 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. The cell density gradually increased until 96 h, indicating that the 2805/pGMF $\alpha$ -HGN strain can grow in the YP-agarose medium by utilizing the galactose that resulted from agarose hydrolysis (Fig. 5). The concentration of ethanol increased as fermentation progressed and eventually increased as high as 1.97 g/l and 1.89 g/l, for C.S and C.C inoculation, respectively, at 96 h of fermentation. The galactose concentration was about 1.2 g/l at the end of fermentation, and other hydrolysis products (AHG and NA2) still remained. Syazni *et. al* reported the direct production of ethanol from neoagarosebiase degraded by using  $\alpha$ -neoagarooligosaccharide (NAOS) hydrolase [30]. Recombinant yeast was inoculated into medium containing 80 % (v/v) NAOS mixture degraded by NAOS hydrolase and then ethanol, at concentration of up to 1.8 g/l,



**Fig. 5.** Comparison of cell growth and ethanol concentration by enzymatic saccharification of agarose and ethanol fermentation in 2805/pGMF $\alpha$ -HGN strain.

Closed circles and open circles indicated cultivated solution (C.S) and cultivated cells (C.C), respectively, of the 2805/pGMF $\alpha$ -HGN strain.

was produced. However, in the present study, saccharification of agarose and ethanol fermentation processes were unified by using single recombinant yeast strain and cultivation. Furthermore, the unified enzymatic saccharification and fermentation (USF) of 10 g/l agarose resulted in maximum 1.97 g/l ethanol concentration. Although it is still difficult for this strain to convert AHG to ethanol, this could be overcome by introducing genes that possess the ability to convert AHG to 2-keto-3-deoxy-galactonate [31]. Therefore, the agarose hydrolysis step should be improved to produce larger quantities of fermentable sugars and thus high concentrations of ethanol from agarose. This USF system was developed as a one-step system that does not require pretreatment of biomass (substrates) for direct production of ethanol from agarose and suggests the possibility of unification and simplification of several processes.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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