J. Microbiol. Biotechnol. (2014), 24(12), 1622–1628
http://dx.doi.org/10.4014/jmb.1405.05019

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Production of DagA, a β-Agarase, by *Streptomyces lividans* in Glucose Medium or Mixed-Sugar Medium Simulating Microalgae Hydrolysate

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Received: May 8, 2014
Revised: July 25, 2014
Accepted: July 31, 2014

First published online
August 1, 2014

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pISSN 1017-7825, eISSN 1738-8872

DagA, a β-agarase, was produced by cultivating a recombinant *Streptomyces lividans* in a glucose medium or a mixed-sugar medium simulating microalgae hydrolysate. The optimum composition of the glucose medium was identified as 25 g/l glucose, 10 g/l yeast extract, and 5 g/l $MgCl_2 \cdot 6H_2O$. With this, a DagA activity of 7.26 U/ml could be obtained. When a mixed-sugar medium containing 25 g/l of sugars was used, a DagA activity of 4.81 U/ml was obtained with very low substrate utilization efficiency owing to the catabolic repression of glucose against the other sugars. When glucose and galactose were removed from the medium, an unexpectedly high DagA activity of about 8.7 U/ml was obtained, even though a smaller amount of sugars was used. It is recommended for better substrate utilization and process economics that glucose and galactose be eliminated from the medium, by being consumed by some other useful applications, before the production of DagA.

Keywords: DagA, *Streptomyces lividans*, mixed-sugar medium, microalgae hydrolysate

Introduction

Enzyme hydrolysis of red algal galactan mainly composed of agarose and agaropectin can be accomplished in several steps [7]. Agarose has alternative D-galactose and 3,6-anhydro-L-galactose [1]. Agaropectin has basically the same structure as agarose, but with side groups of sulfate, pyruvate, *etc*. [9]. Firstly, agarose or agaropectin are broken down to neoagarotetraose and neoagarohexaose by the action of a Type I β-agarase. These oligomers are broken down to neoagarobiose by a Type II β-agarase. Finally, the dimer is degraded by neoagarobiose hydrolase to produce galactose and anhydro-galactose.

DagA is a Type I β-agarase originated from *Streptomyces coelicolor*. The *dagA* gene from *S. coelicolor* A3(2) has been cloned, sequenced, and overexpressed in *S. lividans* [3, 6, 20]. Temuujin *et al*. [24] reported that DagA (Sco3471, 32 kDa), which belongs to the GH16 family, is an endo-type β-agarase that degrades agarose into neoagarotetraose and neoagarohexaose [24]. Neoagarooligomers, including neoagarotetraose and neoagarohexaose, have a potentially high economic value. They are known to have an antioxidative activity, restraining lipid peroxidation and scavenging superoxide anion radicals and hydroxyl free radicals [26]. They have been reported to inhibit bacterial growth, and to slow down the digestion of starch being used in the food industry as a low-calorie additive [12]. Moreover, they have a moisturizing and a whitening effects on skin [18].

In this study, a glucose-based medium was developed for the production of DagA by a recombinant *S. lividans*. Generally, rich and complex media are used for *Streptomyces* strain culture in a laboratory scale. However, using such media for the purpose of enzyme production in a large scale would be economically inappropriate. For this reason, the development of a low-cost medium optimized for DagA production was in need. Key nutrients to be included in the medium were selected, and their concentrations were optimized by employing statistical design approaches of Plackett–Burman design (PBD) [10, 21, 27] and a response surface method (RSM) with the central composite design (CCD) [4, 10, 25], respectively. The feasibility of using a

mixed-sugar medium obtained from the hydrolysis of microalgal biomass was also investigated.

Materials and Methods

Strain and Culture Conditions

S. lividans TK24/pUWL201-DagA was prepared as previously reported [24]. It was grown on R2YE agar plates at 28°C for 4 days. The composition of the R2YE agar medium was 103 g sucrose, 10.12 g $MgCl_2 \cdot 6H_2O$, 10 g glucose, 5 g yeast extract, 0.25 g K_2SO_4, 0.1 g casamino acids, 100 ml of 5.73% TES (pH 7.2), 80 ml of 3.68% $CaCl_2 \cdot 2H_2O$, 15 ml of 20% L-proline, 10 ml of 0.5% K_2HPO_4, 2 ml of trace elements solution, and 22 g agar per liter [16]. For the seed culture, spores from an R2YE plate were inoculated into 20 ml of the R2YE medium in a 125 ml baffled flask and incubated for 60 h. Then, 1% (v/v) of seed culture was transferred to 50 ml of the main culture medium in a 250 ml baffled flask and then incubated at 28°C and 200 rpm for 48 h. The pH of the main culture medium was adjusted to 7.2 with 1 M NaOH solution. It contained 50 μg/ml of thiostrepton. All the experiments were conducted in triplicate.

Analytical Methods

For monitoring cell growth, 5 ml of culture broth sample was centrifuged at 8,000 rpm for 10 min at 4°C, and the pellet was washed twice by distilled water and dried at 70°C for 2 days. The agarase activity of DagA in the supernatant was quantified by using the dinitrosalicylic acid (DNS) method. Twenty-five microliters of the supernatant was mixed with 975 μl of 0.2% agarose solution based on 50 mM Tris-HCl buffer (pH 7.0). After incubation at 40°C for 10 min, the mixture was mixed with 1 ml of DNS reagent solution (6.5 g of dinitrosalicylic acid, 325 ml of 2 M NaOH, and 45 ml of glycerol in 1 L of distilled water) and boiled for 10 min until the color changed. The reaction was stopped by cooling in an ice bath. The absorbance at 540 nm was measured by UV-spectrometry [13]. The DagA activity (U/ml) was defined as the amount of enzyme required to liberate 1 μmol of reducing sugar (D-(+)-galactose) per minute. The concentrations of sugars, including glucose, were measured by high-pressure liquid chromatography (HPLC, Waters, USA) equipped with an evaporative light scattering detector (ELSD, Sedere, France). An Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm; Bio-Rad, USA) and an Asahipak NH2P-50 4E column (250 × 4.6 mm; Shodex, Japan) were used. The Aminex HPX-87H column was maintained at 60°C with a mobile phase (deionized water) flow rate of 0.6 ml/min. The Asahipak NH2P-50 4E was operated at 30°C with a mobile phase (80% ACN) flow rate of 1.0 ml/min.

Results and Discussion

Effect of Carbon Source on DagA Activity

Various carbon sources such as glucose, galactose, xylose, sucrose, agarose, cellulose, starch, and dextrin were tested to identify the optimum carbon source for the production of DagA. Each carbon source was dissolved in the sugar-free R2YE medium. The results indicated that the highest DagA activity was obtained with galactose (6.30 U/ml), followed by xylose (5.77 U/ml), glucose (5.34 U/ml), starch (4.45 U/ml), and dextrin (4.08 U/ml) (Fig. 1). Agarose (2.88 U/ml), sucrose (2.61 U/ml), and cellulose (2.55 U/ml) were found to have no contribution to DagA activity. Although galactose, the best carbon source, produced about 20% higher DagA activity than glucose, its price was considered too high to be used on a large scale. For example, the unit price of reagent-grade galactose is about 12-fold higher than glucose. Xylose also showed a better performance than glucose, but its unit price was also considered to be too high. For this reason, glucose was selected as the carbon source for the cultivation of *S. lividans* TK24/pUWL201-DagA to produce DagA.

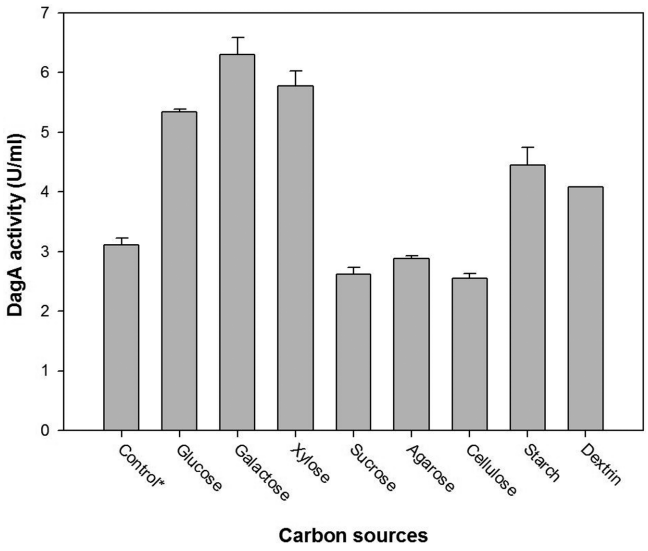

Fig. 1. Effect of carbon source on DagA production by *S. lividans* TK24/pUWL201-DagA (*control: sugar-free R2YE medium).

Screening of Key Nutrients by PBD

The evaluation of seven nutrient sources (glucose as the sole carbon source; three nitrogen sources (yeast extract, malt extract, and bacto peptone); and three mineral sources (K_2SO_4, $MgCl_2 \cdot 6H_2O$, and $CaCl_2 \cdot 2H_2O$)) was carried out in eight combinations organized by PBD [21]. Table 1 illustrates the candidate variables (nutrients) and their ranges used for the screening work, and the responses (enzyme activities) under given conditions. Data were statistically

Table 1. Plackett–Burman design matrix and corresponding responses.

Run	Experimental value							Response
	Glucose (G)	Yeast extract (Y)	Malt extract (Ma)	Bacto peptone (P)	K_2SO_4 (K)	$MgCl_2•6H_2O$ (M)	$CaCl_2•2H_2O$ (Ca)	DagA activity (U/ml)
1	-1	-1	-1	+1	+1	+1	-1	4.052
2	+1	-1	-1	-1	-1	+1	+1	3.533
3	-1	+1	-1	-1	+1	-1	+1	3.973
4	+1	+1	-1	+1	-1	-1	-1	5.800
5	-1	-1	+1	+1	-1	-1	+1	2.826
6	+1	-1	+1	-1	+1	-1	-1	2.876
7	-1	+1	+1	-1	-1	+1	-1	5.140
8	+1	+1	+1	+1	+1	+1	+1	5.859

Positive level (+1): G, 10; Y, 5; Ma, 5; P, 5; K, 3; M, 3; Ca, 3.
Negative level (-1): G, 1; Y, 1; Ma, 1; P, 1; K, 0.5; M, 0.5; Ca, 0.5.

Table 2. Results of key variable screening.

Variable	Effect	Coefficient	DagA activity (U/ml)	
			t-Value	*p*-Value
Intercept		4.258	90.19	0.000*
Glucose	**0.519**	**0.260**	**5.50**	**0.000***
Yeast extract	**1.871**	**0.936**	**19.82**	**0.000***
Malt extract	-0.164	-0.082	-1.74	0.101
Bacto peptone	0.754	0.377	7.99	0.000*
K_2SO_4	-0.134	-0.067	-1.42	0.174
$MgCl_2·6H_2O$	**0.777**	**0.389**	**8.23**	**0.000***
$CaCl_2·2H_2O$	-0.419	-0.210	-4.44	0.000*

R^2 = 0.9731; *Statistically significant at 99% of confidence level.

analyzed by using the software package Minitab 14.0 (Minitab Inc., Pennsylvania, USA). The results as summarized in Table 2 showed that glucose, yeast extract, bacto peptone, and $MgCl_2·6H_2O$ had a significant (p value < 0.001) and positive influence on DagA production. However, $CaCl_2·2H_2O$ was found to have a negative effect, and was thus removed from the nutrients list. In addition, yeast extract was observed to have greater influence (a higher t value) as the nitrogen source than bacto peptone. Based on these observations, glucose as the carbon source, yeast extract as the nitrogen source, and $MgCl_2·6H_2O$ as the mineral source were selected.

Medium Composition Optimization by CCD and RSM

After having selected the three key nutrients glucose, yeast extract, and $MgCl_2·6H_2O$, RSM with CCD was applied to identify the effects of these nutrients on DagA production and thus to determine their optimal concentrations [4]. The dependency DagA activity produced (y) on the key nutrients could be well represented by the following quadratic equation with a correlation coefficient R^2 = 0.9310.

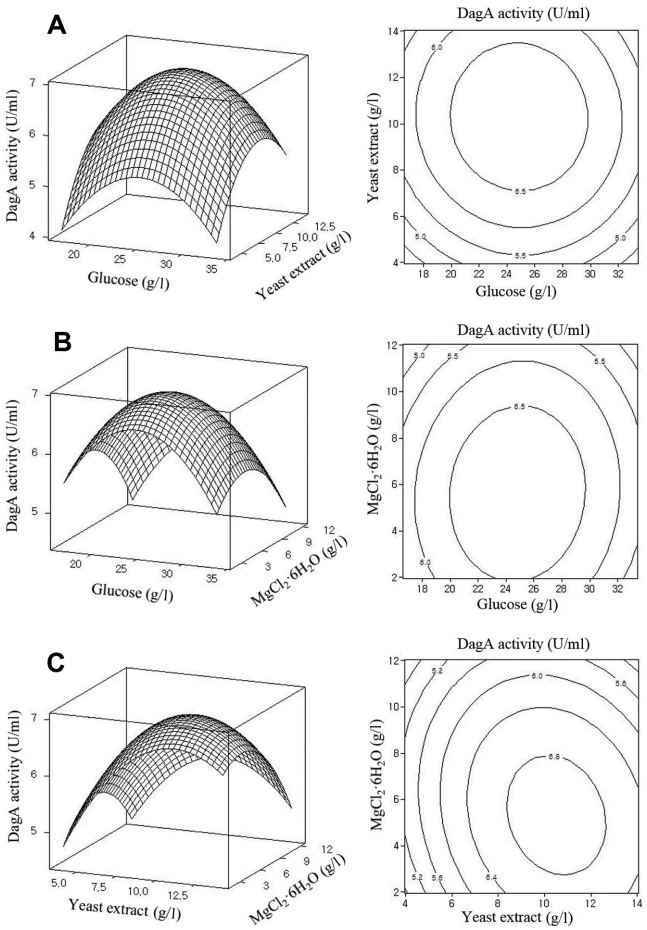

Fig. 2. 3D response surface and contour plots for DagA activity showing the effects of (**A**) glucose and yeast extract; (**B**) glucose and $MgCl_2·6H_2O$; and (**C**) yeast extract and $MgCl_2·6H_2O$.

$$y = 6.835 - 0.013\,G + 0.307\,Y - 0.227\,M - 0.408\,G^2 - 0.355\,Y^2 - 0.246\,M^2 - 0.094\,YM \quad (1)$$

where G, Y, and M denote glucose, yeast extract, and $MgCl_2{\cdot}6H_2O$, respectively.

Three-dimensional response surface curves and their respective contour plots described by the regression model are presented to elucidate the effects of the key nutrients (Fig. 2). Each response surface curve represents the effect of two nutrient factors, holding the other factor at the zero level. The optimum value is indicated by the surface confined in the smallest ellipse in the contour plot [2]. As shown in Fig. 2, each contour plot has an elliptical nature, indicating that mutual interactions among the nutrients are significant and the optimum values for glucose, yeast extract, and $MgCl_2{\cdot}6H_2O$ concentrations are within the tested ranges. The optimum concentrations were calculated based on the regression Eq. (1): 25 g/l of glucose, 10 g/l of yeast extract, and 5 g/l of $MgCl_2{\cdot}6H_2O$. Under the optimized conditions, the predicted response for DagA activity was 6.96 U/ml, whereas the experimentally observed response was 7.25 U/ml.

DagA Production by Using Mixed-Sugar Medium Simulating Microalgae Hydrolysate

Microalgae are gaining interest as an alternative source of energy fuels owing to their fast growth feature and relatively high lipid content [22]. After lipid extraction, the residue of microalgae biomass is generally used as the fertilizer and feedstuff of domestic animals and thrown away. Recently, it was reported that the microalgae residue can be utilized as a fermentation feedstock after being hydrolyzed to fermentable sugars [15]. Microalgae hydrolysate contains glucose, galactose, xylose, rhamnose, mannose, ribose, arabinose, and fucose together [5]. Several studies reported ethanol production, by using *Saccharomyces cerevisiae*, from glucose in microalgae hydrolysate with no utilization of the other sugars mentioned above [14, 15]. To overcome such limited substrate utilization problem, a new approach hiring microbial strains with broad substrate specificity was in need. *Streptomyces* strains, including *S. lividans*, were considered to be a good candidate for this purpose. They are well known to use most of the sugars from hydrolyzed microalgae as carbon sources, except fucose [8, 19]. For this reason, a mixed-sugar medium simulating microalgae hydrolysate, a potentially low-cost alternative carbon source, was used instead of glucose medium for the production of DagA in this study.

The mixed-sugar medium contained 25 g/l of six different monosugars: 7 g/l glucose, 5 g/l galactose, 5 g/l xylose, 3 g/l rhamnose, 3 g/l ribose, and 2 g/l mannose. Its composition was determined by considering that of the acid hydrolysate of *Nannochloropsis oceanica*: 27% glucose, 21% galactose, 20% xylose, 13% rhamnose, 14% ribose, and 5% mannose. Time profiles of DagA activity and cell and sugars concentrations are presented in Fig. 3 together with, for comparison, those in the case when the optimized glucose medium, which contained 25 g/l glucose, was used. When glucose was used as the sole carbon source for DagA production, it was completely consumed in 72 h and the maximum DagA activity was 7.26 U/ml at 48 h; when the mixed-sugar medium was used, 7 g/l of glucose was consumed in 48 h, and galactose and mannose in 60 h. The maximum DagA activity was 4.81 U/ml at 36 h (Table 3), which was approximately 34% lower than the control case

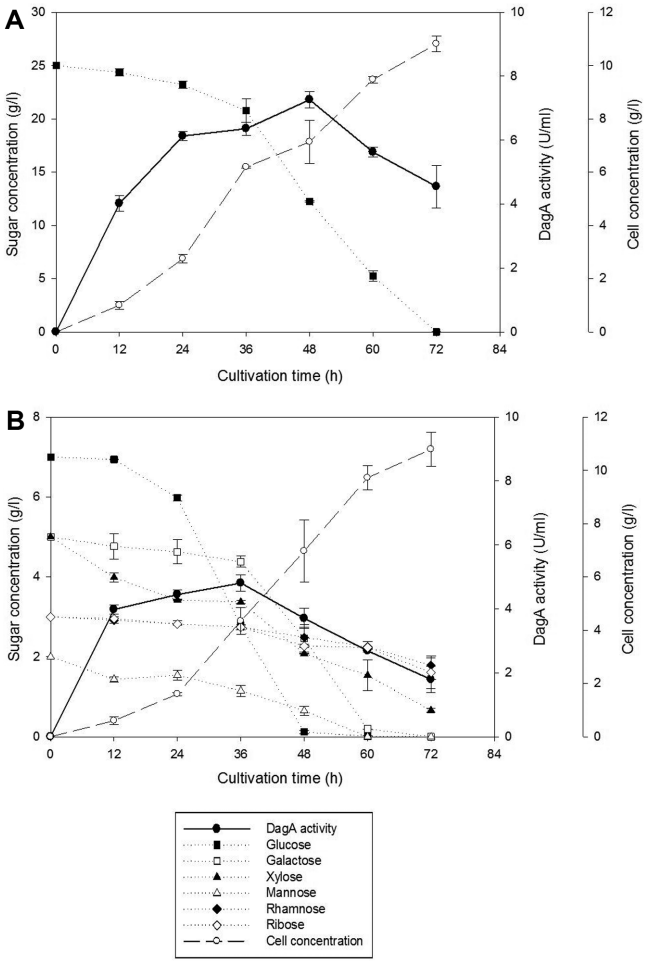

Fig. 3. Time profiles of DagA activity and cell and sugar concentrations: (**A**) 25 g/l mixed-sugars medium; (**B**) optimized glucose medium.

Table 3. Substrate consumed and DagA activity at the time of its maximum activity.

	Carbon substrate (g/l)	Time (h)	Substrate consumed (g/l)	Substrate utilization (wt%)	Dried cell mass (g/l)	Maximum DagA activity (U/ml)	DagA yield (U/ml/g-sugars consumed)
25 g/l Glucose medium	Glucose (25)	48	12.80	51.2	7.13 ± 0.57	7.26 ± 0.18	0.567
25 g/l Mixed-sugar medium	Glucose (7)	36	4.22	31.4	4.43 ± 0.36	4.81 ± 0.18	0.612
	Galactose (5)		0.62				
	Xylose (5)		1.65				
	Rhamnose (3)		0.26				
	Ribose (3)		0.26				
	Mannose (2)		0.85				
	Total (25)		7.86				
18 g/l Mixed-sugar medium	Galactose (5)	48	2.77	55.0	7.21 ± 0.19	8.76 ± 0.06	0.885
	Xylose (5)		2.62				
	Rhamnose (3)		0.74				
	Ribose (3)		1.77				
	Mannose (2)		2.00				
	Total (18)		9.90				
13 g/l Mixed-sugar medium	Xylose (5)	48	2.24	58.4	6.79 ± 0.03	8.63 ± 0.21	1.137
	Rhamnose (3)		1.44				
	Ribose (3)		1.91				
	Mannose (2)		2.00				
	Total (13)		7.59				

with 25 g/l glucose. The decrease in the DagA activity in the later part of the fermentation was speculated (with no clear evidence yet) to be due to protease activity in the culture broth. If we decide to stop the operation when the activity reaches the maximum value, that is, at 36 h in the case with the mixed-sugar medium, the main problem would be a very low substrate utilization efficiency of about 31%. Only glucose was noticeably consumed in 36 h, while the consumption of the other sugars was negligible, probably due to the catabolic repression exerted by glucose (Table 3). In many microorganisms, glucose is quickly utilized and represses the production of enzymes related to the consumption of other carbon substrates [11, 23].

To eliminate such negative effects of glucose, a glucose-free mixed-sugar medium containing 18 g/l of sugars was used, as in the third case in Table 3. As can be seen in Fig. 4A, mannose was completely consumed in 48 h and galactose in 60 h. The maximum DagA activity was 8.76 U/ml at 48 h, which was 82% higher than that in the case of the 25 g/l mixed-sugar medium, and even 21% higher than that of the control case of 25 g/l glucose. In total, 55% of sugars was utilized in 48 h, a much improved substrate utilization. A much higher DagA activity and a slightly smaller amount of cells were obtained with a lower sugars concentrations, in the absence of glucose. Based on these observation it was concluded that glucose had a positive effect on cell growth but a negative effect on enzyme production. Over 93% of sugars could be utilized in 72 h in the absence of glucose.

Galactose was also eliminated from the medium to investigate its effects on cell growth and enzyme production. The resulting medium contained 13 g/l sugars with no glucose and galactose (the fourth case in Table 3). As seen in Fig. 4B, mannose was completely consumed in 48 h. Over 92% of sugars was consumed in 72 h. The maximum DagA activity was 8.63 U/ml at 48 h, which was practically the same as that in the case of the 18 g/l mixed-sugar medium. Without galactose, cell growth decreased slightly. These results indicated that galactose had a positive effect on cell growth and that, however, it had no contribution on enzyme production. Considering the results with glucose-free and glucose and galactose-free media, it would be

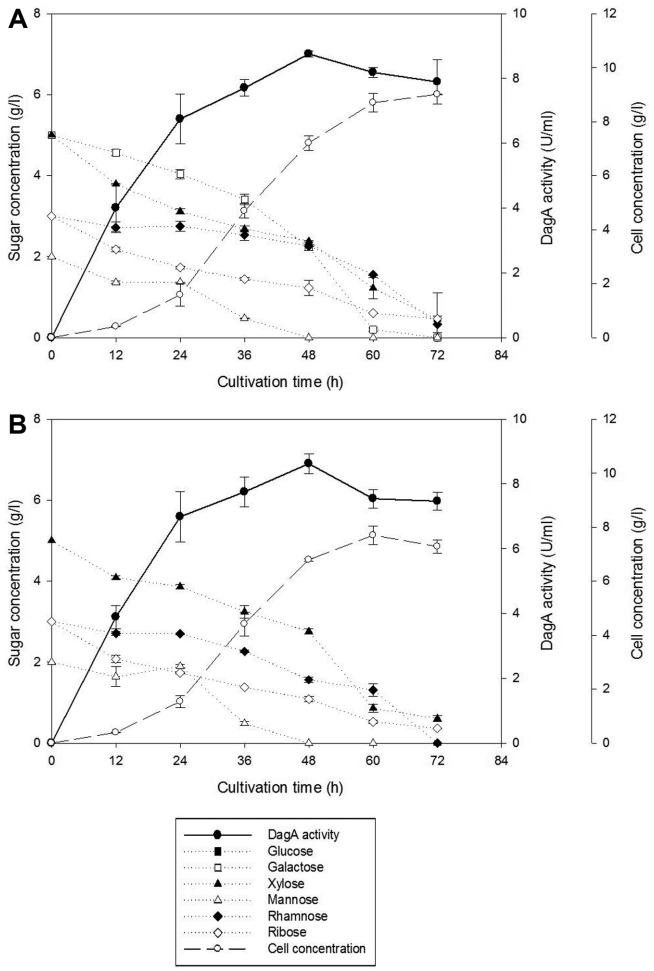

Fig. 4. Time profiles of DagA activity and cell and sugar concentrations: (**A**) 18 g/l glucose-free mixed-sugar medium; (**B**) 13 g/l glucose and galactose-free mixed-sugar medium.

recommendable that these two monosugars be eliminated, before agarase production by the recombinant *S. lividans*, when a microalgae hydrolysate is to be used.

One possible way to eliminate glucose and galactose from a mixed-sugar medium like microalgae hydrolysate for improved substrate utilization and enzyme production would be the utilization of them as substrates to produce some other bioproduct before the production of agarase. If a yeast strain that can take up galactose as well as glucose is used, for example, for this purpose, bioethanol and DagA could be produced in sequence with much better substrate utilization efficiency. Recently, *Saccharomyces cerevisiae* KL17, which was very efficient in utilizing glucose and galactose, was isolated by our group [17].

The results of this study with a synthetic medium simulating microalgal hydrolyste should be compared with those obtained with a real hydrolysate solution in the future. By doing that, the necessity of additional medium development efforts such as neutralization, desalting, and/or nutrient complementation could be identified before the microalgal hydrolysate is used for fermentation.

Acknowledgments

This study was supported by the Advanced Biomass R&D Center (ABC) of Global Frontier Project funded by the Ministry of Education, Science and Technology (ABC-2013-064099).

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