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Efficient synthesis of octyl-β-D-galactopyranoside by *Bacillus* spore-displayed β-galactosidase using an amphiphilic 1,2-dimethoxyethane co-solvent

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ABSTRACT

For enzymatic synthesis of octyl-β-D-galactopyranoside (octyl-gal) from lactose and *n*-octanol, *Escherichia coli* β-galactosidase (β-Gal) was expressed and displayed on the surfaces of *Bacillus subtilis* spores. The spore-displayed β-Gal was found to be stable when an amphiphilic 1,2-dimethoxyethane (DME) was used as a co-solvent; the transgalactosylation efficiency and octyl-gal conversion were optimal at 50% (v/v) DME. In addition, the product was maximally obtained from 100 mM lactose in a phosphate buffer/*n*-octanol/DME (25/25/50, v/v) mixture. By increasing the agitation speed and the amount of spores displaying β-Gal, a yield of 33.7 mM octyl-gal was obtained over 24 h in a batch mode, which is much higher than in other octyl-gal bioconversion processes, such as those involving lipid-coating, reverse micelles, or whole cells. On the other hand, intermittent addition of spore-displayed β-Gal and/or lactose in the reaction medium had no effect on the octyl-gal yield. The synthesized octyl-gal was hydrolyzed by the spore-displayed β-Gal, and a high concentration of octyl-gal competitively inhibited the enzymes (K_i value of 10.8 mM). In summary, we demonstrate that octyl-gal synthesis by spore-displayed β-Gal in non-aqueous medium can be significantly improved with the use of DME as a co-solvent.

1. Introduction

Alkyl galactosides are used industrially as non-ionic surfactants because they exhibit properties of detergency, foaming, wetting, and emulsification, as well as antimicrobial activity [1–3]. They can also be used as solubilizing agents for membrane proteins [4]. Recently, the enzymatic transgalactosylation reaction of alkyl galactosides between lactose donors and alcohol acceptors using β-galactosidase (β-Gal, EC 3.2.1.23) has been shown to be highly favorable in comparison with complex chemical syntheses [5,6]. However, in aqueous-organic biphasic media, the transgalactosylation efficiency has been limited by low enzyme stability in the organic phase and high lactose hydrolytic activity in the aqueous phase. With increasing alkyl chains of primary alcohols, the galactoside yield drastically decreased because the phase-transfer biocatalysis in the biphasic medium was limited. Thus, considerable efforts have been made to improve enzyme stability and the ratio of transgalactosylation to hydrolysis through the use of several immobilization techniques involving lipid coating [7], reverse micelles [8], and whole cells [9]. Specifically, in the synthesis of octyl-β-D-galactopyranoside (octyl-gal), the bioconversion processes increase the product conversion ratio, but it has been shown that the product synthesis rate is not fast and that the octyl-gal yield is very low due to the low lactose solubility and/or slow mass transfer in the medium. Thus, as an alternative to the conventional immobilization of proteins, a CotG-fused β-Gal spore display technique was developed, which involves partitioning of the water-in-oil emulsion at the interface, thus favoring the transgalactosylation reaction [10]. However, these emulsion techniques are not sufficient to produce octyl-gal on a large scale because emulsions are difficult to maintain at such a production scale.

Stevenson et al. reported that short-chain alkyl galactosides could be easily produced using commercial free β-Gals and amphiphilic 1,2-dimethoxyethane (DME) co-solvent [11]. However, lactose was not easily transgalactosylated into non-polar alcohol due to the mass-transfer limitation and low enzyme stability. On the other hand, it has also been reported that high yields of octyl glucoside were produced by reverse hydrolysis using immobilized almond β-glucosidase (β-Glu) in a homogeneous buffer/*n*-octanol/DME mixture [12]. However, the free β-Gal was not stable in the DME co-solvent [13].

In this study, recombinant β-Gal has been expressed in *Bacillus subtilis*, released by autolysis of the cells, and displayed on the surfaces of spores produced during the sporulation phase, as described previously [10,14]. As a co-solvent, DME increases the stability of the spore-displayed β-Gal and the transgalactosylation efficiency in non-aqueous medium. Several reaction parameters, such as co-solvent concentration, lactose concentration, agitation speed,

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and spore concentration, were optimized to increase the octyl-gal yield.

2. Materials and methods

2.1. Materials

Lactose, galactose, glucose, octyl-gal, *o*-nitrophenyl-β-D-galactopyranoside (oNP-gal), and *n*-octanol were purchased from Sigma–Aldrich (St. Louis, MO). DME was obtained from Junsei Chemical Co. (Tokyo, Japan). Organic solvents, including diethyl ether, toluene, cyclohexane, and acetonitrile, were purchased from Merck (Darmstadt, Germany). All other commercial chemicals were of reagent grade.

2.2. Strains and cloning

Protocols for DNA manipulation and analysis were based on standard cloning methods [15]. The *lacZ* gene was amplified by PCR from the genomic DNA of *Escherichia coli* W3110 using two oligonucleotide primers: BamlacZF, 5′-TGGATCCATGACCATGATTACGGATTCACTG-3′; and PstlacZR, 5′-CCCCTGCAGTTATTTTTGACACCAGACCAAC-3′. The primers contained *Bam*HI and *Pst*I restriction sites (underlined sequences). The PCR products were treated with *Bam*HI and *Pst*I enzymes, inserted into the *Bam*HI–*Pst*I sites of the pHPS9Cry3PSTAB vector, and then transformed into *E. coli* DH5α [F⁻ Δ80d*lacZ*ΔM15 Δ(*lac*ZYAargF) U169 *deoR recA*1 *endA*1 *hsdR*17 *phoA supE*44 Δ*thi-1 gyrA*96 *relA*1] [15]. For protein expression and spore display, the resulting pCry3PlacZ plasmid was transformed into *B. subtilis* DB104 (*nprR2 nprE18* Δ*aprA3*) using the method of Cutting and Vander-Horn [16].

2.3. Protein expression and spore purification

Transformed *B. subtilis* DB104/pCry3PlacZ cells were cultivated in a 5-L Erlenmeyer flask with 1 L of Difco sporulation medium (DSM), which was composed of 8 g Bacto-nutrient broth, 1 g KCl, 0.12 g $MgSO_4 \cdot 7H_2O$, and 0.5 mM NaOH supplemented with 1 mM $Ca(NO_3)_4$, 10 μM $MnCl_2$, and 1 μM $FeSO_4$, at 37 °C and 200 rpm for 24 h. The spores displaying β-Gal were efficiently purified using a previously described procedure, with some modifications [14,17]. Briefly, the pellets were collected from 1 L of culture medium by centrifugation (JA-10 rotor, 5000 rpm, 10 min, 4 °C) using Beckman J2-21M/E centrifuge. They were washed with 250 ml of 1 M KCl/0.5 M NaCl and centrifuged (JA-10 rotor, 5000 rpm, 20 min, 4 °C). They were resuspended in 50 ml of 1 M sodium phosphate buffer (pH 7.2). To release the endospores, the resuspended pellets were treated using a Branson Sonifier 450 cell disruptor for 3 min (output 3, duty cycle 30%) on ice. The disrupted cell suspension was then centrifuged (JA-14 rotor, 5000 rpm, 20 min, 4 °C). The pellets were washed twice with 50 mM sodium phosphate buffer (pH 7.2) by centrifugation (JA-14 rotor, 5000 rpm, 10 min, 4 °C). The pellets were resuspended and incubated in 25 ml of lysis buffer (50 mM sodium phosphate buffer, pH 7.2, 500 μg/ml lysozyme, 2 mM $MgCl_2$) for 1 h at 4 °C. The lysed mixture was subsequently washed with 50 mM sodium phosphate buffer (pH 7.2) by centrifugation (JA-14 rotor, 5000 rpm, 10 min, 4 °C). The dry weight of spore-displayed β-Gal was determined by a HB43-S halogen moisture analyzer (Mettler Toledo, Switzerland) with a circular glass fiber filter (HA-F1) and an aluminum sample pan (HA-D90). The sample was heated at 120 °C until the weight was not changed. By this method, the amount of purified spore-displayed β-Gal corresponding to one optical density unit at 600 nm (OD_{600}) in 1 ml suspension was determined to be 87.4 μg. One-ml aliquots of the purified spore-displayed β-Gal (6.25 mg/ml) were stored at −20 °C for use in further experiments.

2.4. Enzyme assay

The enzyme activity of spore-displayed β-Gal was determined by measuring the rate of hydrolysis of oNP-gal using a UV/vis spectrophotometer at 420 nm, as described previously [10]. The enzyme assay mixture was composed of 2 mM $MgCl_2$, 4 mM oNP-gal, and 50 mM sodium phosphate buffer (pH 7.2). The spore-displayed β-Gal was incubated in 1 ml of reaction mixture at 37 °C for 5 min. After the addition of 500 μl of 1 M Na_2CO_3 to the reaction mixture, the reaction was terminated, and the mixture was then centrifuged for 1 min at 10,000 × *g*. The absorption of the supernatant-liberated *o*-nitrophenol was measured at 420 nm. One unit of β-Gal activity was defined as the amount of enzyme that hydrolyzed 1 μmol of oNP-gal per min under the assay conditions.

2.5. Stability test of spore-displayed β-Gal in organic solvents

One hundred microliters of spore-displayed β-Gal (6.25 mg/ml) was incubated in a 1 ml reaction mixture containing 95% (v/v) solvent (acetonitrile, DME, diethyl ether, toluene, and cyclohexane) for 1 h at 37 °C. As a control, 50 mM sodium phosphate buffer (pH 7.2) was used in these assays. In addition, 1 ml of reaction mixture containing 50% (v/v) organic solvent (acetonitrile, DME, diethyl ether, toluene, and cyclohexane) and 30% (v/v) *n*-octanol was incubated at 37 °C for 1 h. The residual β-Gal activity was checked after the sample was washed three times with 50 mM sodium phosphate buffer (pH 7.2) by centrifugation (10,000 × *g*, 1 min).

2.6. Octyl-gal synthesis at different DME concentrations

In this paper, all transgalactosylation reactions were carried out in glass tubes equipped with a magnetic stirrer and block heater in a personal organic synthesizer (ChemiStation; Eyela, Tokyo, Japan). To investigate the effect of DME concentration on transgalactosylation reactions, the experiments were performed in 2 ml of reaction mixture containing 20 mM lactose, 20% (v/v) *n*-octanol, 25% (v/v) 10 mM sodium phosphate buffer (pH 7.2), 0.625 mg/ml spore-displayed β-Gal, and 30–60% (v/v) DME for 12 h at 37 °C and 700 rpm. The conversion of octyl-gal formed from lactose and *n*-octanol was expressed as a molar percentage (mole%).

2.7. Octyl-gal synthesis at different initial lactose concentrations

To produce high yields of octyl-gal, initial lactose concentrations were set at 10 mM, 50 mM, 100 mM, and 150 mM, in 2 ml of 10 mM sodium phosphate buffer (pH 7.2)/*n*-octanol/DME mixture (25/25/50, v/v). Each reaction was initiated by the addition of 1.25 mg/ml spore-displayed β-Gal and was carried out at 37 °C and 700 rpm for 92 h.

2.8. Octyl-gal synthesis at different spore biocatalyst concentrations and agitation speeds

To investigate the effect of agitation speed on octyl-gal synthesis, the transgalactosylation reactions, carried out in 2 ml of reaction buffer containing 100 mM lactose, 25% (v/v) *n*-octanol, and 50% (v/v) DME, were initiated by the addition of spore-displayed β-Gal (1.875 mg/ml) and carried out at three agitation speed values (0 rpm, 700 rpm, and 1400 rpm) for 20 h at 37 °C. To investigate the effect of spore-displayed β-Gal concentration on octyl-gal synthesis, reactions were carried out in 2 ml of medium, containing 100 mM lactose, 25% (v/v) *n*-octanol, and 50% (v/v) DME, with three different spore-displayed β-Gal concentrations (0.625, 1.25, and 1.875 mg/ml) at 37 °C and 1400 rpm for 20 h.

2.9. Octyl-gal synthesis by step-wise addition of lactose and/or spore biocatalyst

Octyl-gal synthesis was carried out by step-wise addition of 50 μl spore-displayed β-Gal (1.25 mg/ml) or 100 μl lactose (1 M) at 6 h, 12 h, 24 h, 48 h, and 72 h in 5 ml of a 10 mM sodium phosphate buffer (pH 7.2)/*n*-octanol/DME mixture (25/25/50, v/v) containing 100 mM lactose for 144 h at 37 °C and 1400 rpm.

2.10. Quantitative determination

The quantitative analyses of lactose, glucose, galactose, and octyl-gal in the transgalactosylation reactions were performed by analytical thin layer chromatography (TLC; 20 × 20 cm, Silica gel 60 F_{254}, Merck, Darmstadt, Germany) using an eluent of acetonitrile, ethyl acetate, *n*-propanol, and water (85/20/20/15, v/v) [18]. Sugars and octyl-gal in the developed TLC plate were visualized by spraying with a solution containing 2.56 g/L 1-naphthol and 10% H_2SO_4 in ethanol. The spot intensity was quantified by densitometric scanning using ImageJ software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij), as previously described [19,20].

2.11. Kinetic analysis

The kinetic parameters (K_m, V_{max}) for the hydrolysis of lactose and octyl-gal by spore-displayed β-Gal were determined at 37 °C in 10 mM sodium phosphate buffer (pH 7.2) using 0.5–10 mM and 0.5–12 mM substrates, respectively. The hydrolysis of lactose was measured using a YSI 2700 SELECT Biochemistry Analyzer (YSI Inc., Yellow Springs, Ohio) and the hydrolysis of octyl-gal was measured by quantitative analysis of TLC spots. The apparent K_i value of octyl-gal was determined at different octyl-gal concentrations (0–20 mM). The apparent K_m and V_{max} values were obtained by fitting the data to the Hanes–Woolf equation, using SigmaPlot 8.0 software (SPSS, Richmond, CA). In addition, the apparent K_i value was estimated by fitting the data to the Lineweaver–Burk equation.

3. Results and discussion

3.1. Effect of organic solvents on the stability of spore-displayed β-Gal

As with the spore display of NADPH-cytochrome P450 oxidoreductase (P450, EC 1.14.14.1) [14], *E. coli* β-Gal was functionally expressed in *B. subtilis* DB104 cells and was displayed on the surface of spores after sporulation. The spores were harvested by autolysis of the host cells. After cultivation of DB104 for 24 h in DSM medium, the activity of the purified spore-displayed β-Gal was determined to be 1.7 U/mg spores. To investigate the stability of spore-displayed β-Gal in organic solvents, the spore biocatalyst was incubated with polar or non-polar organic solvents for 1 h at

Table 1
Effects of organic solvents on activity of the spore-displayed β-Gal.

Reaction medium (v/v)	β-Gal activity (U/mg spores)	Relative activity (%)	Phase system
Buffer (100)	1.73 ± 0.12[a]	100	One
Buffer/acetonitrile (5/95)	1.50 ± 0.05	87	One
Buffer/toluene (5/95)	1.66 ± 0.12	96	One
Buffer/1,2-dimethoxyethane (5/95)	2.66 ± 0.12	154	Two
Buffer/diethyl ether (5/95)	1.43 ± 0.09	83	Two
Buffer/cyclohexane (5/95)	2.01 ± 0.17	116	Two
Buffer/*n*-octanol (70/30)	1.42 ± 0.04	82	Two
Buffer/*n*-octanol/acetonitrile (20/30/50)	0.39 ± 0.06	23	Two
Buffer/*n*-octanol/toluene (20/30/50)	1.32 ± 0.10	76	Two
Buffer/*n*-octanol/1,2-dimethoxyethane (20/30/50)	1.95 ± 0.11	113	Two
Buffer/*n*-octanol/diethyl ether (20/30/50)	1.20 ± 0.12	69	Two
Buffer/*n*-octanol/cyclohexane (20/30/50)	1.17 ± 0.06	68	Two

The spore-displayed β-Gal (0.625 mg/ml) was incubated in 1 ml reaction mixtures for 1 h at 37 °C. The residual β-Gal activity of the spore biocatalyst was checked after three washes with 50 mM sodium phosphate buffer (pH 7.2) by centrifugation. Each reaction was performed in triplicate.

[a] The values are the mean standard deviation.

37 °C. Among the tested organic solvents, both DME and cyclohexane were highly effective in preserving enzyme stability, as shown in Table 1. To evaluate the feasibility of the transgalactosylation reaction for octyl-gal synthesis, the spore-displayed β-Gal was incubated with several organic solvents in the presence of *n*-octanol substrate. Although the spore-displayed β-Gal lost 18% of its activity in the presence of 30% (v/v) *n*-octanol, its activity was not diminished when supplemented with a DME co-solvent, thus forming a buffer/*n*-octanol/DME mixture (20/30/50, v/v). However, the stability was markedly decreased in the other mixtures. Accordingly, DME, which is miscible with both polar and non-polar solvents, was chosen as a co-solvent for octyl-gal synthesis.

In a previous report, most of the commercial β-Gals obtained from bacteria and fungi lost their hydrolytic activity in non-aqueous medium containing 1,4-dioxane, DMSO, DMF, or pyridine solvents [13]. *E. coli* free β-Gal lost about 50% of its activity when incubated with 50% (v/v) DME for 5 min [13]. In contrast, the spore-displayed β-Gal was found to be stable in several non-aqueous media with most of the polar and non-polar organic solvents (Table 1).

3.2. Effect of DME on octyl-gal yield

To determine the optimal DME concentration for octyl-gal synthesis, transgalactosylation reactions were performed in DME concentrations ranging from 30% to 60% (v/v) with two phase systems. As shown in the TLC data in Fig. 1A, octyl-gal and unidentified oligosaccharide spots were detected in the transgalactosylation reaction products using the spore-displayed β-Gal, in addition to the glucose and galactose that formed during the hydrolysis reaction. Quantitative analysis of the TLC spots showed that 4.2 mM octyl-gal was maximally synthesized from 20 mM lactose with 50% (v/v) DME for 12 h; the conversion was determined to be 20.9% (Fig. 1B). The efficiency of the transgalactosylation reaction, the molar ratio of [octyl-gal]/[glucose], increased to 0.14, 0.26, and 0.60 as the DME concentration was increased to 30%, 40%, and 50%, respectively (Fig. 1C). In contrast, it was found that octyl-gal synthe-

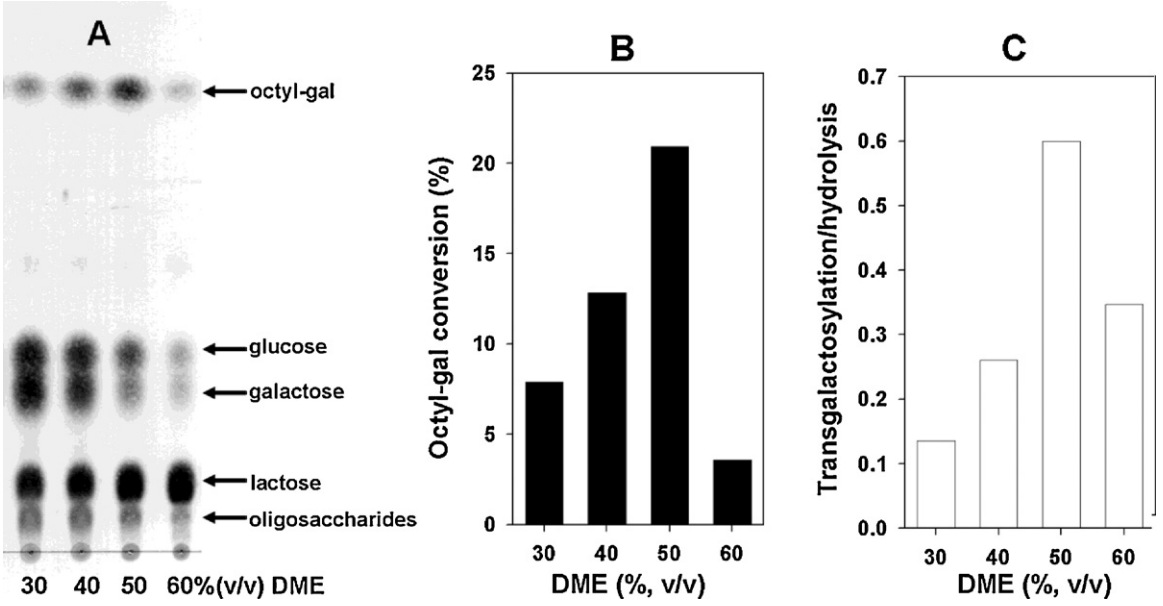

Fig. 1. Effect of DME concentration on octyl-gal yield. The transgalactosylation reactions by spore-displayed β-Gal (0.625 mg/ml) were conducted in mixtures containing 20 mM lactose and 30–60% (v/v) DME in 20% (v/v) *n*-octanol and 25% (v/v) sodium phosphate buffer (10 mM, pH 7.2) for 12 h at 37 °C and 700 rpm. (A) TLC analysis of octyl-gal, glucose, galactose, and oligosaccharides converted from lactose after 12 h of reaction. Spot intensities on the TLC plate were analyzed using ImageJ software. (B) Conversion ratio (%, mM/mM) of octyl-gal converted from lactose. (C) Ratio of [transgalactosylation product (octyl-gal)]/[hydrolysis product (glucose)] after 12 h of reaction.

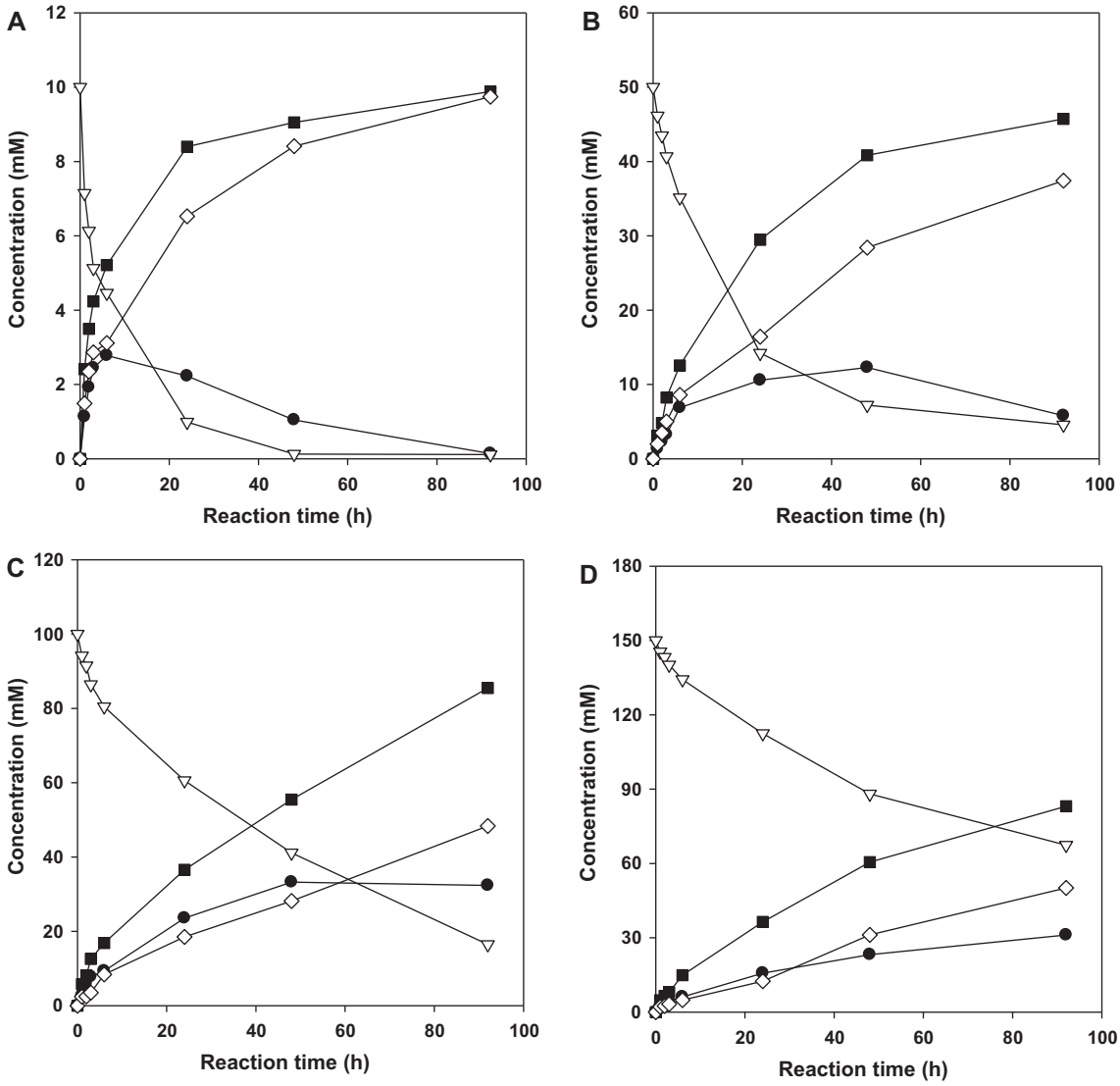

Fig. 2. Octyl-gal synthesis at different lactose concentrations by spore-displayed β-Gal in the presence of DME. Each reaction was conducted by the addition of spore-displayed β-Gal (1.25 mg/ml) in a 10 mM phosphate buffer (pH 7.2)/*n*-octanol/DME (25/25/50, v/v) mixture with initial lactose concentrations of (A) 10 mM, (B) 50 mM, (C) 100 mM, and (D) 150 mM at 37 °C and 700 rpm for 92 h. ●, octyl-gal; ▽, lactose; ■, glucose; and ◇, galactose.

sis drastically decreased to 0.7 mM at 60% (v/v) DME and the molar ratio decreased to 0.35. Indeed, the transgalactosylation reaction catalyzed by the spore-displayed β-Gal was critically affected by the concentration of DME that was used as a co-solvent.

As a spore β-Gal biocatalyst, we previously reported that CotG-fused spore-displayed β-Gal could localize at the water–solvent interface, which increased the phase-transfer catalytic activity and resulted in octyl-gal synthesis of 27.7 mM [10]. As an alternative approach for octyl-gal synthesis, DME co-solvent plays a key role in octyl-gal synthesis because the co-solvent has an amphiphilic property and improves the galactoside yield, as shown in the synthesis of short-chain alkyl galactosides [11]. In the present study, we found that the transgalactosylation activity of spore-displayed β-Gal significantly increased and hydrolytic activity diminished when 50% (v/v) DME was used, although the mixture still formed two separate phases.

3.3. *Effect of lactose concentration on octyl-gal yield*

Generally, in alkyl galactoside synthesis, galactoside yields are affected by lactose solubility in non-aqueous media. In our preliminary experiment, lactose was observed to be soluble up to 100 mM in a buffer/*n*-octanol/DME mixture (25/25/50, v/v). Thus, transgalactosylation reactions were performed at different lactose concentrations ranging from 10 to 150 mM; the data are shown in Fig. 2. When 100 mM lactose was added to the reaction medium, 33.2 mM octyl-gal was obtained after a reaction of 48 h and it was not hydrolyzed until 92 h. However, the lactose was steadily hydrolyzed into glucose and galactose until 92 h, indicating that the spore biocatalyst was still active. In the case of 150 mM lactose, although the octyl-gal concentration steadily increased during the reaction, the octyl-gal synthesis rate was not much faster than that at 100 mM lactose. Many more oligosaccharides were detected by TLC with the use of 150 mM lactose (Supplementary Material, Fig. S1), which might affect the octyl-gal yield.

It was reported that octyl-gal synthesized from a low lactose concentration of 10 mM was rapidly hydrolyzed after 20 h by biocatalysts using whole cells in water/*n*-octanol medium [9]. In contrast, our results show that little hydrolysis of the synthesized octyl-gal occurred at lactose concentrations over 50 mM. However, the octyl-gal synthesized from a low lactose concentration of 10 mM in a buffer/*n*-octanol/DME mixture (25/25/50, v/v) showed degradation patterns similar to those obtained with whole cells [9].

Table 2
Effect of spore-displayed β-Gal concentration and agitation speed on octyl-gal synthesis.

Spores (mg/ml)	Agitation speed (rpm)	Octyl-gal yield (mM)	Relative synthesis ratio (%)
0.625	1,400	12.8	44
1.25	1,400	18.5	64
1.875	1,400	28.7	100
1.875	700	24.5	85
1.875	0	16.6	58

The reactions, containing 100 mM lactose, 25% (v/v) *n*-octanol, 50% (v/v) DME, and 25% (v/v) sodium phosphate buffer (pH 7.2, 10 mM), were initiated by the addition of different spore-displayed β-Gal concentrations at each agitation speed. They were incubated at 37 °C for 20 h.

3.4. Effect of spore concentration and agitation speed on octyl-gal yield

To decrease the reaction time for octyl-gal synthesis, the spore-displayed β-Gal concentration and the agitation speed were increased. As shown in Table 2, the octyl-gal yield improved by about 2.2-fold and 1.7-fold for 20 h by increasing the spore biocatalyst concentration from 0.625 to 1.875 mg/ml at 1400 rpm and by mixing from 0 to 1400 rpm with 1.875 mg/ml spore-displayed β-Gal, respectively. Indeed, the transgalactosylation rate was increased by increasing the agitation speed and the spore-displayed β-Gal concentration in the reaction medium. Based on these results, the spore biocatalyst concentration and the agitation speed were set to 1.875 mg/ml and 1400 rpm in further experiments.

In addition, we tested the same reaction without agitation. Under this condition, most of the spores were observed to be localized at the interface of two separate phases when observed through a microscope; this is probably due to the spores' hydrophobic property [21,22]. In this case, the synthesized octyl-gal yield was about half of that obtained in the case of agitation at 1400 rpm.

3.5. Synthesis of octyl-gal by intermittent addition of spore-displayed β-Gal and/or lactose

In the above experiments, some of the reaction parameters were optimized to increase the octyl-gal yield. We investigated whether the addition of spore-displayed β-Gal and/or lactose into the reaction medium would increase the octyl-gal yield. In batch mode, 33.7 mM octyl-gal was obtained over 24 h and the reaction time decreased by about 2-fold by increasing the agitation speed by 2-fold and increasing the spore biocatalyst concentration by 1.5-fold (Fig. 3A). However, the increase in yield was not significant. When the concentrated spore-displayed β-Gal was intermittently added

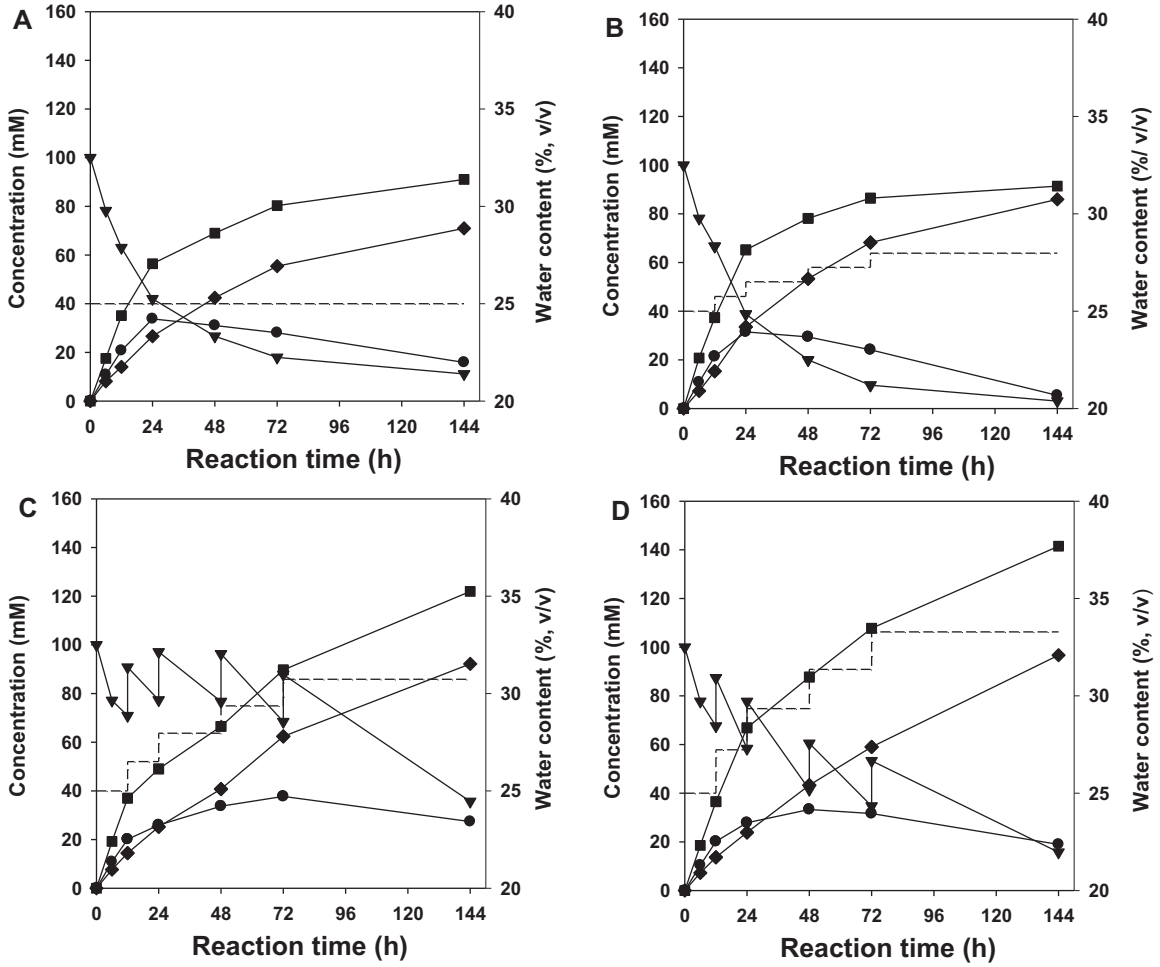

Fig. 3. Octyl-gal synthesis by spore-displayed β-Gal- and/or lactose-feeding in the presence of DME. One hundred microliters of lactose (1 M) and/or 50 μl spore-displayed β-Gal (6.25 mg/ml) were added at 6 h, 12 h, 24 h, 48 h, and 72 h to 5 ml of a 10 mM phosphate buffer (pH 7.2)/*n*-octanol/DME (25/25/50, v/v) mixture with an initial lactose concentration of 100 mM. The reactions were conducted at 37 °C and 1400 rpm for 144 h. (A) Time course of the concentrations of octyl-gal, lactose, glucose, and galactose in batch mode. (B) Time course of the concentrations of octyl-gal, lactose, glucose, and galactose in the spore-displayed β-Gal-feeding mode. (C) Time course of the concentrations of octyl-gal, lactose, glucose, and galactose in the lactose-feeding mode. (D) Time course of the concentrations of octyl-gal, lactose, glucose, and galactose in spore-displayed β-Gal- and lactose-feeding mode. ●, octyl-gal; ▼, lactose; ■, glucose; and ♦, galactose. The dashed lines indicate the water content (%, v/v) of each reaction medium.

Table 3
Comparison of biocatalytic systems for octyl-gal synthesis.

Biocatalyst	β-Gal source[a]	Reaction time	Molar conversion (%)	Initial lactose (mM)	Octyl-gal yield (mM)	Ref.
Lipid coating[b]	Eco	8 days	82	5	0.41	[7]
Reverse micelles[c]	Pca	5 days	45	4.5	2.025	[8]
Whole cells[d]	Bps	20 h	26	10	2.6	[9]
Spore display[e]	Eco	24 h	27.7	100	27.7	[10]
Spore display[f]	Eco	24 h	33.7	100	33.7	This study

[a] Eco, *Escherichia coli*; Pca, *Penicillium canescens*; Bps, *Bacillus pseudofirmus* AR-199.
[b] The conversion was expressed as a molar ratio of octyl-gal converted from 1 mM *n*-octanol in 2-propy ether solution (water phase:organic phase (1:1, v/v)). The initial lactose concentration and octyl-gal yield were calculated based on the whole reaction system in this study.
[c] The conversion was expressed as a molar ratio of octyl-gal converted from 4.5 mM lactose in the whole reaction system. The octyl-gal yield was calculated from the molar conversion and initial lactose concentration in this study.
[d] The conversion was a molar ratio of octyl-gal converted from 10 mM lactose in the whole reaction system. The initial lactose concentration and octyl-gal yield were expressed based on the whole reaction system.
[e] The conversion was a molar ratio of octyl-gal converted from 100 mM lactose in 10 mM phosphate buffer solution (water phase:organic phase (2:7, v/v)). The octyl-gal yield was expressed based on the water phase solution.
[f] The conversion was a molar ratio of octyl-gal converted from 100 mM lactose in the whole reaction system. Octyl-gal yield was measured based on the whole reaction system.

to the reaction medium after 12 h, the water content of 25% (v/v) increased to 28% (v/v) during the reaction, and much more galactose was formed by the hydrolysis of octyl-gal (Fig. 3B). However, the octyl-gal yield was not further increased, despite the step-wise addition of the concentrated spores. By intermittent addition of 1 M lactose, much more lactose was hydrolyzed (122 mM glucose was formed) than in the batch mode (91 mM glucose was formed) at the end of the reaction and the water content increased to 30.7% (v/v) (Fig. 3C). Lactose was transgalactosylated into 33.3 mM octyl-gal after 48 h and was maximally converted into 37.7 mM octyl-gal at 72 h. Thereafter, the lactose was only slightly hydrolyzed into 27.4 mM at 144 h. Although the reaction time was about twice as long as that of the batch mode, the octyl-gal yield was only slightly increased, by about 12%, by the lactose addition. In contrast, the addition of both lactose and the spore biocatalyst was not efficient, because the water content increased by much more, to 33.3% (v/v), at the end of the reaction (Fig. 3D). A yield of 33.3 mM octyl-gal was formed at 48 h and was then hydrolyzed into 18.9 mM after 144 h.

Comparison of the biocatalysts developed for octyl-gal synthesis in batch modes is shown in Table 3. Although several modified biocatalyst techniques involving lipid coating, reverse micelles and whole cells exhibited conversion higher than that of free enzymes in the course of octyl-gal synthesis, it was reported that the yield was very low (<∼3 mM) and a long reaction time was required. Recently, with a spore display technique that increased emulsification capacity, the yield was markedly increased [10]. It is also clear from this study that high octyl-gal yield is obtained by spore display of β-Gal in non-aqueous medium containing a 50% (v/v) DME co-solvent. However, in fed-batch modes, the product yield was only slightly increased by the addition of spores and/or supplementing lactose. First, the water content was increased in the fed-batch reaction mode. In previous studies, water content has been shown to be a critical reaction parameter for determining the transglycosylation/hydrolysis ratio [23–25]. Thus, it can be understood that the addition of lactose and/or spore-displayed β-Gal increased the water content, which might accelerate the hydrolysis of lactose and octyl-gal, which would then affect the product yield. In addition, further study is required to investigate whether octyl-gal acts as an inhibitor of β-Gal at high concentrations of octyl-gal, because octyl glucoside is known to competitively inhibit β-glycosidase, which is an inhibitor in the hydrolysis reaction [26–28].

3.6. Kinetic studies of spore-displayed β-Gal hydrolysis of octyl-gal

Kinetic studies of the hydrolysis of lactose and octyl-gal by spore-displayed β-Gal were investigated in aqueous media. In addition, the inhibition study of octyl-gal over spore-displayed β-Gal was also performed. The apparent V_{max} and K_m values of lactose hydrolysis by spore-displayed β-Gal were observed to be 0.18 μmol/min/mg spores and 0.63 mM, respectively (Supplementary Material, Fig. S3). The apparent V_{max} and K_m values for octyl-gal hydrolysis were 0.02 μmol/min/mg spores and 0.27 mM, respectively. Indeed, lactose hydrolysis was observed to be about 9-fold faster than octyl-gal hydrolysis in aqueous media. More importantly, octyl-gal hydrolysis limits the final yield and titer of the octyl-gal bioconversion process. As in the case that two human β-glucosidases showed competitive inhibition kinetics by alkyl glycosides [29], octyl-gal competitively inhibited spore-displayed β-Gal with a K_i value of 10.8 mM, indicating that the β-Gal activity of the spore biocatalyst is inhibited by a high octyl-gal concentration.

4. Conclusions

For octyl-gal synthesis, β-Gal was expressed in *B. subtilis* and was naturally displayed on the surfaces of spores during the sporulation phase. The spore-displayed β-Gal was stable in a mixture containing DME and *n*-octanol. In addition, DME increased the transgalactosylation efficiency and modifications of several reaction parameters were determined to increase the yield. The present results show that a high octyl-gal yield was obtained by resolving several transgalactosylation reaction barriers, such as enzyme stability, lactose hydrolysis, lactose solubility, and mass transfer, using spore display technology and an amphiphilic DME co-solvent. As alkyl glycosides are surfactants with good biodegradability and low toxicity, there have been considerable studies on its enzymatic synthesis including novel enzyme sources [30,31], reaction system [32,33] efficient glycosyl donor [34], and screening system [35]. We expect that spore display techniques will serve as novel immobilization techniques enabling bioconversion reaction in multi-phase organic solvents.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.enzmictec.2010.11.002.

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