



# Improved production of 2'-fucosyllactose in engineered *Escherichia coli* by expressing putative $\alpha$ -1,2-fucosyltransferase, WcfB from *Bacteroides fragilis*



Young-Wook Chin<sup>a</sup>, Ji-Yeong Kim<sup>b</sup>, Jae-Han Kim<sup>c</sup>, Sang-Min Jung<sup>b</sup>, Jin-Ho Seo<sup>b,\*</sup>

<sup>a</sup> Research Group of Gut Microbiome, Division of Nutrition and Metabolism Research, Korea Food Research Institute, Seongnam 13539, Republic of Korea

<sup>b</sup> Department of Agricultural Biotechnology and Center for Food and Bioconvergence, Seoul National University, Seoul 08826, Republic of Korea

<sup>c</sup> Department of Food and Nutrition, Chungnam National University, Daejeon 34134, Republic of Korea

## ARTICLE INFO

### Article history:

Received 19 September 2016

Received in revised form

30 November 2016

Accepted 30 November 2016

Available online 2 December 2016

### Keywords:

2'-Fucosyllactose

Engineered *Escherichia coli*

$\alpha$ -1,2-Fucosyltransferase

WcfB

## ABSTRACT

2'-Fucosyllactose (2-FL) is one of most abundant oligosaccharides in human milk, which is involved in many biological functions for infant health. Since 2-FL has a great potential in application to functional food materials and pharmaceuticals, several microbial systems for mass production of 2-FL have been developed in recent years. Microbial production of 2-FL was suggested to be influenced by a number of factors including fucosylation activity of  $\alpha$ -1,2-fucosyltransferase. In the present study, the *wcfB* gene coding for  $\alpha$ -1,2-fucosyltransferase from *Bacteroides fragilis* was screened from eleven candidates of putative  $\alpha$ -1,2-fucosyltransferase. Introduction of the *wcfB* gene allows the *lacZ*-deleted strain of *E. coli* expressing the genes for guanosine 5'-diphosphate (GDP)-L-fucose biosynthetic enzymes to produce 2-FL. As a result of fed-batch fermentation, 15.4 g/L extracellular concentration of 2-FL with 2-FL yield of 0.858 g/g lactose and productivity of 0.530 g/L/h were obtained. In addition, the feasibility of industrial production of 2-FL using this microbial system was demonstrated by performing fed-batch fermentation in a 75 L bioreactor.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

2'-Fucosyllactose (2-FL) is one of the most abundant oligosaccharides in human milk (Castanys-Muñoz et al., 2013). In addition to the nutritional importance in infant care, the nutraceutical and pharmaceutical potential of 2-FL necessitates the mass production through chemical or biological processes (Chin et al., 2013; Han et al., 2012). Among the factors governing the microbial production of 2-FL, the activity of  $\alpha$ -1,2-fucosyltransferase is crucial which catalyzes the final synthesis of 2-FL from GDP-L-fucose and lactose (Fig. 1).  $\alpha$ -1,2-Fucosyltransferase, belonging to the glycosyltransferase family 11 (<http://afmb.cnrsmrs.fr/CAZY/fam/acc.GT.html>) can be found in both eukaryotic and prokaryotic organisms. It transfers the L-fucose molecule from GDP-L-fucose to the galactose moiety of acceptor molecules such as N-acetyllactosamine, lacto-N-biose and lactose residues observed in oligosaccharides, glycoproteins, or glycolipids (Ma et al., 2006; Oriol et al., 1999).

Several putative bacterial  $\alpha$ -1,2-fucosyltransferases have been identified to date, including enzymes involved in colanic acid synthesis in *E. coli* K-12, *Salmonella enterica* LT2, in O-antigen synthesis in *Yersinia enterocolitica* O8 (Reeves et al., 2006), the WbsJ in the enteropathogenic *E. coli* O128 strain (Li et al., 2008; Shao et al., 2003), the WbgL in the enteropathogenic *E. coli* O126 (Engels and Elling, 2014), WbnK and WbwK of the pathogenic *E. coli* O86 (Yi et al., 2005) as well as WbiQ from *E. coli* O127 (Pettit et al., 2010) and FucT2 (or FutC) from *Helicobacter pylori* (Wang et al., 1999; Wang et al., 2002). Due to the vast knowledge of its functionality and the characteristics,  $\alpha$ -1,2-fucosyltransferase from *H. pylori* (FucT2) has been widely used for enzymatic- and microbial production of 2-FL (Albermann et al., 2001; Baumgärtner et al., 2013; Drouillard et al., 2006; Petschacher and Nidetzky, 2016). However, the low level of soluble FucT2 expression as well as its activity in recombinant *E. coli* clearly hinder the production of 2-FL, consequently reduce the overall productivity (Lee et al., 2012; Wang et al., 1999).

In our previous research, several attempts have been performed to improve the 2-FL production. To redirect the lactose flux from cell growth to 2-FL production, the endogenous *lac* operon in the host *E. coli* BL21star(DE3) was replaced with the modified *lac* operon bearing *lacZ* $\Delta$ M15 which alleviates the  $\beta$ -galactosidase activity

\* Corresponding author.

E-mail address: [jhseo94@snu.ac.kr](mailto:jhseo94@snu.ac.kr) (J.-H. Seo).

(Chin et al., 2015). FucT2 from *H. pylori* was engineered as well to enhance the solubility and activity by attachment of three aspartate molecules at the N-terminal of FucT2. As results, 2-FL production was increased up to 6.4 g/L in the fed-batch fermentation of the engineered *E. coli* (Chin et al., 2015), however, the 2-FL titer was still insufficient to produce 2-FL industrially.

In this study,  $\alpha$ -1,2-fucosyltransferases from various origins have been evaluated to overcome the critical point for the 2-FL production by the engineered *E. coli* expressing the genes for the *de novo* biosynthesis of GDP-L-fucose. In addition,  $\beta$ -galactosidase activity of the host *E. coli* strain was completely eliminated to enhance 2-FL production further. Finally, fed-batch fermentation was carried out in a laboratory scale as well as a pilot scale bioreactor to assess the feasibility of industrial production of 2-FL.

## 2. Materials and methods

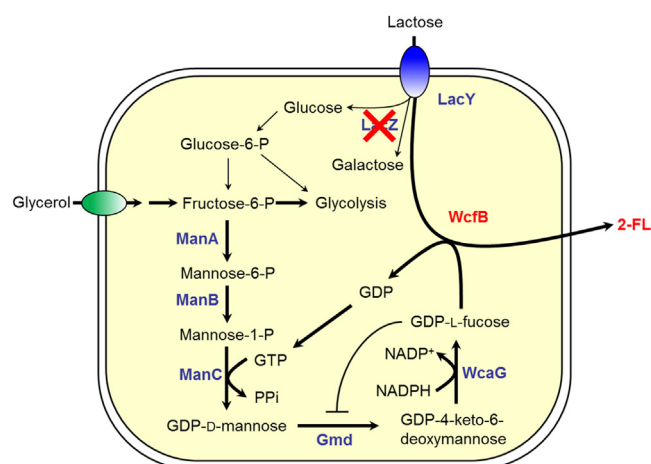
### 2.1. Strains and plasmids

All *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* TOP10 and *E. coli* BL21star(DE3) (Invitrogen, Carlsbad, CA, USA) were used for construction of plasmids and a host strain for production of 2-FL, respectively. Plasmid pBCGW was constructed previously for overexpression of the genes encoding GDP-L-fucose biosynthetic enzymes (ManB, ManC, Gmd and WcaG) (Lee et al., 2009).

The eleven candidate genes coding for  $\alpha$ -1,2-fucosyltransferases were selected by comparing amino acid sequences with FucT2 from *H. pylori* at the carbohydrate-active enzymes database (CAZy) site. The DNA fragments encoding  $\alpha$ -1,2-fucosyltransferases were amplified by PCR from the genomic DNAs of the appropriate sources (Table 1). The PCR products were digested with *Nde*I and *Kpn*I and ligated into plasmid pCOLADuet-1 digested by the same restriction enzymes. All constructs were verified by restriction enzyme digestion and DNA sequencing.

### 2.2. Culture conditions

Batch fermentations were done in a 500 mL baffled flask (Nalgene) containing 100 mL of defined medium [13.5 g/L  $\text{KH}_2\text{PO}_4$ , 4.0 g/L  $(\text{NH}_4)_2\text{HPO}_4$ , 1.7 g/L citric acid, 1.4 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mL trace element solution (10 g/L Fe(III) citrate, 2.25 g/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.35 g/L  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.23 g/L  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 0.11 g/L  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , 2.0 g/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , pH 6.8] with appropriate antibiotics (ampicillin 50  $\mu\text{g}/\text{mL}$  and kanamycin 50  $\mu\text{g}/\text{mL}$ ) at 25 °C. The agitation speed was maintained at 250 rpm. When optical density ( $\text{OD}_{600}$ ) reached 0.8, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and lactose were added at a final concentration of 0.1 mM and 20 g/L, respectively. Fed-batch fermentation was carried out in a 2.5 L bioreactor (Kobitech, Incheon, Korea) containing 1.0 L of defined medium containing 20 g/L glycerol and appropriate antibiotics at 25 °C. After complete utilization of glycerol added initially, feeding solution containing 800 g/L glycerol and 20 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  was fed by a pH-stat mode. At the same time, IPTG and lactose were also added to a final concentration of 0.1 mM and 20 g/L for induction of the T7 promoter-mediated gene expression and for production of 2-FL. For pH-stat feeding, the feeding solution was fed automatically into the bioreactor when the pH rose to a value higher than its set-point due to the depletion of glycerol. The pH of medium was controlled at 6.8 by addition of 28%  $\text{NH}_4\text{OH}$ . Agitation speed elevated up to 1200 rpm for prevention of the deficiency of dissolved oxygen, and air flow rate was maintained at 2 vvm during the fermentation.



**Fig. 1.** Strategy for production of 2-FL in engineered *E. coli*. The names of enzymes are abbreviated as follows; LacY, lactose permease; LacZ,  $\beta$ -galactosidase; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyl-transferase; Gmd, GDP-D-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase; WcfB,  $\alpha$ -1,2-fucosyltransferase. PPi and GTP denote diphosphate and guanosine 5'-triphosphate.

### 2.3. Measurement of concentrations of cell and extracellular metabolites

Dry cell weight (DCW) was determined using optical density and a predetermined conversion factor (0.36). Optical density was measured at 600 nm absorbance using a spectrophotometer (Ultraspac 2000, Amersham Pharmacia Biotech, USA) after the samples were diluted to keep optical density between 0.1 and 0.5. Extracellular concentrations of 2-FL, lactose, glycerol, galactose and acetic acid were measured by a high performance liquid chromatography (HPLC) (Agilent 1100LC, USA) equipped with the Carbohydrate Analysis column (Rezex ROA-organic acid, Phenomenex, USA) and refractive index (RI) detector. The column heated at 60 °C was applied to analyze 20  $\mu\text{L}$  of diluted culture broth. Five millimoles of  $\text{H}_2\text{SO}_4$  solution was used as a mobile phase at a flow rate of 0.6 mL/min.

### 2.4. Identification of 2-FL produced by engineered *E. coli* expressing WcfB

2-FL was readily purified and enriched by solid-phase extraction (SPE) using a porous graphitized carbon cartridge prior to mass spectrometry analysis. Enriched 2-FL was analyzed using a Bruker ultrafleXtreme MALDI-TOF/TOF system with 1000 Hz Smart beam II laser. 2,5-Dihydroxy-benzoic acid (DHB) was used as a matrix (5 mg/100 mL in 50% ACN:H<sub>2</sub>O) and 0.01 M NaCl was added as a cation dopant to increase signal sensitivity. The sample was spotted on a stainless steel target plate, followed by the NaCl dopant and matrix. The spot was dried under vacuum prior to mass spectrometric analysis. To confirm the structure of 2-FL, MALDI-TOF/TOF MS via collision-induced dissociation (CID) was performed. Tandem MS spectra were gained at 1 keV collision energy with argon gas.

## 3. Results

### 3.1. Exploration of putative $\alpha$ -1,2-fucosyltransferase for producing 2-FL

In spite of previous attempts to increase the expression level and activity of FucT2 from *H. pylori*, yet the fucose transfer was suggested to be the rate limiting for microbial production of 2-FL. To

**Table 1**  
List of strains and plasmids used in this study.

Strains/Plasmids	Relevant description	Reference
<b>Strains</b>		
ΔL M15	ΔL <i>Tn7:lacZΔM15YA</i> ( <i>lacZ</i> partially disrupted)	Chin et al. (2015)
ΔL YA	ΔL <i>Tn7:lacYA</i> ( <i>lacZ</i> completely deleted)	Chin et al. (2016)
ΔL M15 BCGW-F	ΔL M15 harboring pBCGW ( <i>manB</i> , <i>manC</i> , <i>gmd</i> , <i>wcaG</i> ) and pF ( <i>fucT2</i> )	Chin et al. (2015)
ΔL M15 BCGW-W	ΔL M15 harboring pBCGW ( <i>manB</i> , <i>manC</i> , <i>gmd</i> , <i>wcaG</i> ) and pBFW ( <i>wcfB</i> )	This study
ΔL YA BCGW-W	ΔL YA harboring pBCGW ( <i>manB</i> , <i>manC</i> , <i>gmd</i> , <i>wcaG</i> ) and pBFW ( <i>wcfB</i> )	This study
<b>Plasmids</b>		
pETDuet-1	Two T7 promoters, pBR322 replicon, Amp <sup>R</sup>	Novagen
pCOLADuet-1	Two T7 promoters, ColA replicon, Kan <sup>R</sup>	Novagen
pBCGW	pETDuet-1 + <i>manC-manB</i> ( <i>NcoI/SacI</i> ) + <i>gmd-wcaG</i> ( <i>NdeI/KpnI</i> )	Lee et al. (2009)
pBFW	pCOLADuet-1 + <i>wcfW</i> (CAH07600.1) from <i>Bacteroides fragilis</i>	This study
pBFB	pCOLADuet-1 + <i>wcfB</i> (CAH06753.1) from <i>Bacteroides fragilis</i>	This study
pECJ	pCOLADuet-1 + <i>wbsJ</i> (AAO37698.1) from <i>Escherichia coli</i> O128: B12	This study
pRPS	pCOLADuet-1 + SPO3391 (AAV96618.1) from <i>Ruegeria pomeroyi</i> DSS-3	This study
pPGP	pCOLADuet-1 + PGA1_c33070 (AF092946.1) from <i>Phaeobacter gallaenciensis</i> DSM 17395	This study
pPHP	pCOLADuet-1 + Phep_1971 (ACU04179.1) from <i>Pedobacter heparinus</i> DSM 2366	This study
pPSP	pCOLADuet-1 + Pedsa_2797 (ADY53338.1) from <i>Pedobacter saltans</i> DSM 12145	This study
pLLL	pCOLADuet-1 + limg_2349 (CAL98913.1) from <i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363	This study
pDFFD	pCOLADuet-1 + Dfer_0178 (ACT91449.1) from <i>Dyadobacter fermentans</i> DSM 18053	This study
pEFH	pCOLADuet-1 + HMPREF0351_11954 (AFK59578.1) from <i>Enterococcus faecium</i> DO	This study
pHSF	pCOLADuet-1 + Fut2;Sec2 (AAC24453.1) from <i>Homo sapiens</i>	This study

overcome the barrier, eleven of putative  $\alpha$ -1,2-fucosyltransferases were cloned from various origins and expressed in *E. coli* ΔL M15 BCGW which harbors a set of genes for the *de novo* synthesis for GDP-L-fucose (*manB*, *manC*, *gmd* and *wcaG*). The eleven candidates were *wcfW* and *wcfB* from *B. fragilis* NCTC9343, *wbsJ* from *E. coli* O128:B12, SPO3391 from *Ruegeria pomeroyi* DSS-3, PGA1\_c33070 from *Phaeobacter gallaenciensis* DSM 17395, Phep\_1971 from *Pedobacter heparinus* DSM 2366, Pedsa\_2797 from *P. saltans* DSM 12145, limg\_2349 from *Lactococcus lactis* subsp. *cremoris* MG1363, Dfer\_0178 from *Dyadobacter fermentans* DSM 18053, HMPREF0351\_11954 from *Enterococcus faecium* DO, Fut2 from *Homo sapiens*. As a result of flask cultures in LB media with 10 g/L lactose and HPLC analysis, a peak matching with the retention time of 2-FL was detected only in the culture of the strain expressing *wcfB* from *B. fragilis* among the candidate  $\alpha$ -1,2-fucosyltransferase genes tested. Although the unknown peaks were also observed in the cultures of the strains expressing PGA1\_c33070 from *P. gallaenciensis* and Phep\_1971 from *P. heparinus*, the retention time of the peaks were different from 2-FL (data not shown). In the case of the cultures of the strains expressing the other genes, a peak was not observed. It might be due to problems with gene expression or the level of 2-FL concentration might be below the detection limit.

### 3.2. Identification of 2-FL produced by engineered *E. coli* expressing *WcfB*

To verify production of 2-FL in the culture of engineered *E. coli* ΔL M15 BCGW-W expressing *wcfB* from *B. fragilis*, a subsequent analysis was performed by MALDI-TOF MS. As shown in Fig. 2a, the ions at 511.16 corresponding to 2-FL ([Hex<sub>2</sub> + Fuc<sub>2</sub> + Na]<sup>+</sup>) were observed as a major oligosaccharide in the culture medium. Hexose series consisting of the degree of polymerization 6–9 were also present as minor oligomers of 2-FL production. Tandem mass spectrometry (MS/MS) using collision-induced dissociation (CID) was further performed to confirm the composition and to elucidate the structure of 2-FL. The representative MS/MS spectrum with the ion at *m/z* 511.16 was shown in Fig. 2b. CID yields the loss of a fucose at *m/z* 511.16, followed by the loss of a second fucose at *m/z* 365.016, and the loss of a hexose at *m/z* 203.032. Indeed, it was clearly verified the presence of 2-FL produced by engineered *E. coli* expressing *WcfB*. Interestingly, di-fucosyllactose was not detected in the cul-

ture of engineered *E. coli* expressing *WcfB* contrary to the case of engineered *E. coli* expressing *FucT2* from *H. pylori* (Fig. 2c and d).

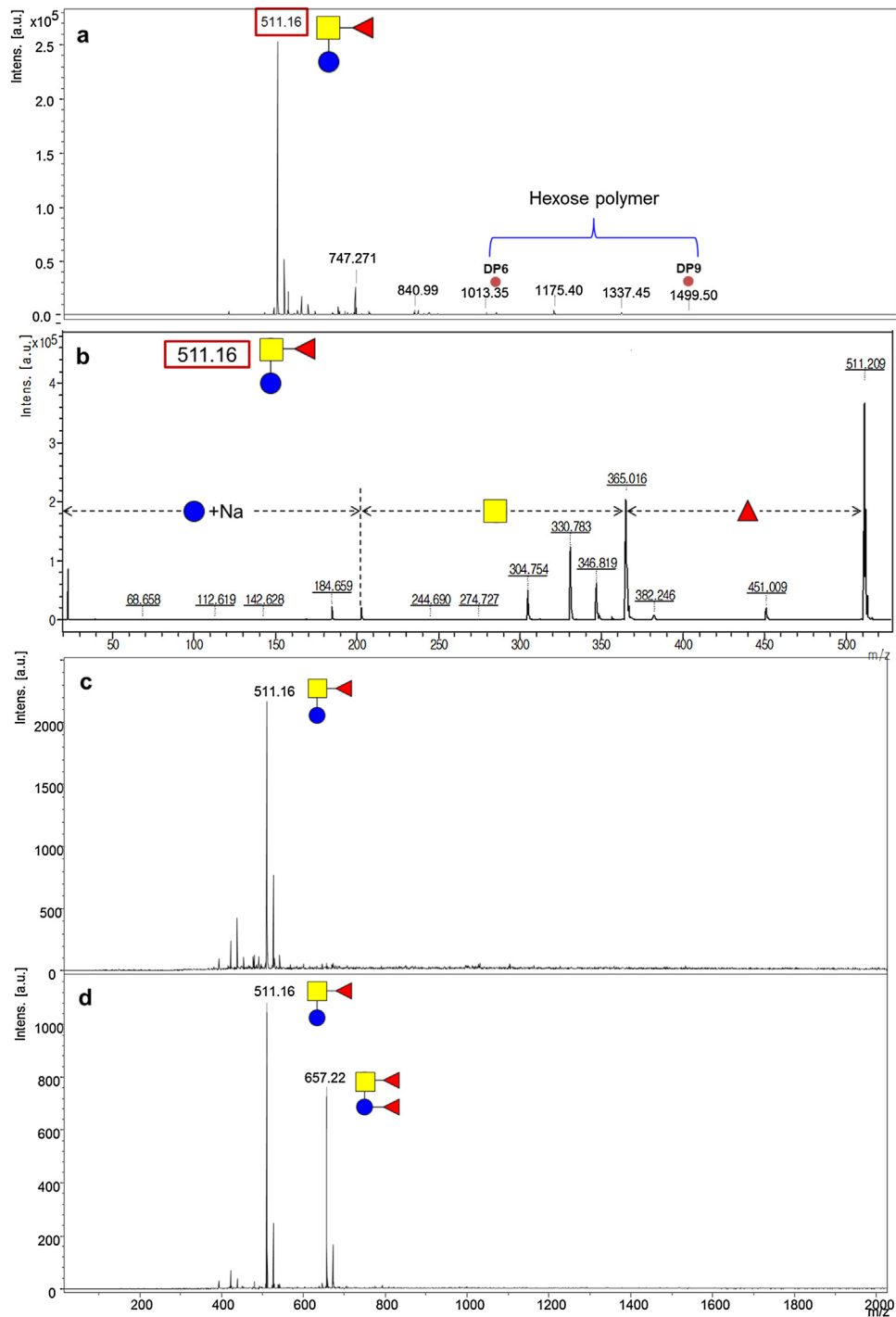
### 3.3. Effects of replacement of *FucT2* with *WcfB* on 2-FL production

After the confirmation of 2-FL production in *E. coli* expressing *WcfB*, flask cultures of the engineered *E. coli* ΔL M15 expressing *FucT2* and the engineered *E. coli* ΔL M15 expressing *WcfB* were performed in defined medium containing 20 g/L glycerol and 10 g/L lactose for comparing the 2-FL production performance. As a result, 0.85 g/L of 2-FL was produced in the culture of the engineered *E. coli* expressing *WcfB* (Fig. 3b). This value was 10 fold higher than that of the engineered *E. coli* expressing *FucT2* (Fig. 3a, b and Table 2). The 2-FL production performance of the engineered *E. coli* ΔL M15 BCGW-W was also assessed in fed-batch fermentation in a 2.5 L bioreactor under the same condition as done for previous research (Chin et al., 2015). By consumption of glycerol, cell mass reached up to 56.2 g/L of final cell concentration. As a result, 10.3 g/L of 2-FL with a yield of 0.53 g 2-FL/g lactose and productivity of 0.255 g/L/h were obtained at the end of fed-batch fermentation (Fig. 4a and Table 3). These values were 4.0, 8.4 and 5.9 fold higher than those of the ΔL M15 BCGW-F.

### 3.4. Host strain improvement: complete deletion of *lacZ*

Ideally, carbon flux toward cell growth and GDP-L-fucose synthesis is provided from the glycerol supplementation, thus a theoretical maximum yield of 2-FL from lactose is predicted to be 1.0 mol 2-FL/mole lactose. However, the experimental yield of 2-FL from the culture of ΔL M15 BCGW-W was only about 37.1% of the theoretical yield. This result indicates that more than 60% of the lactose consumed was used for other purposes such as cell growth and by-products formation. Instead of *LacZ*-attenuation, the *lacZ*-deleted mutant ΔL YA was employed to improve the 2-FL yield from lactose. As a result, *E. coli* ΔL YA BCGW-W (*lacZ*-deleted mutant) produced 1.33 g/L of 2-FL in batch fermentation with a yield of 0.83 mol 2-FL/mole lactose, which are 1.6- and 2.8 times higher than those of the ΔL M15 BCGW-W (*lacZ*-attenuated mutant) strain (Fig. 3b, c and Table 2).

Fed-batch fermentation of *E. coli* ΔL YA BCGW-W was also performed to increase the final concentration of 2-FL (Fig. 4b). The growth pattern and the final dry cell weight of *E. coli* ΔL YA BCGW-W were similar to that of the ΔL M15 BCGW-W. After addition



**Fig. 2.** Representative MS spectrum of 2-FL production from  $\Delta$ L YA BCGW-W identified by MALDI-TOF MS (a), CID tandem MS spectrum of mono-FL ( $[\text{Hex}_2 + \text{Fuc}_1 + \text{Na}]^+$ ) at  $m/z$  511.164 (b), Purified fucosyllactoses from engineered *E. coli* expressing WcfB (c) and expressing FucT2 (d). Symbols for the types of monosaccharides (glucose,  $\bullet$ ; galactose,  $\square$ ; fucose,  $\blacktriangle$ ).

**Table 2**

Comparison of results of batch fermentations of the engineered *E. coli* strains.

Strains	Maximum dry cell weight (g/L)	Maximum 2-FL concentration <sup>a</sup> (g/L)	Yield (mole 2-FL/mole lactose)
$\Delta$ L M15 BCGW-F	5.73 $\pm$ 0.09	0.08 $\pm$ 0.012	0.040 $\pm$ 0.003
$\Delta$ L M15 BCGW-W	6.00 $\pm$ 0.13	0.85 $\pm$ 0.015	0.296 $\pm$ 0.003
$\Delta$ L YA BCGW-W	5.90 $\pm$ 0.28	1.33 $\pm$ 0.022	0.825 $\pm$ 0.012

The values in the table are averages determined from three independent experiments and standard deviations are shown.

<sup>a</sup> Extracellular concentrations of lactose and 2-FL were determined and used for the calculation of lactose consumption and 2-FL production.

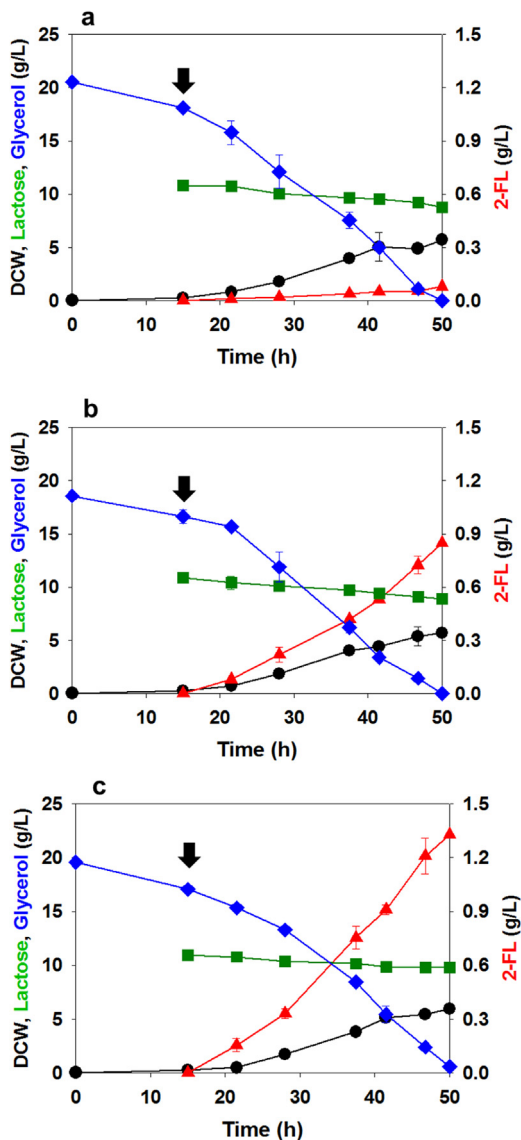
**Table 3**  
Comparison of results of fed-batch fermentations of the engineered *E. coli* strains.

Strains	Maximum dry cell weight (g/L)	Maximum 2-FL concentration <sup>a</sup> (g/L)	Yield (g 2-FL/g lactose)	Productivity <sup>b</sup> (g/L/h)
$\Delta$ L M15 BCGW-F <sup>c</sup>	73.1	2.6	0.063	0.043
$\Delta$ L M15 BCGW-W	56.2	10.3	0.530	0.255
$\Delta$ L YA BCGW-W	57.6	15.4	0.858	0.530
$\Delta$ L YA BCGW-W (75 L)	46.5	12.3	0.591	0.270

<sup>a</sup> Extracellular concentrations of lactose and 2-FL were determined and used for the calculation of lactose consumption and 2-FL production.

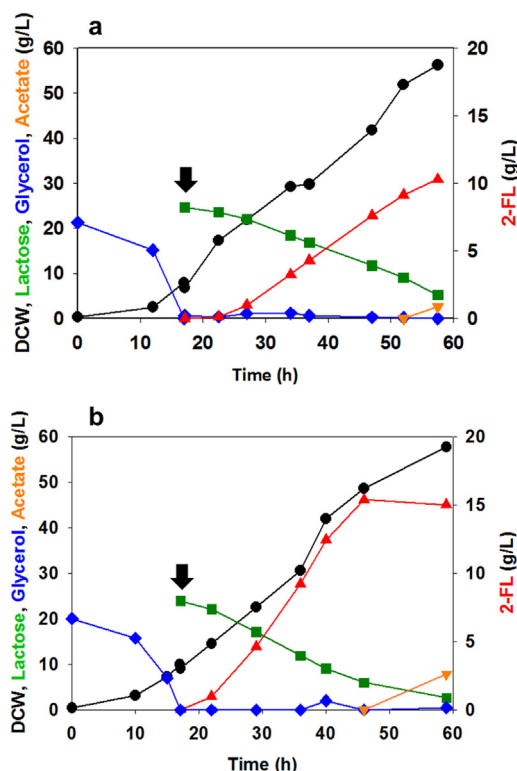
<sup>b</sup> 2-FL productivity was estimated during the 2-FL production period after IPTG induction and lactose dumping.

<sup>c</sup> The result of fed-batch fermentation of engineered *E. coli*  $\Delta$ L M15 expressing *manB*, *manC*, *gmd*, *wcaG* and *fucT2* (from *H. pylori*) was cited in Chin et al. (2015).



**Fig. 3.** Effects of complete deletion of *lacZ* on 2-FL production in batch fermentations of (a)  $\Delta$ L M15 BCGW-F, (b)  $\Delta$ L M15 BCGW-W and (c)  $\Delta$ L YA BCGW-W. When optical density ( $OD_{600}$ ) reached 0.8, IPTG and lactose was added to a final concentration 0.1 mM and 10 g/L, respectively (arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆.

of lactose and IPTG, 2-FL concentration increased sharply up to 15.4 g/L in 46 h culture. The 2-FL yield from lactose and productivity were 0.60 mol/mole lactose and 0.530 g/L/h, which were 1.6-

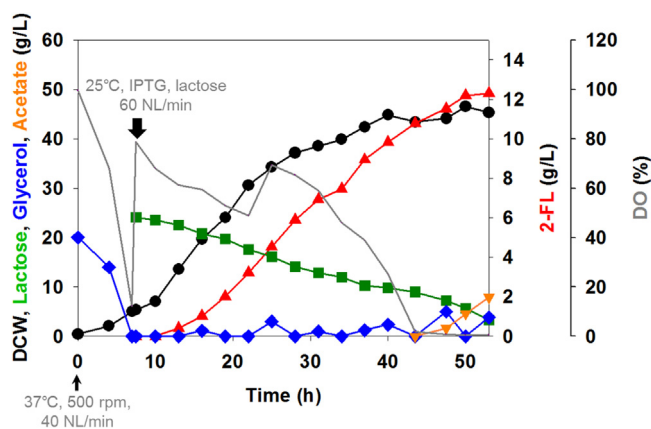


**Fig. 4.** Comparison of 2-FL production of engineered *E. coli*  $\Delta$ L M15 BCGW-W (a) and  $\Delta$ L YA BCGW-W (b) in fed-batch fermentation. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; acetate, ▽.

and 2.1 folds higher than those from the  $\Delta$ L M15 BCGW-F strain, respectively (Table 3).

### 3.5. Scale up: pilot scale production of 2-FL

Fed-batch fermentation of *E. coli*  $\Delta$ L YA BCGW-W was performed in a 75 L bioreactor in order to demonstrate the feasibility of mass production of 2-FL. The key objective was to reproduce 2-FL titer, productivity and yield as obtained in a laboratory scale bioreactor (2.5 L). The fermentation conditions such as media components, inoculum size, feeding strategy and pH were the same as those of the laboratory scale bioreactor. However, a temperature of the main culture before IPTG induction was changed to 37 °C because the cells did not grow well at 25 °C (data not shown). The agitation speed and aeration were controlled manually because the dissolved oxygen (DO) is dependent on cell density. As shown in Fig. 5, the DO value dropped rapidly from 100% to 9.2% during the batch period



**Fig. 5.** Fed-batch fermentation of engineered *E. coli*  $\Delta$ L YA BCGW-W done in pilot scale bioreactor. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). In addition, temperature was shifted from 37 °C to 25 °C. Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; acetate, ▼; dissolved oxygen (DO), —.

(0–8 h) as dry cell weight increased from 0.4 g/L to 5.2 g/L. After depletion of initial 20 g/L of glycerol, the culture temperature was changed to 25 °C and the DO value was restored to 73%. Finally, 12.3 g/L of 2-FL concentration was achieved with a yield of 0.591 g 2-FL/g lactose at the end of fed-batch fermentation. Compared to 2.5 L scale fermentation, 80% of the final concentration of 2-FL was obtained in the fermentation done in a 75 L bioreactor.

#### 4. Discussion

Through a screening process, putative  $\alpha$ -1,2-fucosyltransferase of *B. fragilis*, WcfB was selected to replace FucT2 from *H. pylori*. The *wcfB* gene which is one of the extracellular polysaccharide biosynthesis genes in *B. fragilis* has been predicted as a gene coding for glycosyltransferase, especially fucosyltransferase due to the identity and similarity to  $\alpha$ -1,2-fucosyltransferase in *Yersinia enterocolitica* O8 and *Homo sapiens* (Comstock et al., 1999). By the expression of WcfB in replacement of FucT2, 2-FL concentration and yield were improved by 4.0- and 8.4-folds in fed-batch fermentation (Table 3). It is assumed that the *in vivo* fucosylation activity of WcfB is much higher than that of FucT2 in the engineered *E. coli*. It is noteworthy that WcfB does not catalyze the biosynthesis of difucosyllactose contrary to FucT2 (Fig. 2c and d). It might be due to the difference in enzyme specificity between FucT2 and WcfB. This feature is a great advantage in commercial production of 2-FL since purification is one of the most costly steps in microbial production of value-added materials (Sauer et al., 2008).

It has been reported that 170 mg/L of maximum GDP-L-fucose concentration was obtained in the fed-batch fermentation of the engineered *E. coli* expressing the GDP-L-fucose biosynthetic genes (*manB*, *manC*, *gmd*, *wcaG*) without expressing  $\alpha$ -1,2-fucosyltransferase (Lee et al., 2009). This value is corresponded to only 141 mg/L 2-FL since 1 mol of GDP-L-fucose is needed to produce 1 mol of 2-FL. However, 15.4 g/L 2-FL (corresponded to 18.5 g/L GDP-L-fucose) was produced by expressing WcfB in this study. It was reported that GDP-L-fucose is an intracellular metabolite and causes feedback inhibition to the Gmd enzyme because GDP-L-fucose acts as a competitive inhibitor of GDP-D-mannose (Somoza et al., 2000; Sturla et al., 1997). Thus, achievement of 15.4 g/L 2-FL could be due to alleviation of the feedback inhibition by shifting GDP-L-fucose through the biosynthesis of 2-FL.

Interestingly, the protein band corresponding to the predicted size of WcfB was not observed in SDS-PAGE analysis and no fucosylation activity was detected in an *in vitro* fucosylation assay (data

not shown). This result is consistent with the previous research which reported that the purified WcfB enzyme did not accept lactose as a substrate at all in an *in vitro* fucosylation activity assay (Albermann et al., 2001). Therefore, the WcfB enzyme might be considered to be active only in the cell.

The fermentation performance in a 75 bioreactor is inferior to that in a 2.5 L bioreactor, which is an inherent problem associated with scale-up of fermentation processes. Especially, the 2-FL yield based on lactose consumed decreased to 0.591 g 2-FL/g lactose in a 75 L bioreactor from 0.858 g 2-FL/g lactose in a 2.5 L bioreactor (Table 3). More research will be necessary to reproduce the fermentation performance in a large-scale fermentation system. 2-FL concentration, yield and productivity were enhanced further by 50%, 62% and 100% through the complete deletion of *lacZ*. This results is consistent with our previous research done using the engineered *E. coli* strain able to produce 2-FL from fucose, lactose and glycerol via the *salvage* GDP-L-fucose biosynthetic pathway (Chin et al., 2016). It might be due to a shift of the lactose flux from cell growth to 2-FL production. Although  $\beta$ -galactosidase activity was completely eliminated, the percent yield (experimental yield/theoretical yield) was 60%. This result suggests that approximately 40% lactose consumed might be used for by-products formation. Characterization and engineering of WcfB as well as eliminating by-products formation would lead to an improvement of 2-FL titer and yield further.

#### Acknowledgements

This work was supported by The Advanced Biomass R&D Center (ABC) of Global Frontier Project (2011-0031359) funded by the Ministry of Science, ICT and Future Planning and Danish Research Council (0652-20140008).

#### References

- Albermann, C., Piepersberg, W., Wehmeier, U., 2001. Synthesis of the milk oligosaccharide 2'-fucosyllactose using recombinant bacterial enzymes. *Carbohydr. Res.* 334, 97–103.
- Baumgärtner, F., Seitz, L., Sprenger, G.A., Albermann, C., 2013. Construction of *Escherichia coli* strains with chromosomally integrated expression cassettes for the synthesis of 2'-fucosyllactose. *Microb. Cell Fact.* 12, 40.
- Castany-Muñoz, E., Martin, M.J., Prieto, P.A., 2013. 2'-Fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. *Nutr. Rev.* 71 (12), 773–789.
- Chin, Y.W., Park, J.B., Park, Y.C., Kim, K.H., Seo, J.H., 2013. Metabolic engineering of *Corynebacterium glutamicum* to produce GDP-L-fucose from glucose and mannose. *Bioprocess Biosyst. Eng.* 36 (6), 749–756.
- Chin, Y.W., Kim, J.Y., Lee, W.H., Seo, J.H., 2015. Enhanced production of 2'-fucosyllactose in engineered *Escherichia coli* BL21star (DE3) by modulation of lactose metabolism and fucosyltransferase. *J. Biotechnol.* 210, 107–115.
- Chin, Y.W., Seo, N., Kim, J.H., Seo, J.H., 2016. Metabolic engineering of *Escherichia coli* to produce 2'-fucosyllactose via Salvage pathway of guanosine 5'-diphosphate (GDP)-L-fucose. *Biotechnol. Bioeng.* 113 (11), 2443–2452.
- Comstock, L.E., Coyne, M.J., Tzianabos, A.O., Pantosti, A., Onderdonk, A.B., Kasper, D.L., 1999. Analysis of a capsular polysaccharide biosynthesis locus of *Bacteroides fragilis*. *Infect. Immun.* 67, 3525–3532.
- Drouillard, S., Driguez, H., Samain, E., 2006. Large-scale synthesis of H-antigen oligosaccharides by expressing *Helicobacter pylori*  $\alpha$ -1,2-fucosyltransferase in metabolically engineered *Escherichia coli* cells. *Angew. Chem.* 118, 1810–1812.
- Engels, L., Elling, L., 2014. Wbgl: a novel bacterial  $\alpha$ 1,2-fucosyltransferase for the synthesis of 2'-fucosyllactose. *Glycobiology* 24, 170–178.
- Han, N.S., Kim, T.J., Park, Y.C., Kim, J., Seo, J.H., 2012. Biotechnological production of human milk oligosaccharides. *Biotechnol. Adv.* 30, 1268–1278.
- Lee, W.H., Han, N.S., Park, Y.C., Seo, J.H., 2009. Modulation of guanosine 5'-diphosphate-d-mannose metabolism in recombinant *Escherichia coli* for production of guanosine 5'-diphosphate-L-fucose. *Bioresour. Technol.* 100, 6143–6148.
- Lee, W.H., Pathanibul, P., Quarterman, J., Jo, J.H., Han, N.S., Miller, M.J., Jin, Y.S., Seo, J.H., 2012. Whole cell biosynthesis of a functional oligosaccharide, 2'-fucosyllactose, using engineered *Escherichia coli*. *Microb. Cell Fact.* 11, 48.
- Li, M., Shen, J., Liu, X., Shao, J., Yi, W., Chow, C.S., Wang, P.G., 2008. Identification of a new  $\alpha$ 1,2-fucosyltransferase involved in O-antigen biosynthesis of *Escherichia coli* O86: B7 and formation of H-type 3 blood group antigen. *Biochemistry* 47, 11590–11597.

- Ma, B., Simala-Grant, J., Taylor, D., 2006. Fucosylation in prokaryotes and eukaryotes. *Glycobiology* 16, 158R.
- Oriol, R., Mollicone, R., Cailleau, A., Balanzino, L., Breton, C., 1999. Divergent evolution of fucosyltransferase genes from vertebrates, invertebrates, and bacteria. *Glycobiology* 9, 323–334.
- Petschacher, B., Nidetzky, B., 2016. Biotechnological production of fucosylated human milk oligosaccharides: prokaryotic fucosyltransferases and their use in biocatalytic cascades or whole cell conversion systems. *J. Biotechnol.* 235, 61–83.
- Pettit, N., Styslinger, T., Mei, Z., Han, W., Zhao, G., Wang, P.G., 2010. Characterization of WbiQ: an  $\alpha$  1,2-fucosyltransferase from *Escherichia coli* O127: K63 (B8) and synthesis of H-type 3 blood group antigen. *Biochem. Biophys. Res. Commun.* 402, 190–195.
- Reeves, P.R., Hobbs, M., Valvano, M., Skurnik, M., Whitfield, C., Coplin, D., Kido, N., Klena, J., Maskell, D., Raetz, C., 2006. Application of a New Nomenclature for Bacterial Polysaccharide Genes. [http://sydney.edu.au/science/molecular\\_bioscience/BPGD/big\\_paper.pdf](http://sydney.edu.au/science/molecular_bioscience/BPGD/big_paper.pdf).
- Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. *Trends Biotechnol.* 26, 100–108.
- Shao, J., Li, M., Jia, Q., Lu, Y., Wang, P.G., 2003. Sequence of *Escherichia coli* O128 antigen biosynthesis cluster and functional identification of an  $\alpha$ -1,2-fucosyltransferase. *FEBS Lett.* 553, 99–103.
- Somoza, J., Menon, S., Schmidt, H., Joseph-McCarthy, D., Dessen, A., Stahl, M., Somers, W., Sullivan, F., 2000. Structural and kinetic analysis of *Escherichia coli* GDP-mannose 4,6 dehydratase provides insights into the enzyme's catalytic mechanism and regulation by GDP-fucose. *Structure* 8, 123–135.
- Sturla, L., Bisso, A., Zanardi, D., Benatti, U., De Flora, A., Tonetti, M., 1997. Expression, purification and characterization of GDP-d-mannose 4,6-dehydratase from *Escherichia coli*. *FEBS Lett.* 412, 126–130.
- Wang, G., Boulton, P.G., Chan, N.W.C., Palcic, M.M., Taylor, D.E., 1999. Novel *Helicobacter pylori*  $\alpha$ 1,2-fucosyltransferase, a key enzyme in the synthesis of Lewis antigens. *Microbiology* 145, 3245–3253.
- Wang, G., Rasko, D.A., Sherburne, R., Taylor, D.E., 2002. Molecular genetic basis for the variable expression of Lewis Y antigen in *Helicobacter pylori*: analysis of the  $\alpha$  (1, 2) fucosyltransferase gene. *Mol. Microbiol.* 31, 1265–1274.
- Yi, W., Shao, J., Zhu, L., Li, M., Singh, M., Lu, Y., Lin, S., Li, H., Ryu, K., Shen, J., 2005. *Escherichia coli* O86 O-antigen biosynthetic gene cluster and stepwise enzymatic synthesis of human blood group B antigen tetrasaccharide. *J. Am. Chem. Soc.* 127, 2040–2041.