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Inulooligosaccharide Production from Inulin by *Saccharomyces cerevisiae* Strain Displaying Cell-Surface Endoinulinase

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Abstract The endoinulinase gene (*inu1*) from *Pseudomonas mucidolens* was expressed on the cell surface of *Saccharomyces cerevisiae* by fusing with Aga2p linked to the membrane anchored protein, Aga1p. The *inu1* gene of *P. mucidolens* was subcloned into the surface display vector, pCTcon (*GAL1* promoter). The constructed plasmid, pCTENIU (8.5 kb), was then introduced to *S. cerevisiae* EBY100 cells and the yeast transformants selected on synthetic defined media lacking uracil and inulin-containing media. The *inu1* gene under the control of the *GAL1* promoter was successfully expressed in the yeast transformants, and the surface display of endoinulinase confirmed by immunofluorescence microscopy, along with its enzymatic ability to form inulooligosaccharides (IOSs) from inulin. The total endoinulinase activity reached about 2.31 units/ml when the yeast transformants were cultivated on a YPDG medium. To efficiently hydrolyze the inulin, various reaction conditions were examined, including the pH, temperature, and inulin source. The optimized conditions were then determined as follows: pH, 7.0; temperature, 50°C; inulin source, Jerusalem artichoke. Under the optimized condition and 46 units of endoinulinase per g of inulin, IOSs started to be produced after 10 min of enzymatic reaction. The highest yield, 71.2% of IOSs, was achieved after 30 h of reaction without any significant loss of the initial enzyme activity. As a result of the reaction with inulin, IOSs consisting of inulobiose (F2), inulotriose (F3), inulotetraose (F4), and inulopentaose (F5) were produced, and F4 was the major product.

Key words: Endoinulinase, *Saccharomyces cerevisiae*, cell-surface display, inulooligosaccharide, inulin

The inulooligosaccharides (IOSs) produced from inulin by endoinulinase have been suggested to exhibit similar physiological functions to fructooligosaccharides [28]. Many kinds of oligosaccharide have become increasingly important, because of their beneficial functionalities, including being low caloric, noncariogenic, and acting as a growth factor for beneficial microorganisms in the intestinal flora [27, 29, 31, 32, 34, 39]. Hydrolysis of the internal linkages of inulin yields several oligosaccharides that are soluble dietary fibers and/or functional sweeteners [3, 25, 38]. Several bacteria and fungi have already been reported as producers of the endoinulinase enzyme. Among them, the soil bacterium *Pseudomonas mucidolens* was isolated, and the endoinulinase gene (*inu1*) cloned from the strain constitutively expressed in *E. coli* for the production of IOSs [37].

The display of heterologous proteins on the cell surface of microorganisms, such as yeast and bacterial cells, has become one of the most interesting research areas with applications in the production of live vaccines, antibody libraries, and whole-cell biocatalysts and absorbents [1, 7, 10, 23, 24]. Furthermore, the expression of proteins on the surfaces of bacteriophages and bacteria has also been actively studied [2], and these systems are expected to be useful for the segregation of polypeptides and construction of microbial biocatalysts. Nonetheless, the expression of proteins on the cell surface of *Saccharomyces cerevisiae* offers more advantages than other microbial systems. First, since *S. cerevisiae* is widely used in the industrial production of proteins and chemicals, enzyme-coated yeast cells can be used as novel whole-cell biocatalysts, because the surface-immobilized proteins are covalently linked to glucan in the cell wall, rendering them resistant to extraction. Second, *S. cerevisiae* is safe for oral use in food and pharmaceutical products. Third, it can be cultivated to a high cell density using relatively inexpensive media. To immobilize protein on the cell surface of *S. cerevisiae*, α-agglutinin, a yeast cell-wall-anchored protein, has been widely utilized [30].

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α-Agglutinin is a mannoprotein involved in the sexual adhesion of mating-type a *S. cerevisiae* cells [19]. It also has a glycosyl-phosphatidylinositol (GPI) anchor attachment signal that is involved in anchoring cell-wall proteins [20], and has already been combined with signals for secreted enzymes using recombinant DNA techniques. The 73-kDa Aga1p subunit of α-agglutinin is anchored to the cell wall via a β-glucan linkage, and a 69 amino-acids binding subunit Aga2p is linked to Aga1p by two disulfide linkages [6].

A number of studies have already focused on the expression of various enzymes on the cell surface of *S. cerevisiae* using α-agglutinin [11, 16, 21, 24, 36]. However, the properties of the yeast cell surface, such as its hydropathy and charges, may also support the accessibility and affinity between enzymes and substrates in an organic solvent. One of the advantages of enzyme-displaying whole-cell biocatalysts is that the enzymes are immobilized on the yeast cell surface by genetically modified anchor systems, which not only minimizes the risk of inactivation and inhibition, but also improves the performance of the displayed enzyme.

Accordingly, this paper described the development of a recombinant *S. cerevisiae* strain anchoring *P. mucidolens* endoinulinase on the cell surface using Aga2p as the fusion partner and examined its application as a novel whole-cell biocatalyst for the production of IOSs.

MATERIALS AND METHODS

Strains and Media

E. coli DH5α (*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZΔM15*) was used as the host strain for the recombinant DNA manipulation. The yeast *S. cerevisiae* EBY100 (*trp1 leu2Δ1 his3Δ200 pep4::HIS2 prbΔ1.6R can1 GAL*) [4] was used for the expression and surface display of *P. mucidolens* endoinulinase [9]. The *E. coli* was grown in an LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 50 μg/ml ampicillin. A YNBCAD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.5% casamino acids) was used for the selection of the yeast transformants and for the seed culture. To induce yeast cells displaying endoinulinase on their surface, the yeast transformants were grown in a fermentor (KoBiotech Co., Korea) containing a YPDG medium (1% yeast extract, 2% peptone, 1% glucose, and 1% galactose) at 20°C and pH 6.0.

Construction of Expression Plasmid

The endoinulinase gene, *inu1*, consisting of 2.3 kb from *P. mucidolens* was amplified by a PCR using pENV1 [9] as the template. The sequences of the two primers were [(5'-3') ACGGCTAGCATGCATAATACCGAAGATACCGGG and (5'-3') GGGATCCTTTGGTTTGGACTCCGTCTGCGTGCTG]. The underlined sequences denote the NheI and BamHI restriction enzyme sites. After the PCR amplification, the *inu1* gene was digested with NheI and BamHI, and then subcloned into the surface display vector, pCTcon [5], resulting in the pCTENIU plasmid.

Yeast Transformation

The transformation of the plasmid pCTENIU into *S. cerevisiae* EBY100 was carried out using the lithium acetate method [12]. The resulting transformant was named EBY100/pCTENIU.

Immunofluorescence Microscopy

The immunofluorescence microscopy was performed as described previously [18], and the immunostaining conducted as follows: the cells were cultivated in the YPDG medium at 20°C for 48 h, collected by centrifugation at 8,000 rpm for 10 min, and washed with phosphate-buffered saline (pH 7.4, PBS). The cell suspension was then adjusted to OD_{600}= 20 with PBS, and 100 μl of the cell suspension collected in a microtube. The cells and 100 μl of the primary antibody were further incubated in PBS containing 1% BSA for 1.5 h on ice. The primary antibody used was c-myc monoclonal antibody (mAb) 9E10 (Covance Inc., U.S.A.) at a dilution rate of 1:500. The cells were then washed with PBS and incubated for 1 h on ice at a dilution rate of 1:100 with 100 μl of the secondary antibody, fluorecein-isothiocyanate (FITC)-labeled anti-mouse IgG (H+L) (KPL Co., U.S.A.). After washing the cells again with PBS, the fluorescence image was observed under a confocal laser scanning microscope (Carl Zeiss, LSM 510 META, Germany).

Enzymatic Reaction

The enzymatic reaction was carried out in a 50 mM phosphate buffer (pH 7.0). Inulins from chicory, dahlia, and Jerusalem artichoke (Sigma Co., U.S.A.) were used as the substrates. The *S. cerevisiae* EBY100/pCTENIU and control strain *S. cerevisiae* EBY100 were incubated in the YPDG medium for 48 h at 20°C, harvested by centrifugation, and washed twice with PBS. The yeast cells were then resuspended in the reaction buffer at a final concentration of OD_{600}=20 (2.3 unit/ml) for the enzymatic reaction. The endoinulinase activity was assayed by incubating 100 μl of the cell suspension with 100 μl of 5% (w/v) inulin at 50°C for 20 min. The reaction was stopped by heating at 100°C for 10 min. One unit of endoinulinase activity was defined as the amount of enzyme liberating 1 μmol of reducing sugar (fructose equivalent) from the dahlia inulin per min at 50°C, where the mean molecular weight of inulin was estimated as 5,000 Da after an acid-hydrolysis analysis. The reaction products were analyzed by TLC and HPLC.

Thin Layer Chromatography

A TLC analysis was performed to identify the IOSs produced by the recombinant *S. cerevisiae* EBY100/pCTENIU.

After activating the TLC plate (60 F_{254}, Merck Co., Berlin, Germany) for 1 h at 110°C, aliquots (5 μl) of the reaction mixtures were spotted and then developed in a solvent [*n*-butanol:isopropanol:water=3:12:4 (v/v)]. The products were detected by spraying the plate with a urea reagent (93.22 ml of *n*-butanol, 6.78 ml of phosphoric acid, 5 ml of ethanol, and 3 g urea) and heating at 110°C for 10 min [13]. A standard set of fructooligosaccharides, including 1-kestose, nystose, and 1^F-fructosylnystose (Wako, Japan), and reagent-grade sugars, such as glucose, fructose, and sucrose (Junsei, Japan), were used to identify the reaction products. The color images were analyzed using an image analyzer (FluorChem 5500, Alpha InnoTech., U.S.A.).

High Performance Liquid Chromatography

The HPLC analysis of the end products after the enzymatic reaction was performed using an RI detector (Waters 410, U.S.A.), and the column used for the separation was a Sugar-Pak I (6.5×300 mm, Waters, U.S.A.). The HPLC (Alliance 2690, Waters, Milford, MA, U.S.A.) was operated at 90°C with 0.5 ml/min of 0.1 mM Ca-EDTA as the mobile phase.

Analytical Methods

The residual sugar concentration in the medium was measured using the dinitrosalicylic acid method [22], where glucose was used as the standard. In the assay of endoinulinase activity, fructose was used as the standard. The quantitative analyses of the carbohydrates, including the IOSs, were carried out by HPLC. The total amount of IOSs was estimated as the sum of inulobiose (F2) and other oligofructosides (F_n and some GF_n), where the degree of polymerization (DP) ranged from DP3 to DP7. The yield of IOSs was expressed as the ratio of the total amount of IOSs to the amount of inulin. The total amount of IOSs produced from the Jerusalem artichoke inulin was taken as 100%, then the relative amounts (%) of IOSs from the other inulins were calculated. The overall carbohydrate content in the reaction mixture was determined using the phenol-sulfuric acid method [8].

RESULTS AND DISCUSSION

Construction of Cell-Surface Expression Plasmid

A PCR amplification was carried out to obtain a 2.3 kb DNA fragment of *P. mucidolens* endoinulinase, which was then digested with the NheI and BamHI restriction endonucleases, and placed in a frame with the Aga2p gene in the pCTcon vector to construct the pCTENIU (8.5 kb) plasmid. Aga2p was employed as a fusion partner to secrete and localize the endoinulinase on the cell surface of *S. cerevisiae*. The expression of the Aga2p/endoinulinase fusion protein was under the control of the *GAL1* promoter.

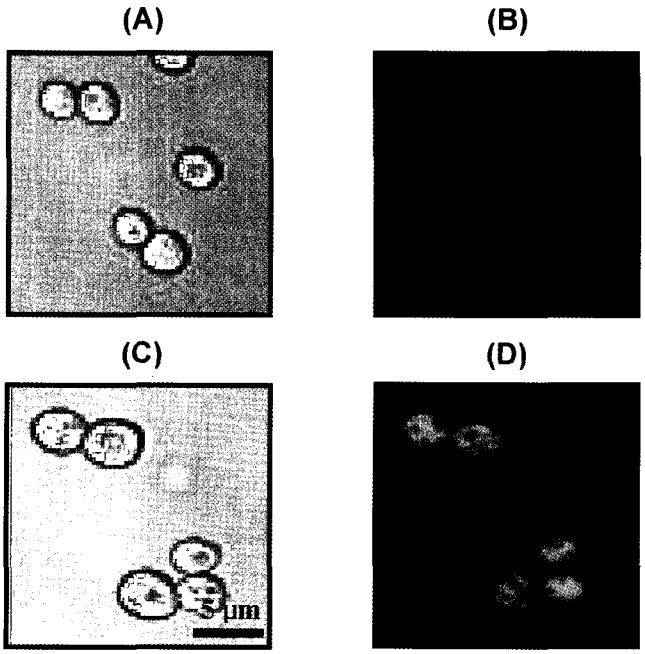

Fig. 1. Confocal microscopic images of yeast *Saccharomyces cerevisiae* EBY100 (**A**, **B**) and EBY100/pCTENIU (**C**, **D**).
Optical micrographs (**A**, **C**) and confocal laser scanning micrographs (**B**, **D**).

Immunofluorescence Microscopy

Immunofluorescence microscopy was used to confirm the expression of the *P. mucidolens* endoinulinase on the cell surface of *S. cerevisiae*. Thus, immunofluorescent labeling of the recombinant yeast cells was performed using anti-c-myc as the primary antibody and FITC IgG (mouse) as the secondary antibody. The *S. cerevisiae* EBY100 and *S. cerevisiae* EBY100/pCTENIU cells were observed under a confocal laser scanning microscope. As shown in Fig. 1, the *S. cerevisiae* EBY100/pCTENIU was labeled by FITC and its fluorescence was observed on the cells, whereas no fluorescence was observed on the cells of the control strain, *S. cerevisiae* EBY100. Therefore, this observation confirmed the successful expression and localization of the endoinulinase on the cell surface of *S. cerevisiae*.

Localization of Endoinulinase in *S. cerevisiae*

The patterns of the cell growth and expression of the surface-displayed endoinulinase on the recombinant *S. cerevisiae* were examined at various incubation temperatures and initial medium pHs to optimize the culture conditions. The optimum pH for the medium was identified as pH 6.0 (data not shown). Meanwhile, the specific growth rate did not vary much from 25 to 30°C, and the maximum endoinulinase expression was observed at 20°C. Incubation at higher temperatures can lead to poor surface expression, possibly due to inefficient protein folding and retention in the endoplasmic reticulum, as observed with CGTase [16].

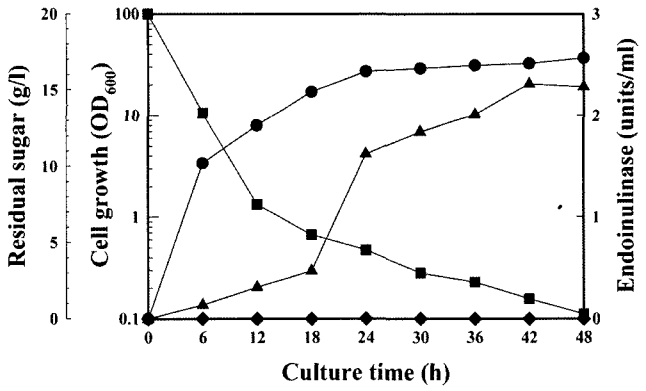

Fig. 2. Time profiles of cell growth, sugar consumption, and endoinulinase expression during batch fermentation of *S. cerevisiae* EBY100/pCTENIU on YPDG medium.
Symbols: (●), cell growth; (■), residual sugar; (▲), endoinulinase activity of cell pellet; (◆), endoinulinase activity of culture supernatant.

To determine whether the endoinulinase was secreted in the culture medium or retained on the cell surface, the endoinulinase activity of EBY100/pCTENIU was examined in both the culture supernatant and the cell pellet fractions. A seed culture grown in the YNBCAD medium was transferred to a fermentor containing the YPDG medium at 20°C, pH 6.0 for 48 h, and then separated into the culture supernatant and the cell pellet. The endoinulinase activities of both fractions were then measured using 5% dahlia inulin as the substrate at 50°C and pH 7.0. As shown in Fig. 2, the total endoinulinase activity in the cell pellet of *S. cerevisiae* EBY100 harboring pCTENIU was 2.31 unit/ml, whereas no endoinulinase activity was detected in the culture supernatant, indicating that the endoinulinase was retained on the cell surface in an active form.

Effect of pH and Temperature

To evaluate the features of the endoinulinase displayed on the cell surface, the effects of pH (4.0–10.0) and temperature (30–70°C) on the endoinulinase activity of EBY100/pCTENIU were examined. The optimal pH and temperature for the endoinulinase activity were found to be pH 7.0 and 50°C, respectively (Fig. 3 and Fig. 4). As previously reported, the optimal pH and temperature for the *P. mucidolens* endoinulinase expressed in *E. coli* were pH 7.5 and 50°C, respectively [37]. Furthermore, over 75% and 50% of the endoinulinase activity remained after incubation for 1 h at pH 5.0 to pH 7.0 and 30°C to 70°C, respectively.

Effect of Inulin Source

To determine the optimal inulin source, 5% each of three kinds of inulin (chicory inulin, dahlia inulin, and Jerusalem artichoke inulin) were hydrolyzed at 50°C and pH 7.0. The highest IOS production was observed with the Jerusalem artichoke inulin (Fig. 5), and the total amount of IOSs produced (1.78 g from 2.5 g inulin) was taken as 100%. The amounts of IOSs produced from the chicory and dahlia inulins then corresponded to 1.48 g (83%) and 1.67 g (94%), respectively. Therefore, the preferable substrate for IOS production was in the following order: Jerusalem artichoke > dahlia > chicory. These results also suggest that the Jerusalem artichoke inulin may have industrial potential for IOS production. As previously reported, when considering the average degree of polymerization (DP) at 33.6 for chicory and 40.3 for dahlia, a much higher DP value would seem to be appropriate for Jerusalem artichoke [26]. As such, this high DP for Jerusalem artichoke may result in an increased cell growth, leading to an elevated production of IOS or ethanol [15, 33]. The optimal IOS production by endoinulinase has already been analyzed

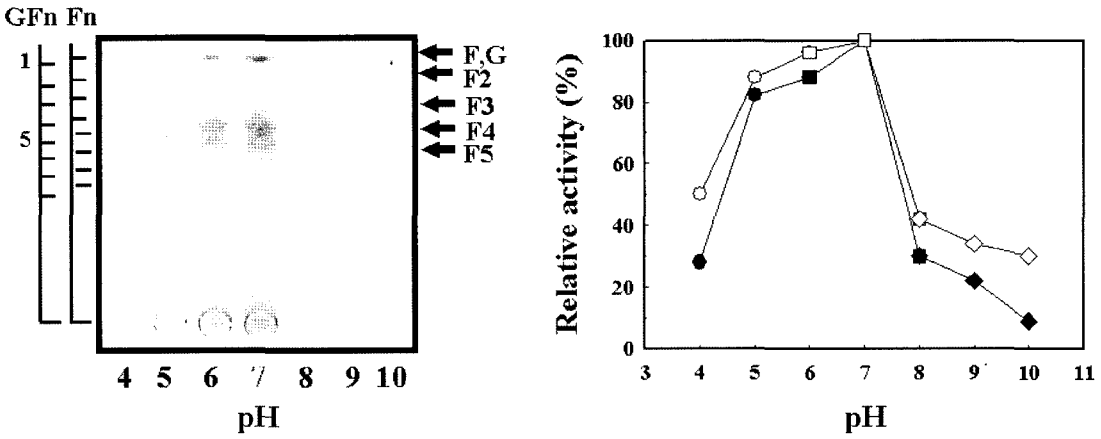

Fig. 3. Effects of pH on the activity and stability of recombinant endoinulinase.
Dahlia inulin (5%) was used as the substrate. The enzyme activity was measured in a 50 mM acetate buffer (●), 50 mM phosphate buffer (■), and Tris buffer (◆) at 50°C at the indicated pHs. Meanwhile, the pH stability was measured in a 50 mM acetate buffer (○), 50 mM phosphate buffer (□), and Tris buffer (◇) by incubating the enzyme at the indicated pHs for 1 h at 50°C, andthen the residual activity was measured in a 50 mM phosphate buffer (pH 7) at 50°C. F, fructose; G, glucose; F2, inulobiose; F3, inulotriose; F4, inulotetraose; F5, inulopentaose; GFx, glucofructooligosaccharides; Fx, inulooligosaccharides.

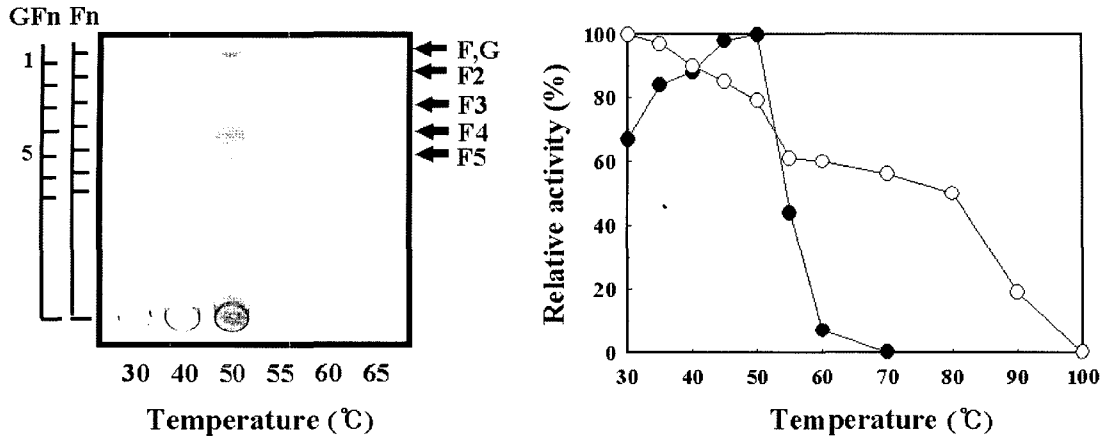

Fig. 4. Effects of temperature on the activity and stability of recombinant endoinulinase.
Dahlia inulin (5%) was used as the substrate. The enzyme activity (●) was measured in a 50 mM phosphate buffer (pH 7) at the indicated temperatures. Meanwhile, the thermostability (○) was measured by incubating the enzyme at the indicated temperatures for 1 h in a 50 mM phosphate buffer (pH 7), then the residual activity was measured at 50°C. F, fructose; G, glucose; F2, inulobiose; F3, inulotriose; F4, inulotetraose; F5, inulopentaose; GFn, glucofructooligosaccharides; Fn, inulooligosaccharides.

with different substrates, including Jerusalem artichoke inulin, Jerusalem artichoke powder, and Jerusalem artichoke juice [14], where the maximum IOS yield (79.8%) was achieved after 72 h with Jerusalem artichoke juice as the substrate, indicating that Jerusalem artichoke juice was the optimal substrate for IOS production with the *Aspergillus ficuum* endoinulinase.

However, in this work, the optimized conditions for the complete hydrolysis of inulin were determined as follows: pH, 7.0; temperature, 50°C; inulin source, Jerusalem artichoke.

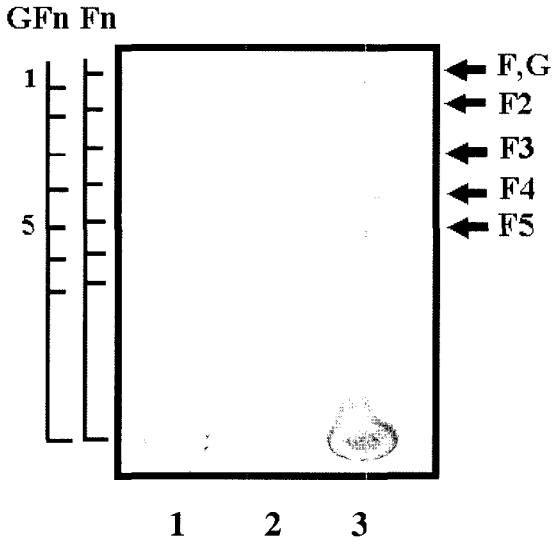

Fig. 5. Effect of inulin source on the production of inulooligosaccharides.
Lane 1, Dahlia; 2, chicory; 3, Jerusalem artichoke. F, fructose; G, glucose; F2, inulobiose; F3, inulotriose; F4, inulotetraose; F5, inulopentaose; GFn, glucofructooligosaccharides; Fn, inulooligosaccharides.

Analysis of Inulin Hydrolysates

To study the change in product composition during the hydrolysis, the enzyme reactions were carried out under the optimal conditions. During the course of the dahlia inulin hydrolysis by the endoinulinase, aliquots of the reaction mixture were periodically withdrawn and the hydrolysis products analyzed by TLC. After 18 h of culture time, the IOSs in the reaction products from the inulin included inulobiose (F2), inulotriose (F3), inulotetraose (F4), and inulopentaose (F5), where F4 was the major product (Fig. 6A). As shown in the product profile relative to the reaction time, IOSs started to be produced after 10 min of enzymatic reaction (Fig. 6B). An HPLC analysis of the product compositions was also performed to identify the final reaction products at the reaction time when the IOS production was maximal. As shown in Fig. 7, it was confirmed that F4 was the major product. The carbohydrates composition of the reaction products catalyzed by the cell-surface-displayed endoinulinase is shown in Table 1. The predominant product was an IOS of DP and the total percentage of IOSs reached 71.2%. This maximum yield of IOSs was achieved with the Jerusalem artichoke inulin after 30 h (Fig. 8).

In previous reports, the maximum IOS yields for the wild-type endoinulinase from *Pseudomonas* sp. and its recombinant form expressed in *E. coli* were 75.6% and 78.0%, respectively [15, 37]. When the wild-type endouinulinase or *E. coli* cells harboring the endoinulinase gene were immobilized on polystyrene carriers, the IOS yields reached 83% and 77.2%, respectively, within 50 h of reaction, based on 25 or 50 units of endoinulinase per g of inulin [37, 38]. In addition, the major products were found to be F2 and F3, and their ratio differed considerably with

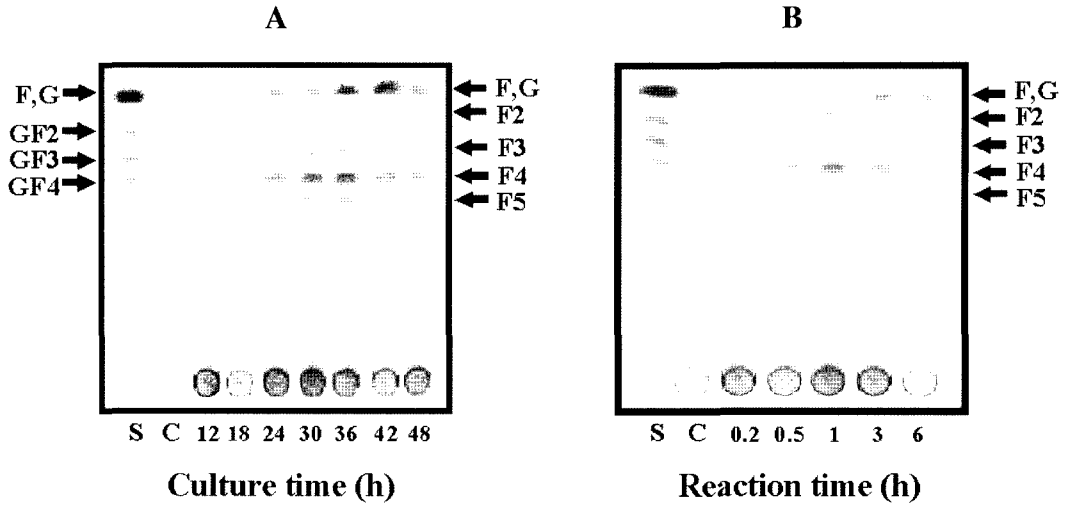

Fig. 6. Culture time profile (**A**) and reaction time profile (**B**) of reaction products from dahlia inulin when using surface-displayed endoinulinase.
S, inulin hydrolysate; C, inulin without enzyme; F, fructose; G, glucose; GF2, 1-kestose; GF3, nystose; GF4, 1^F-fructosylnystose; F2, inulobiose; F3, inulotriose; F4, inulotetraose; F5, inulopentaose.

the type of enzyme used. In the present work, yeast cells corresponding to about 46 units of endoinulinase per g of inulin were used. The immobilization of endoinulinase on the yeast cell surface avoids the tedious purification process of the enzyme used in conventional immobilization and reuse. In particular, the present study achieved a relatively high IOS yield (71.2%) and productivity of 1.19 g/l·h within a short reaction time, 30 h. Furthermore the predominant degree of polymerization in this IOS production system was 4 (DP4, F4). Consequently, since the length of the oligosaccharide chains has a significant effect on the sweetness of their solutions, and the sweetness of oligosaccharides above DP7 is imperceptible [35], the IOSs of DP4 produced in this work can be used as functional sweeteners.

The current study developed a *S. cerevisiae* system anchored with the *P. mucidolens* endoinulinase to produce IOSs from inulin. Further research is also in progress to improve the secretion efficiency of bacterial endoinulinase on *S. cerevisiae*, select a clone harboring the maximum endoinulinase activity via fluorescence-activated cell sorting (FACS), compare product profiles for a whole-cell biocatalyst, and finally optimize the reactor configurations for IOS production.

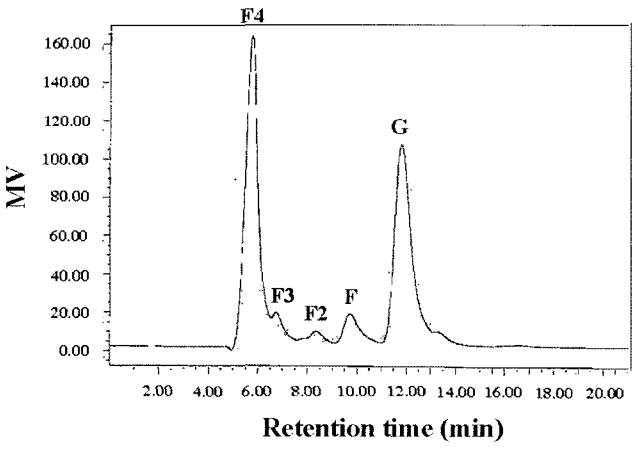

Fig. 7. HPLC analysis of dahlia inulin hydrolysate when using surface-displayed endoinulinase.
G, glucose; F, fructose; F2, inulobiose; F3, inulotriose; F4, inulotetraose.

Table 1. Carbohydrate composition of inulin hydrolysate from Jerusalem artichoke inulin when using surface-displayed endoinulinase.[a]

Carbohydrate	Composition (%)
Inulin	1.8
Glucose	20.1
Fructose	5.1
Sucrose	0.8
Inulooligosaccharides[b]	
DP2	4.1
DP3	8.9
DP4	54.8
>DP4	3.4
Total Inulooligosaccharides	71.2

[a]The carbohydrate composition was analyzed at the reaction time when the oligosaccharide production was maximal. The initial concentration of inulin was 50 g/l. The endoinulinase activity added was 46 units per g of inulin.
[b]DP, degree of polymerization.

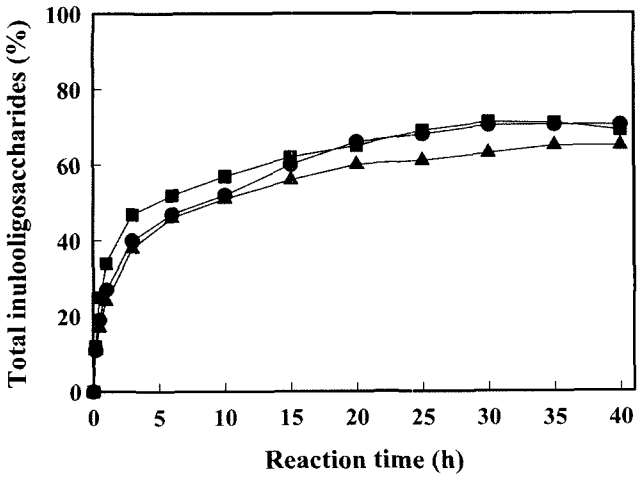

Fig. 8. Time profile of the total inulooligosaccharides yield from three different inulins when using surface-displayed endoinulinase. Symbols: (▲) chicory; (●) dahlia; (■) Jerusalem artichoke.

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