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ORIGINAL ARTICLE

Nonomuraea sp. ID06-A0189 inulin fructotransferase (DFA III-forming): gene cloning, characterization and conservation among other *Nonomuraea* species

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The inulin fructotransferase (DFA III-forming)(EC 4.2.2.18) gene in *Nonomuraea* sp. ID06-A0189 was amplified from genomic DNA, sequenced and expressed in *Escherichia coli*. The 1326-bp gene, designated as *Nsp-ift,* encodes a protein composed of a putative 37-amino-acid signal peptide and 404-amino-acid mature protein. A putative ribosomal binding sequence was identified 12 bases upstream from the start codon. However, a typical bacterial promoter could not be found by *in silico* analysis. The deduced amino-acid sequence of the enzyme was most similar to that of inulin fructotransferase (DFA I-forming) in *Frankia* sp. EAN1pec. Phylogenetic analysis of deduced amino-acid sequences indicated that *Nonomuraea* sp. ID06-A0189 and *Frankia* sp. EAN1pec inulin fructotransferases formed a distinct clade from those from *Arthrobacter* sp. H65-7, *A. globiformis* and *Bacillus* sp. snu-7 that showed 57, 56 and 56% identity to that of *Nsp-ift*, respectively. The *Nsp-ift* without a putative signal peptide was successfully expressed in *E. coli* and partially purified using His-tag affinity chromatography. The recombinant enzyme displayed optimum temperature between 65 and 70 °C, optimum pH between 5.5 and 6.0 and remained stable up to 70 °C. The properties were identical to those of the original enzyme. Of 10 *Nonomuraea* species tested by Southern hybridization, enzyme activity measurements and PCR, only *Nonomuraea* sp. ID06-A0189 has the *Nsp-ift* gene, suggesting that *Nsp-ift* is not highly conserved in this genus.
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INTRODUCTION

Inulin fructotransferase [EC 4.2.2.18], the enzyme that converts inulin into di-D-fructofuranose 1, 2′:2, 3′ dianhydride (DFA III) and a small amount of oligosaccharides, was initially discovered in *Arthrobacter ureafaciens*.[1] The main product of this enzyme reaction, DFA III, was first established as a food ingredient for the prevention of osteoporosis because of its ability to stimulate calcium absorption in rats and humans.[2–5] Subsequently, DFA III has been found to inhibit secondary bile acid formation in rats and humans,[6–7] thus it is also a good candidate for use as a prebiotic for the prevention of colon cancer. The health benefits of DFA III make inulin fructotransferase (DFA III-forming) an important enzyme for the food industry. Japan has used it for industrial production of DFA III since 2004.[8]

To date, inulin fructotransferases from species of *Arthrobacter*,[9–13] *Bacillus*,[14] and *Leifsonia*[15] have been studied, the corresponding genes from three of them have been cloned and expressed in heterologous hosts.[16–18] For a better understanding of this enzyme in another genus, the gene that encodes inulin fructotransferase in *Nonomuraea* sp. ID06-A0189 was characterized and expressed in this study. Previously, we selected and described *Nonomuraea* sp. ID06-A0189 that could produce inulin fructotransferase (DFA III-forming) in the culture broth.[19] The crude enzyme showed promising characteristics for mass production of DFA III. The objectives of this work were to clone and express the gene that encodes inulin fructotransferase in *Nonomuraea* sp. ID06-A0189 and to compare it to previously studied genes. In addition, we used Southern hybridization and PCR to determine whether this gene is conserved among other members of the genus *Nonomuraea*. This study will provide molecular aspects of the enzyme and aid in improving mass production of DFA III.

MATERIALS AND METHODS

Bacterial strains and culture condition

This study used 10 different species of the genus *Nonomuraea*. One, *Nonomuraea* sp. ID06-A0189, was isolated from Indonesian soil[20] and the other nine type strains for *Nonomuraea* species, *N. africana* JCM 3109^T, *N. ferruginea* JCM 3283^T, *N. fastidiosa* JCM 3321^T, *N. longicatena* JCM 11136^T,

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N. polychroma JCM 6834^T, *N. pusilla* JCM 3144^T, *N. roseoviolaceae* subsp. *carminata* JCM 9946^T, *N. roseoviolaceae* subsp. *roseoviolaceae* JCM 3145^T and *N. rubra* JCM 3234^T, were purchased from the Japan Collection of Microorganisms (JCM). These type strains were chosen because they represented different clades of the *Nonomuraea* phylogenetic tree.[19] *Nonomuraea* sp. ID06-A0189 was maintained on International Streptomyces Project 2 agar medium[21] and incubated at 30 °C for 7 days, whereas the strains from the JCM were cultured according to their catalog. For DNA extraction, the strains were cultivated in International Streptomyces Project 2 liquid medium at 30 °C with shaking of 150 r.p.m. for 3 days. The cells were collected by centrifugation (8000 × *g*, 20 min) and washed twice with 0.95% NaCl solution. Total genomic DNA was extracted using the ISOPLANT II DNA extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction.

Amplification of inulin fructotransferase gene partial fragments

For PCR amplification of inulin fructotransferase genes from *Nonomuraea* sp. ID06-A0189, conserved regions of the known inulin fructotransferase genes from *A. globiformis* C11-1 (D38528), *Arthrobacter* sp. H65-7 (D84399), *Bacillus* sp. (DQ112363) and *Frankia* sp. (CP000820) were used to design forward and reverse primers named N4F and N7R, respectively (Table 1). To determine the conservation of *Nsp-ift* among the genus *Nonomuraea* by PCR, N4F and N7R primers were used to amplify inulin fructotransferase genes of the 10 chosen *Nonomuraea* strains. PCR was performed in 50 μl reaction mixtures using the Colorless GoTaq Flexi PCR Kit (Promega, Madison, WI, USA) according to the protocol provided. PCR was performed in a Gene Amp PCR system 9600 (Applied Biosystems, Foster City, CA, USA) thermal cycler. The PCR consisted of an initial denaturation at 95 °C for 90 s then 30 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 50 s. The PCR product was purified using a SUPREC-PCR Kit (Takara Bio Tokyo, Japan) and sequenced using the ABI Prism Big Dye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems) and ABI 3100 DNA sequencer (Applied Biosystems). The obtained data were analyzed using an Auto assembler software from Applied Biosystems.

Inverse PCR and bioinformatics analysis

Genomic DNA of *Nonomuraea* sp. ID06-A0189 was digested with *Kpn*I (Takara Bio, Ohtsu, Japan), and self-ligated using T4 DNA ligase (NEB, Ipswich, MA, USA). The ligated product was used as template for inverse PCR using primers F1 and R1 (Table 1), which were designed based on the partial sequence of inulin fructotransferase genes of *Nonomuraea* sp. ID06-A0189 obtained by PCR. Inverse PCR was conducted using a KOD-FX Kit (Toyobo, Osaka, Japan) according to the protocol provided. Thermal cycler conditions used were initial denaturation at 94 °C for 2 min followed by 5 cycles at 98 °C for 10 s and 74 °C for 6 min; 5 cycles at 98 °C for 10 s and 72 °C for 6 min; 5 cycles at 98 °C for 10 s and 70 °C for 6 min; 25 cycles at 98 °C for 10 s and 68 °C for 6 min and 68 °C for 7 min. The inverse PCR products were purified using a SUPREC-PCR Kit (Takara Bio) and sequenced using a primer-walking strategy. The fragment sequences were assembled using Auto Assembler software (Applied Biosystems) and analyzed using Genetyx-MAC (Genetyx, Tokyo, Japan). The signal peptide cleavage site in the deduced amino-acid sequence was predicted using SignalP-3.0.[22] BLAST was used to compare of the deduced amino-acid sequence to those in the NCBI database. The phylogenetic tree was constructed using the neighbor-joining method in CLUSTAL X.

Cloning and expression of inulin fructotransferase gene

Primers pETIFTF2 (5′-GGGGAATTCGCAGGCCGGGCCGTCCA-3′) and pETIFTR2 (5′-GGGGCGGCCGCTCAGGGAGTGGCGCGGA-3′) were used to amplify the gene that encodes the mature protein of inulin fructotransferase. PCR was performed in 50 μl reaction mixtures using the Colorless GoTaq Flexi PCR Kit (Promega) according to the protocol provided. PCR was performed in a Gene Amp PCR system 9600 (Applied Biosystems) thermal cycler. The PCR consisted of an initial denaturation at 95 °C for 90 s then 30 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 50 s. The PCR product was gel purified using the Wizard SV Column Kit (Promega). The purified PCR product and the vector pET28(b) (Merck, Darmstadt, Germany) were digested with *Eco*RI and *Not*I, and ligated with T4 DNA ligase (NEB). The recombinant plasmid (pETIFT) was introduced into *E. coli* BL21(DE3). The transformant was cultured in Luria-Bertani liquid medium containing 50 μg ml^{-1} kanamycin with shaking at 37 °C and *Nsp-ift* was induced with isopropyl β-D-thiogalactoside at a final concentration of 0.1 mM, during OD_{600} was 0.4–0.6. Following additional shaking cultivation at 30 °C for 24 h, cells were harvested by centrifugation. Cell-free extract was obtained by beads disruption and purified with His-tag affinity chromatography (HisTrap HP, GE Healthcare, Buckinghamshire, UK). Fractions were desalted with Econo-Pac 10DG (Bio-Rad, Hercules, CA, USA) and used for inulin fructotransferase activity assay.

Table 1 Primers used for PCR reaction

Name	*Sequence*	*Forward/reverse*
N4F	5′-CGACAACWTSATCGCCGAATG-3′	Forward
N7R	5′-GAVGCBGKGSCGCTGTC-3′	Reverse
F1	5′-CACGCGAGGCACGAAGGTG-3′	Forward
R1	5′-CCCGCGCCGACATGGTT-3′	Reverse

Production of inulin fructotrasferase and measurement of enzyme activity

Single colony of each type strain of *Nonomuraea* was inoculated into 10 ml of inulase induction medium, containing 10 g inulin, 2 g $NaNO_3$, 0.5 g $MgSO_4$ 7 H_2O, 0.5 g KCl, 0.01 g $FeSO_4$ 7 H_2O, 0.2 g yeast extract and 0.5 g KH_2PO_4 per liter (pH 7.0) and incubated at 30 °C with shaking (140 r.p.m.) for 5 days. The culture was centrifuged to remove the biomass and the supernatant was assayed for its inulin fructotransferase activity. Culture supernatant (0.5 ml) was mixed with 0.5 ml of 10% inulin solution in 20 mM citric acid–NaOH buffer (pH 5.6) and incubated at 60 °C for 1 h then boiled in a water bath for 5 min to stop the reaction. The reaction mixture was applied to a TLC silica gel plate (Merck) and exposed to a mixture of 1-butanol-2-propanol-water (10: 5: 4, v/v). DFA III spot was detected by spraying with *p*-anisaldehyde-H_2SO_4-ethanol (1: 1: 18, v/v) followed by heating at 120 °C for 30 s. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of DFA III per minute at 65 °C and at a pH of 5.5 in 10 min.

Inulin fructotransferase gene detection in *Nonomuraea* species by Southern hybridization

To determine the conservation of *Nsp-ift* among the genus *Nonomuraea,* genomic DNA of the 10 chosen *Nonomuraea* strains were digested with *Eco*RV, *Cla*I, *Kpn*I and *Sma*I (Takara Bio), separated in a 1% (w/v) agarose gel then blotted onto a Hybond-N$^+$ membrane (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. Hybridization was performed using the AlkPhos Direct Labeling and Detection System using the CDP-Star detection (GE Healthcare). The hybridization probe was prepared from the partial inulin fructotransferase gene of *Nonomuraea* sp. ID06-A0189 obtained by PCR. To obtain sufficient probe DNA, before labeling, the PCR fragment was cloned using the pGEMT-easy vector system according to the manufacturer's instructions (Promega). Hybridization and subsequent membrane washing were conducted at 62 °C. When the probe did not hybridize, the temperatures were lowered to 55 °C to increase sensitivity. Hybridization signals were detected using a Luminescent Image Analyzer LAS-400 (Fuji Film, Tokyo, Japan).

RESULTS AND DISCUSSION

Molecular cloning and characterization of inulin fructotransferase gene

Primers N4F and N7R, which were designed based on the conserved regions of previously reported inulin fructotransferase (DFA III-forming) genes,[16–18] produced a PCR product from *Nonomuraea* sp. ID06-A0189 with the expected size (~600 bp) based on the inulin fructotransferase gene of *Arthrobacter* sp. H65-7 (Figure 1a). This partial fragment was designated as NSP17 and the highest identity of the deduced amino-acid sequence was 72% to *Frankia* sp. EAN1pec

inulin fructotransferase (DFA I-forming). Hybridization of a labeled probe made from this fragment to *Nonomuraea* sp. ID06-A0189 genomic DNA indicated that the gene was carried on *Eco*RV- and *Cla*I-digested DNA fragments ~8.5–9.0 kb, 6.0 kb *Kpn*I fragment and 3.0 kb (the strong band) *Sma*I fragment (Figure1b). A second weak hybridization band in *Sma*I-digested DNA was likely a nonspecific signal resulting from the low stringency temperatures used for hybridization and washing, 55 °C. The 6.0-kb *Kpn*I fragment was large enough to contain the whole inulin fructotransferase gene, therefore, *Kpn*I was chosen for genomic DNA digestion for inverse PCR.

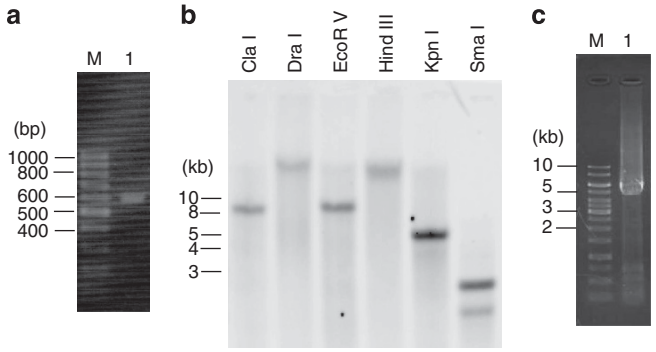

Figure 1 Amplification of inulin fructotransferase gene from *Nonomuraea* sp. ID06-A0189. (**a**) Amplification of partial inulin fructotransferase gene from genomic DNA of *Nonomuraea* sp. ID06-A0189. (lane 1); M indicates 100 bp DNA ladder, (**b**) Southern hybridization of *Nonomuraea* sp. ID06-A0189 genomic DNA using the inulin fructotransferase gene fragment (NSP17) as a probe. The restriction enzyme used for digestion of genomic DNA is indicated above each lane, (**c**) inverse PCR product amplified from *Kpn*I-digested chromosomal DNA. (lane 1); M indicates 1 kbp DNA ladder.

Inverse PCR produced a 6.0-kb fragment that corresponded to the 6.0-kb *Kpn*I fragment identified by hybridization (Figure 1c). A total of 1665 bp of sequence for the fragment was obtained. A 1326-bp open reading frame appeared to code the inulin fructotransferase gene. This open reading frame encoded a protein composed of a putative 37-amino acids signal peptide for secretion and a 404-amino acids mature protein. A putative ribosomal binding sequence, AGGAGG, was identified 12 bases upstream from the start codon. However, typical bacterial promoter sequences were not found upstream of the ribosomal binding sequence by *in silico* analysis. This open reading frame was named *Nsp-ift*, for the *Nonomuraea* sp. putative inulin fructotransferase gene. The nucleotide sequence of *Nsp-ift* has been deposited in the GenBank database under accession number AB775898.

Sequence similarity comparisons indicated that the deduced amino-acid sequence of *Nsp-ift* was 72% identical to inulin fructotoransferase in *Frankia* sp. EAN1pec. It was less similar to the deduced sequences from *Arthrobacter* sp. H65-7 (BAA18967.1), *A. globiformis* and *Bacillus* sp. snu-7 with 57, 56 and 56% identity, respectively. The lowest sequence similarity 54% was to *Arthrobacter* sp. H65-7 (BAD06469.1). The sequence identity among these inulin fructotransferases provides a means to determine interaction between active sites and substrates as well as the circulation mechanism of the difructose moiety as proposed by Jung *et al.*[23] Two amino-acid residues, Asp-233 and Glu-244 that were identified in *Bacillus* sp. snu-7 inulin fructotransferase (DFA III-forming), were predicted to have important roles in the cleavage of glycosidic linkage between the difructose moiety to adjacent fructose unit in the formation of dioxane ring of DFA III. Both residues were identified as strictly

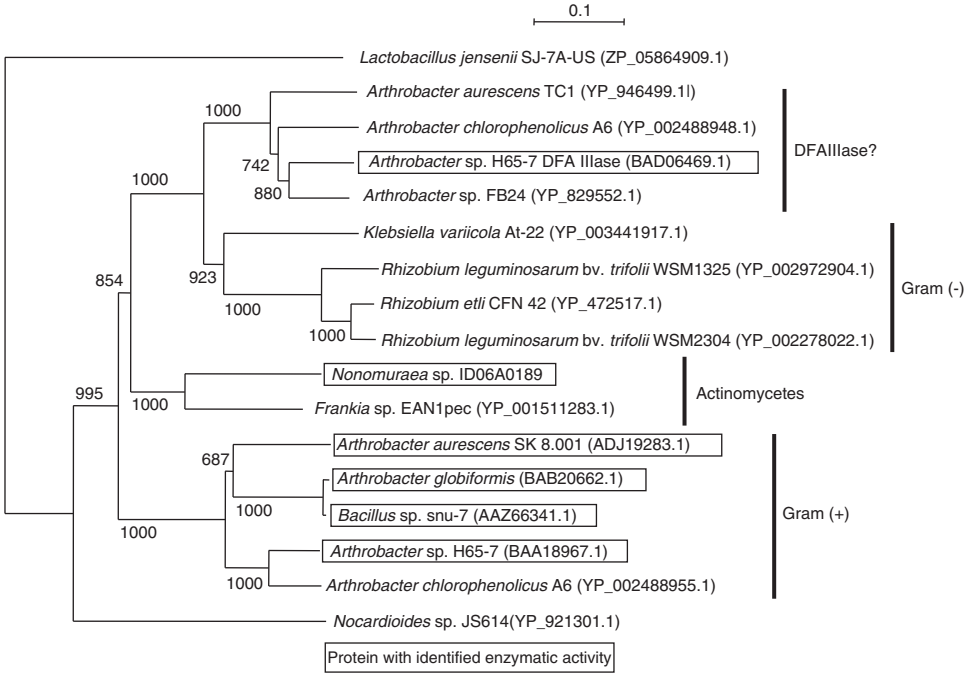

Figure 2 A neighbor-joining phylogenetic tree based on amino-acid sequences of known inulin fructotransferases and putative enzymes annotated in genome analyses of other bacterial species. Amino-acid sequences of *Nonomuraea* sp. ID06-A0189 and other enzymes are aligned and a neighbor-joining tree was constructed without sequence positions with gaps. Accession numbers are indicated in parentheses after sequence names. Bootstrap values calculated on 1000 iterations were indicated on corresponding clades. Known enzymes with identified activity are indicated by boxes. Clade names are indicated on the right side of corresponding clades.

conserved sequences (data not shown) in all five known enzymes (marked with boxes), as shown in Figure 2.

Phylogenetic analysis indicated that the deduced inulin fructotansferases and related sequences made four distinct clades (Figure 2). The putative *Nsp-ift* protein sequence made a distinct clade with the deduced inulin fructotransferase (DFA I-forming) of *Frankia* sp. EAN1pec. The other previously studied inulin fructotansferases made a second clade with the exception of *Arthrobacter* sp. H65-7 (BAD06469.1) that made a third clade with hypothetical enzymes found in the genomes of other *Arthrobacter* species. These three *Arthrobacter* enzymes were identified only by sequence annotation and not functionally tested; however, because of their sequence similarity, it is possible that they have DFA IIIase activity. The fourth clade consists of four hypothetical enzymes from Gram-negative bacteria, in which inulin fructotransferase has not been reported. Existence of a similar gene in *Rhizobium* species is interesting for future studies on the potential role of inulin fructotransferase-related genes in plant–microbe interactions. The deep branching of the deduced *Nsp-ift* amino-acid sequence from other inulin fructotransferases[16–18] indicates the distinction of *Nsp-ift* protein primary structure that contributes to its greater DFA IIIase activity. This makes it ideal for the development of DFA III production by accumulation in the medium along with the observation that *Nonomuraea* sp. ID 06-A0189 does not assimilate DFA III as carbon source.

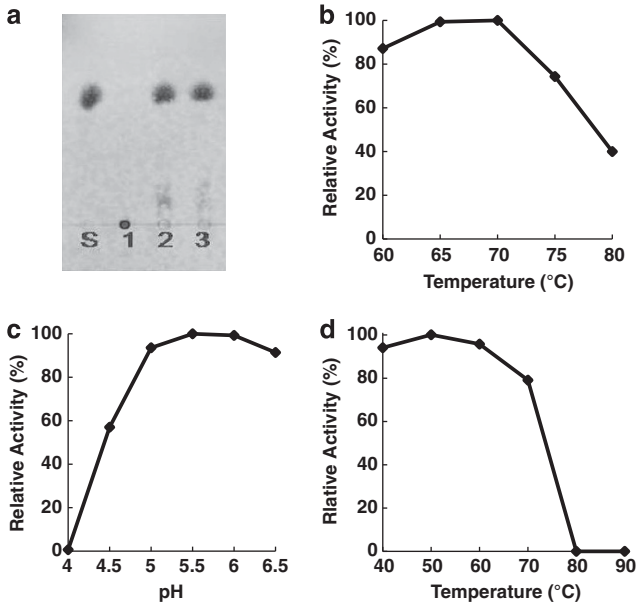

Figure 3 Activity of *Nonomuraea* sp. ID06-A0189 recombinant inulin fructotransferase. (**a**) Thin-layer chromatogram of enzyme reaction product from inulin. S indicates DFA III standard; 1 Inulin reacted with crude extract of pET28(b) blank vector for 10 min; 2 Inulin reacted with crude extract of recombinant *Nonomurea* sp. ID06-A0189 inulin fructotransferase for 10 min; 3 Inulin reacted with purified recombinant *Nonomurea* sp. ID06-A0189 inulin fructotransferase for 10 min. (**b**) Effect of temperature on enzyme activity. The enzyme reaction was performed in 10 mM citric acid–NaOH buffer at a pH of 5.6, at temperatures ranging from 60–80 °C for 1 h. (**c**) Effect of pH on enzyme activity. The enzyme reaction was performed at different pH values (4.0–6.5) at 65 °C for 1 h. (**d**) Thermal stability of enzyme. The enzyme was heated to various temperatures (40–90 °C) for 20 min. The residual activity was measured at 65 °C and pH of 5.5 for 1 h. Relative activity (%) for optimum pH and temperature was calculated based on the highest enzyme activity, which is shown as 100% of the relative activity. For thermal stability, the residual activity was compared with the activity before heating.

Expression, purification and properties of recombinant inulin fructotransferase

The recombinant enzyme without a signal sequence was expressed in *E. coli* BL21(DE3) and the cell-free extract produced DFA III from inulin (Figure 3a) with enzyme activity of 543 U ml^{-1}. This value was ninefold higher than that produced by the wild strain (60.3 U ml^{-1}).[19] Purification using His-tag affinity chromatography increased the purity of recombinant enzyme two fold higher than that of its crude extract with specific activity of 1991 U per mg protein. The purity was confirmed by SDS-PAGE, in which even though many other protein bands still existed, the major band corresponding to inulin fructotransferase was clearly observed with an estimated MW of 43 kDa (data not shown).

Optimal activity of the purified recombinant enzyme was observed over a temperature range of 65–70 °C (Figure 3b) and a pH range of 5.5–6.0 (Figure 3c). The enzyme was stable up to 70 °C and retained 79% its activity after treatment for 20 min (Figure 3d). Those properties were almost identical to those of the native enzyme[19] and generally did not significantly differ from other similar enzymes (Table 2). The recombinant enzyme showed somewhat higher activity at sub-optimum pH as compared with the native enzyme but this might be due to the influence of contaminated protein in the native enzyme, which was characterized as crude enzyme.[19] As can be seen in Table 2, the production level of recombinant inulin fructotransferase from *Nonomuraea* sp. ID-A0189 was higher than those of other similar enzymes reported from *Arthrobacter* sp. H65-7 (180 000 U l^{-1}),[16] *A. globiformis* C11-1 (1500 U l^{-1})[17] and *A. aurescens* SK 8.001 (81 000 U l^{-1}).[24]

Table 2 Properties of recombinant inulin fructotransferases from *Nonomuraea* sp. ID-06A0189 and other bacteria

				Thermostability		
Microorganism	*Enzyme activity (U l^{-1})*	*Optimal pH*	*Optimal temperature (°C)*	*(°C)*	*Time (min)*	*Reference*
Nonomuraea sp. ID-A0189	543 000	5.5–6.0	65–70	70	20	This work
Arthrobacter sp. H65-7	180 000					Yokota *et al.*[11]
Arthrobacter sp. A-6		6.0	70	70	300	Kim *et al.* [25]
A. globiformis C11-1	1500	5.0	50–60	75		Haraguchi *et al.*[17]
Bacillus sp. snu-7	1 735 000	5.5–6.0	40	60	10	Kim *et al.*[18]
A. aurescens SK 8.001	81 000	6.0	55	60	240	Zhao *et al.*[24]

Even though the production level of *Nonomuraea* sp. ID-A0189 recombinant inulin fructotransferase was lower than that produced by *Bacillus* sp. snu-7, the thermostability was higher. Because of high thermostability and conversion yield,[19] this enzyme can be applied for the production of DFA III in large scale where inulin fructotransferase with high activity is needed. This expression system seems appropriate for satisfying this need. Besides that, information on characteristics of the purified recombinant enzyme is necessary for optimization of DFA III production to get high conversion yield. Further, for more efficient and massive production of this intracellular recombinant enzyme, development of fermentation process and technology is required.

As shown in Figure 3, it seems that fructose was not produced from inulin by the enzyme. In our previous work, we found that the native crude enzyme not only converted inulin into DFA III (the major product) and small amounts of 1-kestose (GF2), 1-nystose (GF3) and 1-fructosylnystose (GF4) but also a very small amount of fructose.[19] This finding suggested that the strain might also secrete other inulin-degrading enzyme to produce fructose. Thus, removing the inulin-degrading gene by cloning technology increased productivity of inulin fructotransferase.

Conservation of *Nsp-ift* among *Nonomuraea* species

Conservation of *Nsp-ift* among *Nonomuraea* species was investigated by Southern hybridization and PCR amplification of *ift* gene and enzyme activity measurements. All results indicated that *Nsp-ift* was present in *Nonomuraea* sp. ID06-A0189 but none of the other *Nonomuraea* species tested. This finding indicates that *Nsp-ift* is a specific gene in *Nonomuraea* sp. ID06-A0189.

In conclusion, inulin fructotransferase from *Nonomuraea* sp. ID-A0189 was cloned and characterized in this study. The gene indicated weak DFA IIIase activity that makes it possible for the development of DFA III production by accumulation in the medium and the enzyme has potential properties for large-scale production of DFA III. Further study on this enzyme should contribute for molecular characterization of the enzymatic reaction and efficient production of DFA III.

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