**技術報告**

# Production and Immobilization of an Inulin Fructotransferase (DFA Ⅰ-producing) from *Arthrobacter ureafaciens* A51-1.

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## Abstract

A bacterium strain A51-1 producing an inulin fructotransferase (DFA Ⅰ-producing) [EC 2.4.1.200] in the culture supernatant was isolated. The strain A51-1 was identified as *Arthrobacter ureafaciens*. The maximum enzyme activity (1.1 U/ml) was obtained with a medium containing 1.5 % yeast extract. The enzyme showed maximum activity at pH 5.5 and 40℃. The enzyme was tolerant up to 70℃. An immobilized enzyme of an inulin fructotransferase (DFA Ⅰ-producing) was prepared using Chitopearl BCW3510 as a carrier, for the first time. The immobilized enzyme can be used eight times without a significant loss of the activity.

## Introduction

Inulin is a polysaccharide contained in chicory, dahlia, Jerusalem artichoke and other plants. The chemical structure of inulin is a β-2, 1 linked fructose polymer terminated with a sucrose residue. In the course of study of inulin decomposing enzymes, inulinase [EC 3.2.1.7] from *Penicillium* was reported[1]. Subsequently, a new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered [2]. The enzyme converted inulin into an oligosaccharide DFA Ⅲ (di-D-fructofuranose 1, 2 : 2, 3 ' dianhydride) and a small amount of other oligosaccharides. This enzyme was designated as inulin fructotransferase (DFA Ⅲ-producing) [EC 2.4.1.93]. Since then, there have been several reports on the inulin fructotransferase (DFA Ⅲ-producing) from *Arthrobacter* species[3),4),5),6)]. Kang et al.[7)] reported the enzyme from *Bacillus* sp.

We have found another type of inulin decomposing enzyme[8)] from *Arthrobacter globiformis* S14-3; the enzyme converted inulin into oligosaccharide DFA Ⅰ (di-D-fructofuranose 1, 2 : 2, 1 ' dianhydride) and a small amount of other oligosaccharides. This enzyme was designated as inuliun fructotransferase (DFA Ⅰ-producing) [EC 2.4.1.200]. Other enzymes producing DFA Ⅰ from inulin have been reported from *Streptomyces* sp.[9)] and *Arthrobacter* sp.[10)]. The DFA Ⅰ has half the sweetness of sucrose. Recently, Goto[11)] reported that DFA Ⅰ did not cause an increase of blood sugar after assimilation in rats. Therefore, DFA Ⅰ has potential as a new type of a low calorie sweetener.

Recently, we isolated a microorganism, strain A51-1, which produced an inulin fructotransferase (DFA Ⅰ-producing) in the culture supernatant. In this paper, we describe the identification of the microorganism, the conditions for the enzyme production, properties of a crude enzyme, and the immobilization of the enzyme. This is the first report on successful immobilization of the inulin fructotransferase (DFA Ⅰ-producing).

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## Materials and Methods

### Microorganism and identification

We isolated a bacterium, strain A51-1, from a soil sampled from Hokkaido, Japan. For the extraction of genome DNA of the microorganism, Prepman method (Applied Biosystems, Co. Ltd., USA) was used. The amplification of 16S rDNA was performed using PCR system 9600 (Applied Biosystems Co. Ltd.). The sequencing of 16S rDNA (500bp) was performed with ABI PRISM 3100 DNA sequencer (Applied Biosystems Co. Ltd.). The homology analysis of the 16S rDNA was performed using Microseq bacterial 500 library v.0023 (Applied Biosystems Co. Ltd.).

### Cultivation of microorganism

The microorganism was cultured in a 500 ml shaking flask at 30℃ for 24 h. The medium was composed of 0.4% $Na_2HPO_4 \cdot 12H_2O$, 0.1% $KH_2PO_4$, 0.1% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% yeast extract (Difco), and 0.3% inulin, pH was adjusted to 7.0. This culture was used as a pre-culture when needed. For the investigation of the effect of culture conditions, the pre-culture (5 ml) was introduced to a 500 ml shaking flask containing 100 ml of a basal medium and incubated for 24h at 30℃. The basal medium was composed of 0.4 % $Na_2HPO_4 \cdot 12H_2O$, 0.1 % $KH_2PO_4$, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.001 % $CaCl_2 \cdot 2H_2O$, 0.001 % $FeSO_4 \cdot 7H_2O$, 0.3 % inulin, and a nitrogen source (adjusted to pH 7.0). After cultivation, the cells were removed by a centrifugation (8000 xg, 30 min) and the supernatant was used as a crude enzyme solution.

### Standard assay methods

For the measurement of the enzyme activity, 0.1M citrate buffer, pH5.5 (0.5 ml), the enzyme solution (0.2 ml), water (0.3 ml), and 2% inulin (1.0 ml) were mixed. The enzyme reaction was performed at 40℃ for 30 minutes and the reaction was stopped by heating at 100℃ for seven minutes. The DFA Ⅰ produced was determined by HPLC (column, Shim-pack CLC ODS, 4.6 mm x 25 cm (Shimadzu Co. Ltd., Kyoto); mobile phase, water; detector, refractive index detector). One unit of the enzyme was defined as the amount of enzyme which can produce 1 μmole of DFA Ⅰ per min at pH5.5 and 40℃.

### Preparation of immobilized enzyme

For the preparation of an immobilized enzyme, Chitopearl BCW3510 (5g, Fuji Bohseki, Japan) was suspended in 10 mM citrate buffer, pH 5.5 (50 ml) to which, the crude enzyme solution (10 ml, 1 unit/ ml) was added and incubated at 30℃ for one hour with shaking. The prepared immobilized enzyme was washed once with water. The washed immobilized enzyme was treated with 2.5% glutalaldehyde with shaking for one hour at 30℃ to stabilize the enzyme activity. The treated enzyme was washed thoroughly with water and used as the immobilized enzyme.

### Repeated use of immobilized enzyme

The immobilized enzyme sample (2.0 g) was put in a 50 ml Erlenmeyer flask, and the reaction was started with the addition of 1 % inulin (5.0 ml) containing 10 mM citrate buffer, pH 5.5. The enzyme reaction was performed at 40℃ for 30 minutes with shaking. After the reaction, the reaction mixture was moved to a test tube and heated at 100℃ for five minutes to stop the enzyme reaction. After cooling, DFA Ⅰ produced was determined by HPLC (column: Shim-pack CLC ODS, mobile phase: water). To the Erlenmeyer flask containing the immobilized enzyme, a new inulin solution was added and the enzyme reaction was performed repeatedly.

## Results and discussion

### Identification of microorganism.

Table 1 summarizes the taxonomic characters of strain A51-1. The strain was a gram-positive non-spore forming aerobic rod. It was catalase positive and oxidase negative.

**Table 1 Taxonomic characters of strain A51-1**

| | |
|---|---|
| shape and size | rod: 0.7 x 1.0~1.2 μm |
| Gram staining | positive |
| spore formation | − |
| motility | − |
| catalase | + |
| oxidase | − |
| O-F test | − |
| alkaline phosphatase | + |
| β-galactosidase | + |
| α-glucosidase | + |
| reduction of nitrate | − |
| urease | − |
| gelatin hydrolysis | + |

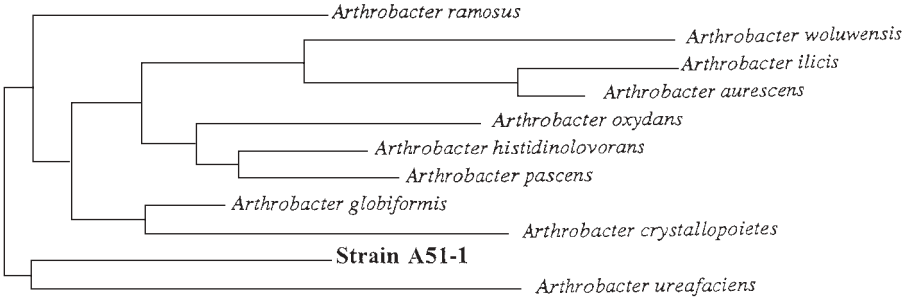


**Fig. 1 Molecular genealogical analysis of 16S rDNA.**

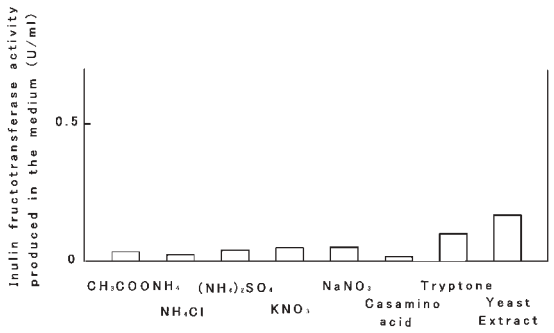


**Fig. 2 Effect of nitrogen source on the enzyme production.**

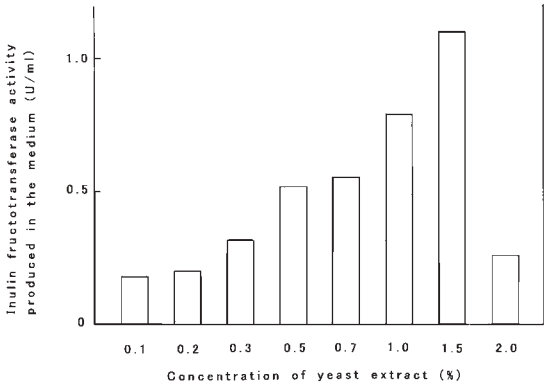


**Fig. 3 Effect of yeast extract on the enzyme production.**

From these results, the strain A51-1 was assigned to be a coryneform bacterium. The 16S r DNA sequence (500 bp) of the strain A51-1 was determined. Fig. 1 shows the result of molecular genealogical tree analysis of 16S rDNA. The strain A51-1 formed the same cluster as the type strain of *Arthrobacters ureafaciens*. From these results, the strain A51-1 was identified as *Arthrobacter ureafaciens* A51-1.

## Effect of nitrogen source for the production.

The pre-culture (5 ml) was introduced to the basal medium containing various nitrogen sources (0.2 %) and cultured at 30℃ for 24 h. After cultivation, the cells were removed by centrifugation and the enzyme activity in the culture supernatant was measured. As shown in Fig. 2, the medium containing yeast extract as a nitrogen source gave rise to the highest activity.

## Effect of concentration of yeast extract for enzyme production

The pre-culture (5 ml) was introduced to the basal medium containing various concentrations of yeast extract and cultured at 30℃ for 24 h. After cultivation, the enzyme activity in the culture supernatant was measured. As shown in Fig. 3,

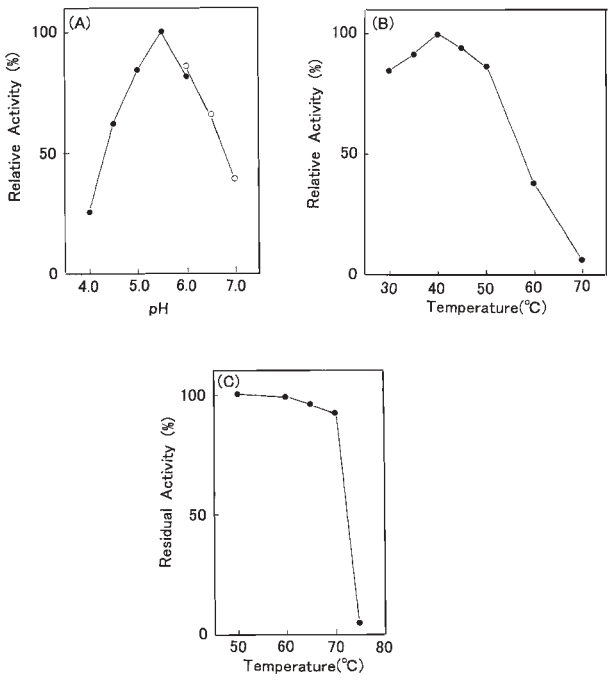


**Fig.4 (A) Effect of pH on the enzyme activity. (●), citrate buffer; (○), phosphate buffer.**
**(B) Effect of temperature on the enzyme activity.**
**(C) Thermal stability of the enzyme.**

the maximum activity (1.1 U/ml) was obtained when the concentration of yeast extract was 1.5 %. We previously reported that the activity of crude enzyme from *Arthrobacter globiformis* S14-3 was 0.2 U / ml[8]. The enzyme activity obtained from *Arthrobacter ureafaciens* A51-1 was therefore over 5 times of that of *A. globiformis* S14-3.

### Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was investigated in the pH range 4.0-7.0 at 40℃.

As shown in Fig. 4(A), maximum activity was detected at pH 5.5. Then, the enzyme reaction was performed in the range 30-70℃ at a fixed pH of 5.5. As shown in Fig. 4(B), the temperature for maximum activity was 40℃.

### Thermal stability of enzyme

The enzyme solution was heated at various temperatures for 30 minutes at pH 5.5. After the treatment, the residual activities were measured at pH 5.5 and 40℃. As shown in Fig.4 (C), the enzyme was tolerant up to 70℃ but its activity ceased when treated at 75℃.

### Repeatability of immobilized enzyme

The prepared immobilized enzyme (5g) had an activity of 0.38 units/g and the original free enzyme used was 10 units. Therefore, the recovery of the enzyme activity on immobilization was about 20 %. Fig. 5 shows the results of repeated use of the immobilized enzyme. The reactions were performed eight times and there was no significant loss of the enzyme activity even after the repeated use of the immobilized enzyme. This result suggests that a bio-reactor using the immobilized enzyme may be applicable for the large scaled production of oligosaccharide DFA Ⅰ.

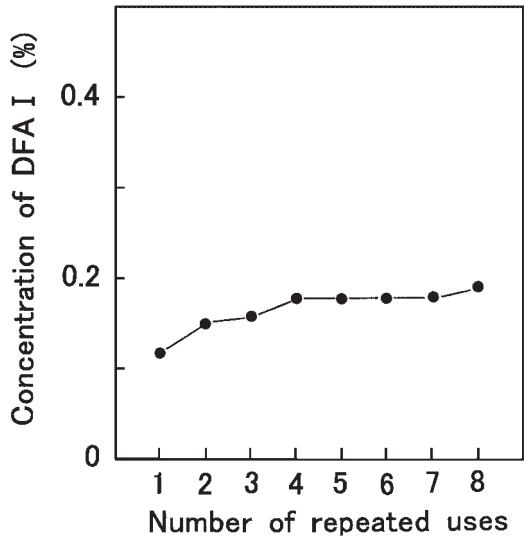


**Fig. 5 The repeated use of the immobilized enzyme.**

### Conclusion

In this report, we described the identification of the new microorganism producing inulin fructotransferase (DFA Ⅰ-producing), together with conditions for the enzyme production, properties of the enzyme, and for the first time, the immobilization of the enzyme. The prepared immobilized enzyme was demonstrated to be usable eight times without a significant loss of the activity. Our result suggests that a bio-reactor is applicable for the big scaled production of oligosaccharide DFA Ⅰ.

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## *Arthrobacter ureafaciens*のInulin fructotransferase (DFAⅠ-producing)の生産と固定化

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培養上清中にイヌリンからオリゴ糖DFAⅠを生成する酵素の生産菌（A51-1）株を新たに分離した。

分類学的な検討の結果、本菌株は*Arthrobacter ureafaciens* と同定された。酵素の生産条件について検討したところ、窒素源として酵母エキスを用いその濃度が1.5%のときにもっとも高い酵素活性が得られた。本酵素はpH5.5, 40℃で最大の高い活性を示し、70℃まで安定であった。キトパールBCW3510を担体とした固定化酵素の調製についても検討した。これはDFAⅠ合成酵素の固定化に関する初めての報告である。本固定化酵素の繰り返し使用を行ったところ、８回の使用後も酵素活性の低下はみられなかった。