Technical note

# Production and Immobilization of Thermostable Oligosaccharide DFA III Producing Enzyme from *Arthrobacter* sp. L68-1.

Kazutomo Haraguchi* and Ken ichi Ohtsubo
National Food Research Institute, 2-1-12 Kannondai, Tsukuba-shi, Ibaraki 305-8642, Japan

## Abstract

A bacterium producing a thermostable inulin fructotransferase (DFAIII-producing) in a culture supernatant was isolated. Through taxonomical studies the strain was identified as *Arthrobacter* sp. L68-1. The inulin fructotransferase (DFAIII-producing) of the strain showed maximum activity at pH 5.5 and 55℃. The enzyme activity was stable up to 80℃ after 30 min heat treatment. This heat stability was the highest of the inulin fructotransferases (DFAIII-producing) reported to date. The maximum enzyme activity (5.0 U/ ml) was obtained with a medium containing 0.5% Tryptone. An immobilized enzyme was prepared using Chitopearl BCW3510 as a carrier. The immobilized enzyme was able to be used eight times without a significant loss of the enzyme activity.

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## Introduction

Inulin is a storage 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 studies of inulin decomposing enzymes, inulinase [EC 3.2.1.7] from Penicillum sp. was reported[1] (Nakamura & Hoashi 1969). Subsequently, a new type of inulin decomposing enzyme produced by *Arthrobacter ureafaciens* was discovered[2] (Uchiyama et al. 1973). 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]. Subsequently, there have been several reports on the inulin fructotransferase (DFA III-producing) from *Arthrobacter* species[3), 4), 5), 6)] (Haraguchi et al. 1988; Kawamura et al. 1988; Yokota et al. 1991; Haraguchi et al. 2002). Kang et al. reported[7)] on the enzyme from *Bacillus* sp..

Chicory is a possible candidate for an alternative crop to sugar beet and it contains inulin. The DFA III, which can produce from chicory, has half the sweetness of sucrose. Recently, Goto[8)] reported that DFA III did not cause an increase in blood sugar after assimilation (using rats). Therefore, DFA III has the potential as a new type of a low calorie sweetener.

Recently, we isolated a microorganism, strain L68-1, which produced a thermostable inulin fructotransferase (DFAIII-producing) in a culture supernatant. In this paper, we describe the identification of the microorganism, the properties of the crude enzyme, conditions for the enzyme production, and the immobilization of the enzyme.

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*Corresponding author: Kazutomo Haraguchi
Fax: +81-0298-38-7996, e-mail: haraguti@nfri.affrc.go.jp

## Materials and methods

### Microorganism and identification

We isolated a bacterium, strain L68-1, from a soil sampled in Ibaraki prefecture, Japan. For the extraction of genome DNA, the Prepman method (Applied Biosystems, Co. Ltd., USA) was used. The amplification of 16 S rDNA was performed using PCR system 9600 (Applied Biosystems Co. Ltd.). The sequencing of DNA was performed with a ABI PRISM 3100 DNA sequencer (Applied Biosystems Co. Ltd.). The homology analysis was performed using the Microseq bacterial 500 library v.0023 (Applied Biosystems Co. Ltd.).

### Cultivation of microorganism

The microorganism was cultured in a 500 ml shaking flask with 100 ml of medium at 30℃, for 24h. 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 7.0. This culture was used as a pre-culture when needed. For the preparation of a crude enzyme, the cells were removed by centrifugation (8000 x g, 30 min) and the supernatant was used as a crude enzyme solution. For the investigation of effect of culture conditions, the pre-culture (5 ml) was inoculated to a 500 ml shaking flask containing 100 ml of a basal medium and cultured 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, pH7.0. After cultivation, the cells were removed by a centrifugation (8 000 x g, 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 55℃, for 30 min, and the reaction was stopped by heating at 100℃, for 7 min. The DFAⅢproduced 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 μ mole of DFAⅢ per min at pH5.5 and 55℃.

### Preparation of immobilized enzyme

For the preparation of immobilized enzyme, Chitopearl BCW3510 (5 g, Fuji Bohseki, Japan) was suspended in 10 mM citrate buffer, pH5.5 (50 ml). The crude enzyme solution (20 ml, 5 units/ ml) was added to it and incubated at 30℃ for 1h while shaking. Afterwards, the prepared immobilized enzyme was washed once with water. The washed immobilized enzyme was treated with 2.5% glutalaldehyde for 1h at 30℃ to stabilize the activity. The treated enzyme was washed thoroughly with water, and used as the prepared immobilized enzyme.

### The enzyme reaction using immobilized enzyme

The immobilized enzyme (3.0 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 55℃ for 30 min while shaking. After the reaction, the reaction mixture was moved to a test tube and heated at 100℃ for 5 min to stop the enzyme reaction. After cooling, the DFAⅢ produced was determined by HPLC (column: Shim-pack CLC ODS, mobile phase: water). To the Erlenmeyer flask containing the immobilized enzyme, the new inulin solution was added and the enzyme reaction was performed repeatedly.

## Results and discussion

### Identification of microorganism.

Table 1 summarizes the taxonomic characteristics of strain L68-1. The strain L68-1 was a gram-positive non-spore producing aerobic rod. And the strain was catalase positive and oxidase negative. From these results, the strain L68-1 was

**Table 1 Taxonomic characteristics of strain L68-1**

| | |
|---|---|
| shape and size | rod： 0.6-0.7 x 1.5-2.0 μm(24h)<br>0.5-0.6 x 1.0-1.2 μm(72h) |
| Gram staining | positive |
| spore formation | − |
| motility | ＋ |
| catalase | ＋ |
| oxidase | − |
| alkaline phosphatase | ＋ |
| β-galactosidase | ＋ |
| α-glucosidase | ＋ |
| reduction of nitrate | − |
| urease | − |
| gelatin hydrolysis | − |
| O-F test | − |

estimated to be a coryneform bacterium. Table 2 shows the result of homology analysis of 16S rDNA. The 16 S rDNA sequence (500 bp) of strain L68-1 showed homology of 97.22% with the strain of *Arthrobacter aurescens*. Fig.1 shows the result of molecular genealogical tree analysis. The strain L68-1 formed a single branch and did not form the same cluster with any type of strain of *Arthrobacters*. From these results, the strain L68-1 was identified as *Arthrobacter* sp. L68-1.

### Effect of pH and temperature on enzyme activity

The effect of pH on the enzyme activity was investigated in the pH range 4.0-8.0 at 55℃. As shown in Fig.2 (A), maximum activity was obtained at pH5.5. The enzyme reaction was performed in the range 30-70℃ at pH5.5. As shown in Fig.2 (B), maximum activity was obtained at 55℃. For the industrial application of the enzyme, it is desirable react over 60℃ because of sanitation. This enzyme retains more than 90% of the maximum activity at 60℃ Therefore, this enzyme is suitable for the large scale production of oligosaccharide DFAⅢ.

**Table 2 The result of 16S rDNA sequence homology analysis**

| | |
|---|---|
| 97.22 % | *Arthrobacter aurescens* |
| 96.62 % | *Arthrobacter ilicis* |
| 95.02 % | *Arthrobacter histidinolovorans* |
| 94.43 % | *Arthrobacter woluwensis* |
| 93.84 % | *Arthrobacter ramosus* |
| 93.24 % | *Arthrobacter agilis* |
| 91.85 % | *Arthrobacter oxydans* |
| 91.45 % | *Arthrobacter pascens* |
| 91.05 % | *Arthrobacter protophormiae* |
| 90.85 % | *Arthrobacter crystallopoietes* |

### Thermal stability

The enzyme solution was heated at various temperatures for 30 min at pH5.5. After the treatment, the residual activities were measured at pH5.5 and 55℃. As shown in Fig.2 (C), the enzyme was stable up to 80℃, but it was inactivated at 85℃. This heat stability was highest in the inulin fructotransferase (DFA Ⅲ-producing) reported to date. For the industrial application of the enzyme, heat stability is one of the most important factors. Therefore, it would be advantageous to use this enzyme for the large scale production of oligosaccharide DFAⅢ. Table 3 summarizes the properties of the inulin fructotransferases (DFAⅢ-producing) from various microorganisms.

### Effect of nitrogen source for enzyme production.

The pre-culture (5 ml) was inoculated to the basal medium containing various nitrogen sources (0.1%) and cultured at 30℃ for 24h. After cultivation, the cells were removed by centrifugation and the enzyme activity in the culture supernatant was measured. As shown in Fig.3, the medium containing Tryptone as a nitrogen source gave the highest activity.

### Effect of concentration of Tryptone for enzyme production

The pre-culture (5 ml) was inoculated to the basal medium containing various concentrations of Tryptone and cultured at 30℃ for 24h. After cultivation, the enzyme activity in the culture supernatant was measured. As shown in Fig.4, the

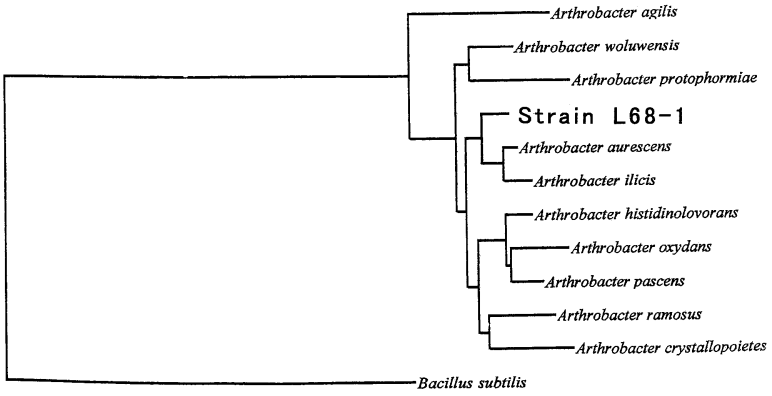


**Fig.1 Molecular genealogical tree analysis of 16S rDNAs**

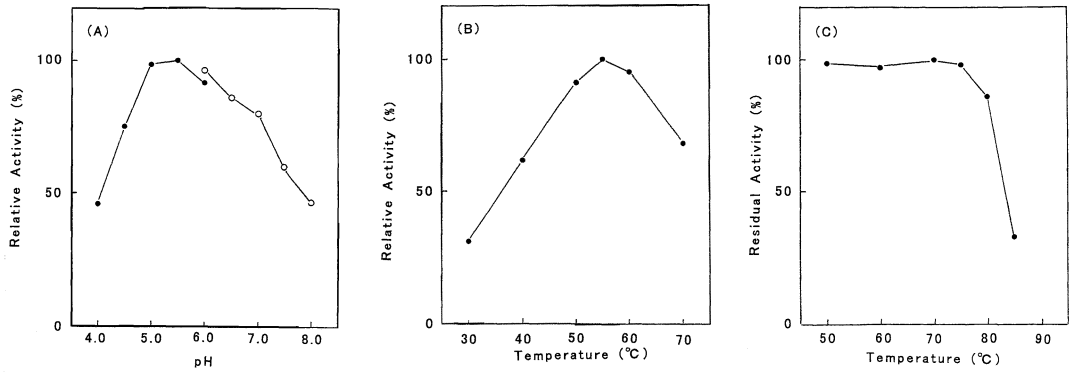


**Fig.2 (A) Effect of pH on enzyme activity ●, Citrate buffer; ○, Phosphate buffer**
**Fig.2 (B) Effect of temperature on enzyme activity**
**Fig.2 (C) Thermal stability of enzyme**

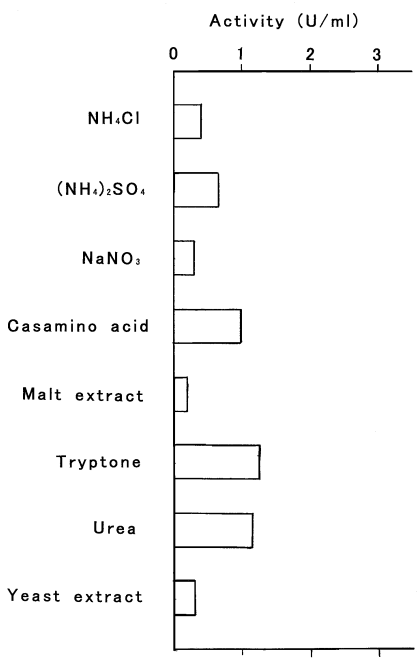


**Fig.3 Effect of nitrogen source on enzyme activity The concentration of nitrogen sources is 0.1%.**

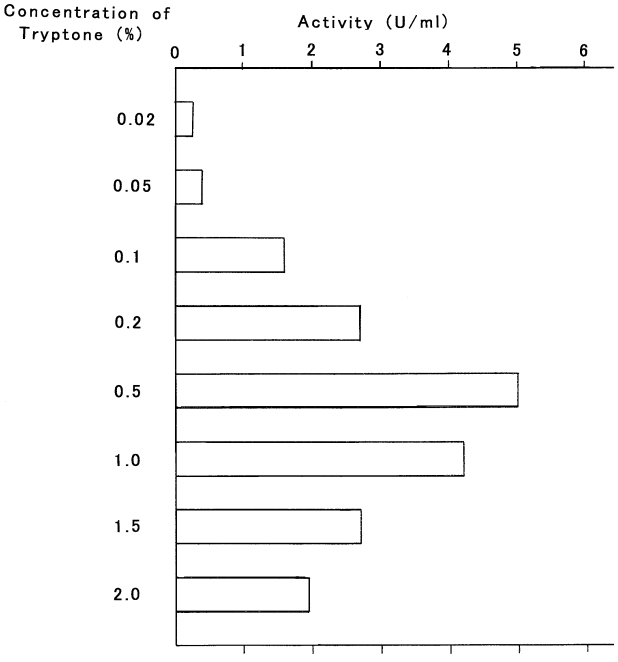


**Fig.4 Effect of concentration of Tryptone on enzyme activity**

**Table 3 Comparison of properties of inulin fructotransferase (DFA III-producing) from different microorganisms**

| Microorganisms | Optimum | | Heat stability | References |
|---|---|---|---|---|
| | pH | ℃ | ℃ (time) | |
| *Arthrobacter sp.* L68-1 | 5.5 | 55 | 80 (30 min) | This work |
| *A. ureafaciens* | 6.0 | 50 | 50 (30 min) | Uchiyama et al.(1973) |
| *A. globiformis* C11-1 | 5.0 | 55 | 75 (20min) | Haraguchi et al. (1988) |
| *A. ilicis* OKU17B | 5.5 | 60 | 70 (30 min) | Kawamura et al. (1988) |
| *Arthrobacter* sp. H65-7 | 5.5 | 5.5 | 70 (20 min) | Yokota et al. (1991) |
| *A. pascens* T13-2 | 5.5-6.0 | 50 | 75 (20 min) | Haraguchi et al.(2002) |
| *Bacillus* sp. snu-7 | 6.0 | 40 | 60 (10 min) | Kang et al. (1998) |

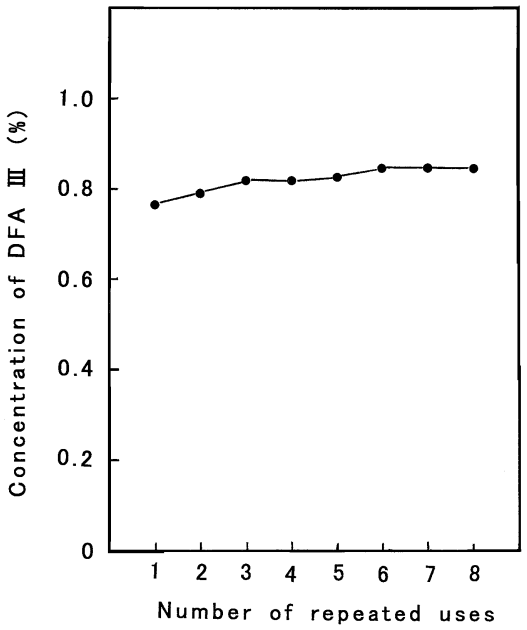


**Fig.5 Repeated uses of immobilized enzyme**

maximum activity (5.0 U/ml) was obtained when the concentration of Tryptone was 0.5%.

### Reaction with immobilized enzyme

The prepared immobilized enzyme had an activity of 2.6 units/ g. Therefore, the recovery of the enzyme activity on immobilization was about 13%. 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 enzyme activity during the repeated use of the immobilized enzyme. This result suggests that a bio-reactor using the immobilized enzyme is applicable for the large scale production of oligosaccharide DFAⅢ.

## Conclusion

In this report, we described the identification of the microorganism, properties of the crude enzyme, conditions for the enzyme production, and the immobilization of the enzyme. The enzyme was stable up to 80℃ and this heat stability was highest in the enzymes reported to date. The prepared immobilized enzyme was able to use eight times without a significant loss of the activity. This result suggests that a bio-reactor is applicable for the large scale production of oligosaccharide DFAⅢ.

## References

1) Nakamura, T. and Hoashi, S. Culture conditions for inulase production by *Penicillum. Nippon Nogeikagaku Kaishi* ,43, 599-605 (1969) .

2) Uchiyama T., Niwa S. and Tanaka K. Purification and properties of *Arthrobacter ureafaciens* inulase Ⅱ. *Biochim. Biophys.* Acta 315: 412-420 (1973) .

3) Haraguchi, K., Kishimoto, M., Seki, K., Kobayashi, S. and Kainuma, K. Purification and properties of inulin fructotransferase (depolymerizing) from *Arthrobacter globiformis* C11-1. *Agric. Biol. Chem.* , 52, 291-292 (1988) .

4) Kawamura, M., Takahashi, S. and Uchiyama T. Purification and some properties of inulin fructotransferase (depolymerizing) from *Arthrobacter ilicis. Agric. Biol. Chem*., 52, 3209-3210 (1988) .

5) Yokota A., Enomoto K. and Tomita F. Purification and properties of an inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. H65-7. *J . Ferment. Bioeng*., 72: 262-265 (1991) .

6) Haraguchi, K., Yamanaka, T. and Ohtsubo, K. Purification and properties of a heat stable inulin fructotransferase (DFAⅢ-producing) from Arthrobacter pascens T13-2. *Carbohydrate Polymers,* 52, 117-121 (2002) .

7) Kang, S., Kim, W., Chang, Y. and Kim, S. Purification and properties of inulin fructotransferase (DFAⅢ-producing) from *Bacillus* sp. snu-7. *Biosci. Biotech. Biochem*., 62, 628-631 (1998) .

8) Goto, K. Fructo-oligosaccharides from yacon (in Japanese). *The abstracts of "Bio-Renaissance program"*, ministry of agriculture, forestry and fisheries, Japan, 9-10 (2002) .

# *Arthrobacter* sp.L68-1の耐熱性DFAIIIオリゴ糖合成酵素の生産と固定化

原口和朋・大坪研一
独立行政法人食品総合研究所

耐熱性のDFAⅢオリゴ糖合成酵素を培養上清中に生産する細菌が分離された。分類学的検討の結果本菌株は*Arthrobacter* sp. L68-1と同定された。本菌株の生産するDFAⅢオリゴ糖合成酵素はpH5.5, 55℃において最高の活性を示した。本酵素は30分間の加熱に対して80℃まで安定であった。この耐熱性はこれまでに報告されたDFAⅢ合成酵素の中で最高であった。酵素の生産条件について検討すると0.5%のトリプトンを含有する培地を用いた場合に最も高い活性が得られた。キトパールBCW3510を担体として固定化酵素を調製した。この固定化酵素を８回繰り返し使用したが、活性の低下は見られなかった。