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Production of Inulin Fructotransferase(depolymerizing) from *Flavobacterium* sp. LC-413

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Abstract

A bacterial strain LC-413, producing extracellular inulin fructotransferase which converts inulin into di-D-fructofuranose dianhydride(DFAIII) and small amount of oligosaccharides, was isolated from soil and presumed as *Flavobacterium* sp. LC-413. The enzyme production was induced by inulin as carbon source and enhanced by the addition of 0.3% malt extract and 0.2% $NaNO_3$ as nitrogen source. The enzyme activity in the culture supernatant reached at the maximum, 78.6units/ml, after 11 hours of cultivation in the medium composition of 1.5% inulin, 0.2% $NaNO_3$, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, a trace amount of $FeSO_4 \cdot 7H_2O$, and 0.3% malt ext. at 30°C. The oligosaccharide produced by enzyme reaction from inulin was identified as DFAIII by TLC and ^{13}C-NMR spectroscopy.

Key words: *Flabobacterium* sp. LC-413, inulin fructotransferase, DFA III

INTRODUCTION

Inulin is a linear β-2,1 linked polyfructan terminated by a sucrose residue and contained as reserve carbohydrate in Jerusalem artichoke, dahlia, chicory and so on.

Among inulin-decomposing enzymes, inulinases(EC 3. 2. 1. 7) which split β-2,1 fructofuranosidic linkages of inulin through either exo- or endo-type reaction have been reported in several microorganisms(1-3). Another type of inulin-decomposing enzyme is inulin fructotransferase(EC 2. 4. 1. 93). This enzyme was originally found in the culture broth of a bacterium, *Arthrobacter ureafaciens*, and has been shown to convert inulin into di-D-fructofuranose-1,2′ : 2,3′-dianhydride(DFAIII) and small amount of oligosaccharides *via* an intramolecular transfructosylation reaction(4).

Interest in enzymatic formation of difructoanhydrides has been stimulated by the potential function of oligosaccharides as a food additive, thus several reports on enzymatic formation of DFA I(5) and DFA V(6), and other isomeric difructoanhydrides produced by dilute acid hydrolysis of inulin, as well as DFAIII(7), have been published.

At present, difructoanhydrides are expected to be used as a non-calorie or anti-tooth decaying sweetener, and known to have growth promoting activity for such enteric bacterium as *Bifidobacterium*(8). It has been also studied whether they have other peculiar physiological properties(9).

In this paper, we report the isolation of a new microbial strain and its classification, the cultural conditions for the enzyme production, and the identification of oligosaccharide produced by the enzyme. Further experiments for the purification and measuring enzymatic properties are under study.

MATERIALS AND METHODS

Medium

A basal medium containing inulin(Difco) as the sole carbon and energy source was used for the isolation and cultivation of inulin fructotransferase-producing strains. The medium for screening was composed of 10.0g inulin, 2.0g $NaNO_3$, 0.5g K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g KCl, a trace amount of $FeSO_4 \cdot 7H_2O$, and 3.0g yeast extract per liter of distilled water. The pH of the medium was around 7.0 without adjustment. The PDA(potato dextrose agar, Difco, U.S.A) slant was used for the preservation of isolates.

Isolation and classification of inulin fructotransferase-producing strain

Inulin fructotransferase producers were isolated from

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soil samples by enrichment culture. A 0.2g soil sample was added into 3.0ml of the basal medium in a test tube(14×150mm) and cultivated on a reciprocal shaker of 120rpm at 30°C for 24 hours. The culture broth(0.5ml) was transferred into 3.0ml fresh medium, cultivating further in the same condition. The culture broth was then spread on an agar plate of the same medium. Following incubation at 30°C for 2 days, every single colony was picked up, and inoculated into PDA slant for preservation. After liquid culture of the selected strains, the inulin fructotransferase activity in the culture broth was assayed by TLC analysis. Among the isolated inulin fructotransferase-producing isolates, the strain which exhibited the highest enzyme activity was selected, and used for the further studies.

The strain was identified in accordance with Bergy's manual of systematic bacteriology(10).

Preparation of crude enzyme solution

The inulin fructotransferase-producing strain was precultured in 10ml of the basal medium in a test tube (24×200mm) at 30°C for 14 hours on a reciprocal shaker at 120rpm and was transferred into a 500ml shaking flask containing 100ml medium. After cultivation in the same condition, the culture broth was centrifuged at 10,000g, 4°C for 20 min, and the supernatant was used as a crude enzyme solution.

Enzyme assay

Inulin fructotransferase activity was assayed by measuring the amount of reaction product after complete removal of residual inulin by acetone precipitation(11). The reaction mixture was composed of 0.2% inulin solution dissolved in 0.1M phosphate buffer(pH 5.6) and 0.2ml of enzyme solution. Following incubation at 40°C for 30min, the reaction was stopped by boiling at 100°C for 5min. Immediately 0.4ml of 0.8 M Clark & Lubs solution(pH 8.0), 0.02ml of 3.2M $CaCl_2$ and 3ml of chilled acetone were added to the reaction mixture, and mixed well. After standing in an ice-bath for 10min, the mixture was centrifuged at 10,000×g and 0°C for 15min. One mililiter aliquot was taken from the supernatant and evaporated quickly to be a thick syrup. It was dissolved in 1.0ml of distilled water, and the amount of ketohexose was measured by Resorcinol-HCl method(12) using fructose as a standard. For a control, 0.1M phosphate buffer without inulin was added. One unit of enzyme activity was defined as the amount of the enzyme which produced 1μmol of DFA per min under this assay condition. Protein concentration was determined by measuring the absorbance at 280nm with a spectrophotometer.

Analytical methods

TLC was carried out using a Silica gel plate(Silica gel 60, Merck) with a solvent system of butanol-ethanol-water(2/1/1). Spots were detected with 50% sulfuric acid. Bacterial growth was determined by measuring the optical density of the culture broth at 660nm after appropriate dilutions. GC was performed with the following conditions : column, 5% OV-101 packard glass column; temperature of oven, 100~280°C; temperature of injector, 300°C; temperature of detector, 300°C; detector, flame ionization; flow rate, 30ml/min; sample volume, 0.5μl.

^{13}C-NMR spectrometry was carried out at 300 MHz with Varian Unity Plus-300 spectrometer(using acetone as an internal standard).

RESULTS AND DISCUSSION

Classification of the isolate

A photomicrograph of the strain LC-413 is shown in Fig. 1, and morphological and biochemical properties of the strain are summarized in Table 1. The bacterium is a Gram-negative, non-sporeforming, which is a rod with round end, and does not have any flagella. After 2 days' culture on nutrient agar at 30°C, the cells form a circular, entire, wet and yellow colony.

Fig. 1. Electron micrograph of the negative stained isolate LC-413. (×20,000)

Table 1. Morphological and biochemical properties of the isolated strain LC-413

Morphological properties

Contents	Properties
Shape	Rod with round end
Cell size(μm)	0.5-0.6×1.0-1.5
Motility	Nonmotile
Gram stain	Negative
Acid-fast stain	Negative
Colonies	Circular, entire, convex, wet
Colony surface	Smooth
Colony color	Yellow
Colony opacity	Opaque
Colony viscosity	Moderate

Biochemical properties

Contents	Strain LC-413
Catalase test	+
Oxidase test	+
Oxidation-Fermentation test	F
Glucose acidification test	−
Growth in MacConkey medium	−
Indole production test	−
Nitrate reduction test	−
H_2S production test	−
Starch hydrolysis test	−
Cellulose hydrolysis test	−
Casein hydrolysis test	−
Gelatin liquefaction test	+
Esculin hydrolysis test	+
Urease test	+
KCN tolerance test	−
β-galactosidase test(PNPG)	+
Arginine dihydrolase test	−
Lysine decarboxylase test	+
Ornithine decarboxylase test	−
Gluconate utilization	+
Citrate utilization	+
Caprate utilization	−
Adipate utilization	−
Phenyl-acetate utilization	+
N-acetyl-glucosamine utilization	+

This strain is biochemically positive in the tests of catalase, oxidase, esculin hydrolysis, β-galactosidase, urease, etc., and negative in the tests of indole production. H_2S production, nitrate reduction, starch hydrolysis, casein hydrolysis, cellulose hydrolysis and so on. According to Bergy's manual of systematic bacteriology (10), the strain LC-413 was similar to those of Genus *Flavobacterium*.

Cultural conditions for inulin fructotransferase production

In order to optimize the cultural condition, various components of the basal medium were examined. The enzyme production was carried out for 12 hours as described under "Materials and Methods" using basal medium.

The effect of carbon sources :

Table 2 shows the effect of carbon sources on the enzyme production. It was found that the production of inulin fructotransferase was markedly induced by inulin in this strain. Although low levels of enzyme activities were detected with such carbon sources as fructose, galactose, sorbose, maltose, lactose, mannitol, and soluble starch, the enzyme is supposed to be not constitutive but inducible. This strain in almost all the carbon source except bacterial levan showed good growth levels comparable to that in case of inulin. The inulin concentration on the enzyme production was most effective in 1.5 percent, but the activity was declined with increasing concentration of inulin.

The effect of nitrogen sources :

Table 3 shows the effect of organic and inorganic nitrogen sources on enzyme production. Each organic nitrogen source was added to the basal medium containing 1.5% inulin as the concentration of 0.1% and 0.3%, respectively. The addition of 0.3% malt extract. enhanced the enzyme activity. And it was shown that $NaNO_3$ as inorganic nitrogen source was the optimal one.

Table 2. Effect of carbon sources on inulin fructotransferase production *by Flabobacteium*

Carbon sources (1.0%)	Final pH	Growth (O.D. at 660nm)	Enzyme activity (units/ml)
Glucose	5.08	5.9	0.0
Fructose	6.09	4.6	5.4
Galactose	6.84	4.0	1.7
Arabinose	6.90	3.7	0.0
Sorbose	6.94	3.0	2.8
Xylose	6.67	4.1	0.0
Sucrose	5.35	4.9	0.0
Maltose	5.25	4.2	3.7
Lactose	7.16	3.6	1.1
Sorbitol	6.34	4.1	0.0
Mannitol	6.75	4.2	8.4
Starch	7.15	3.5	1.4
Levan	7.00	0.9	0.0
Inulin	6.82	4.5	38.0

Table 3. Effect of nitrogen sources on inulin fructotransferase production

Nitrogen sources(%)		Final pH	Growth (O.D. at 660nm)	Enzyme activity (units/ml)
Bactopeptone	0.1	6.96	6.1	30.1
	0.3	6.56	3.8	43.2
Polypeptone	0.1	6.88	4.5	46.6
	0.3	6.89	4.7	34.6
Beef ext.	0.1	7.04	6.1	43.6
	0.3	6.95	5.5	69.4
Malt ext.	0.1	7.02	7.7	50.3
	0.3	7.10	5.7	84.8
Yeast ext.	0.1	6.90	5.5	61.1
	0.3	6.78	5.0	20.7
NH_4Cl	0.2	4.02	5.5	41.6
NH_4NO_3	0.2	4.13	4.5	55.4
$(NH_4)_2SO_4$	0.2	4.14	5.3	38.7
KNO_3	0.2	7.15	5.3	79.3
$NaNO_3$	0.2	7.07	5.2	98.5

Table 4. Effect of initial pH of medium on inulin fructotransferase production

Initial pH	Final pH	Growth (O.D. at 660nm)	Enzyme activity (units/ml)
5	6.3	3.5	0.0
6	6.3	3.4	16.3
7	6.8	4.3	51.7
8	7.0	4.4	59.6
9	7.0	4.0	77.3
10	7.9	3.9	65.5

The effect of initial pH of medium :

Finally, in order to examine the effect of initial pH of medium, pH of the culture medium, containing 1.5% inulin, 0.2% $NaNO_3$, 0.3% malt ext. and the same inorganic salt composition of the basal medium, was adjusted to the indicated pHs. As shown in Table 4, the enzyme activity of 77.3units/ml was obtained at pH 9.0, but the activity was decreased at pH 10.0.

From these results and other experimental data, the optimum culture condition for enzyme production was determined as follows: 1.5% inulin, 0.2% $NaNO_3$, 0.05 % K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, a trace amount of $FeSO_4 \cdot 7H_2O$, and 0.3% malt ext.(pH 9.0).

Time course of inulin fructotransferase production

The time course of the enzyme production was investigated using the optimal medium. At suitable time intervals during cultivation in the optimal medium, small amounts of culture broth was withdrawn and enzyme activity in the supernatant was assayed along with changes in cell growth and broth pH. As shown in Fig. 2, the enzyme production was maximal after 11 hours of cultivation, having the enzyme activity as high as 78.6units/ml, and dramatically declined after 24 hours. The growth rate of this strain was increased sharply in proportion to enzyme activity, reaching to the maximum after 18 hours. The pH was gradually decreased to below 7 with the progress of cultivation.

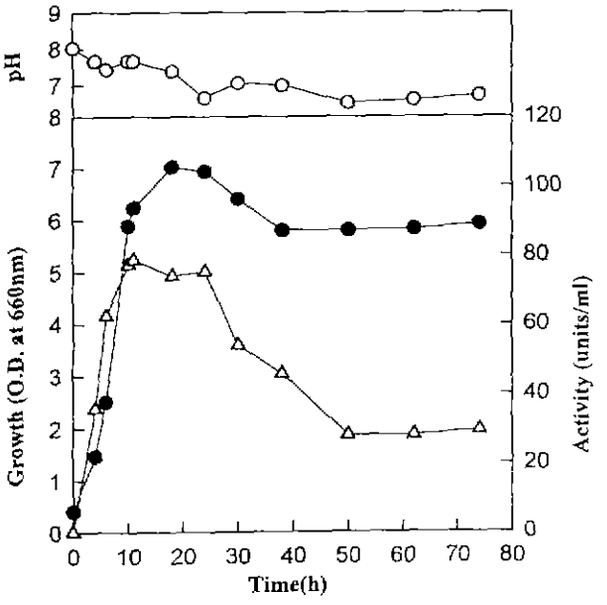

Fig. 2. Time course of cell growth, pH change, and inulin fructotransferase production.
-○- : pH, -●- : Growth, -△- : Inulinase II activity

Preparation of the reaction product

To identify the main product of the enzyme reaction, the inulin was digested exhaustively with the enzyme for 6 hours, then the reaction mixture was purified by the following procedure. The enzyme reaction was stopped by heating at 100°C for 10min, and then baker's yeast was added to the reaction mixture to remove the oligosaccharide, kestose, and incubated at 30°C for 8 hours. The yeast cells were removed by centrifugation (10,000×g, 20min) and the supernatant was condensated *in vacuo* at 60°C to thick syrup. The condensate was applied on Sephadex G-25 column(20×900mm) equilibrated with distilled water and eluted with water. The fractions containing the main reaction product were collected and concentrated with a rotary evaporator. Ten times volume of chilled acetone was then added to

Fig. 3. TLC and GC chromatograms of the purified compound produced by the enzyme reaction.
Solvent systems, A, ethanol : butanol : H_2O(1/2/1); B, butanol : isopropanol : acetic acid(7/5/2); C, chloroform : methanol : H_2O : acetic acid(15/12/3/1).

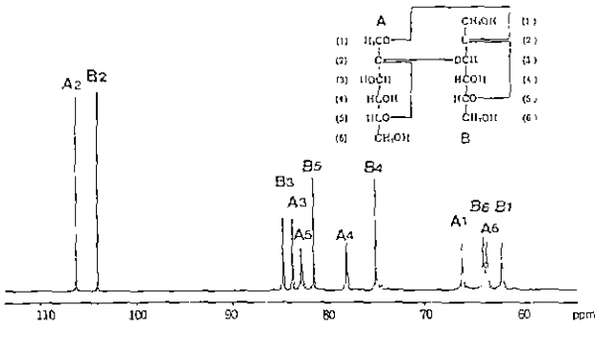

Fig. 4. ^{13}C-NMR spectrum of the compound produced from inulin by the enzyme reaction.

the thick syrup, and insoluble material was removed by centrifugation(10,000×g, 0°C, 15min). The supernatant was concentrated and freeze-dried to obtain colorless material. The purified oligosaccharide was detected as one spot on TLC chromatogram and also one peak on GC as shown in Fig. 3, suggesting that it should be homogeneous.

Table 5. ^{13}C-NMR chemical shifts of DFA produced by *Flavobacterium* sp. LC-413

Assignment (carbon atom number)	Chemical shifts of ^{13}C resonance of	
	α-D-fructofuranose	β-D-fructofuranose
2	106.2(106.0)[1)]	103.9(103.8)
3	83.7 (83.6)	84.7 (84.4)
4	77.0 (77.9)	75.0 (74.8)
5	82.8 (82.6)	81.5 (81.4)
1	66.0 (65.8)	62.0 (61.4)
6	63.5 (63.2)	63.9 (63.5)

[1)]Data in parenthesis are from Reference # 13

Identification of the reaction product

To identify the purified compound produced by enzyme reaction, ^{13}C-NMR spectrometry was performed. The spectrum is shown in Fig. 4, and ^{13}C-NMR chemical shifts of this material are presented in Table 5. These values agreed well with those of DFAIII reported previously(13). This compound also showed no reducing power, and the molecular weight was approximately 330 by gel filtration which is similar to the size of DFA III, confirming that the enzyme was an inulin fructotransferase converting inulin into DFAIII.

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