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Production of Cycloinulooligosaccharide Fructanotransferase (CFTase) from *Bacillus* sp. CFC1

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A bacterial strain CFC1, which produced an extracellular cycloinulooligosaccharide fructanotransferase (CFTase), was isolated from soil. The isolated strain was identified as a strain of *Bacillus* sp. The synthesis of CFTase by the bacterium was found to be induced by inulin which was added to the culture medium as a carbon source. The highest activity of CFTase was observed at pH 7.5 and 37°C in the medium containing 4% inulin and 0.5% peptone as a carbon source and a nitrogen source, respectively. Under the optimal conditions, the enzyme activity in the culture supernatant reached the highest level of 85 munits/ml after 96 h cultivation.

Inulin is a polyfructan, consisting of a linear β-(2→1)-linked polyfructose chain having a terminal glucose residue. It is found as a reserve carbohydrate in various plants, such as chicory, dahlia, and jerusalem artichoke. Inulin can be decomposed by various kinds of microbial enzymes, and then it is of growing interest as a renewable carbohydrate raw material for the sugar, food, fuel and other industries. The inulin-decomposing enzymes reported so far are 2,1-β-D-fructan fructanohydrolase (inulinase) (EC 3.2.1.7), inulin fructotransferase (depolymerizing) (EC 2.4.1.93), inulin fructotransferase (DFA I-producing) (EC 2.4.1.200) (2-4, 10). Recently, Kawamura and Uchiyama reported a new type of inulin-decomposing enzyme produced by a strain of *Bacillus circulans*, and designated the enzyme as cycloinulooligosaccharide fructanotransferase (CFTase) (7).

CFTase converts inulin into novel cycloinulooligosaccharides (cyclofructan) of β-(2→1)-linked D-fructofuranose. Cycloinulohexaose (CF6) is known to be the major product of the CFTase reaction, and cycloinuloheptaose (CF7) and cycloinulooctaose (CF8) are also produced in small quantities (8). Cyclofructan has a characteristic crown ether in the central part of the molecule and is then expected to bind cationic molecules via charge-dipole electrostatic interactions (6). In this study, we isolated a bacterial strain from soil which produced an extracellular CFTase. The isolated strain, CFC1, was identified as a strain of *Bacillus* sp. In addition, we investigated optimal conditions for the production of CFTase from the *Bacillus* sp. CFC1.

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MATERIALS AND METHODS

Chemicals

Inulin (Dahlia) was purchased from Sigma Co., St. Louis, Mo. Other chemicals used were of first grade and available commercially.

Media

The selective medium used in this work contained 20 g inulin, 5 g peptone, 10.6 g K_2HPO_4, 6.1 g $NaH_2PO_4 \cdot 2H_2O$, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 3 mg $FeSO_4 \cdot 7H_2O$, and 20 g agar per liter with initial pH 7.0. Modified Kushibe medium (9) was used for production of CFTase. The production medium composed of 20 g inulin, 5 g peptone, 2 g $NaNO_3$, 4 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.5 g KCl per liter with initial pH 7.5 adjusted with NaOH. All these media were autoclaved at 121°C for 15 min before use.

Isolation and Identification of Bacterial Strains

To isolate a bacterial strain capable of producing CFTase, soil samples were diluted with sterilized water, and inoculated to plates of the selective medium. After incubation at 30°C for 7 days, the colonies which had formed clear-zone around them were isolated. The isolated bacterial strains were then inoculated into the liquid selective medium and cultivated at 30°C for 3 days. Cyclofructan in the supernatant of the cultures was detected by paper chromatography and also by high performance liquid chromatography (HPLC) as described under Analytical Methods. The bacterial strain which produced large amounts of cyclofructan was selected and identified on the basis of

Bergey's manual of systematic bacteriology (5).

Preparation of Crude Enzyme

One loopful of the bacterial cells was inoculated to a 20 ml of the CFTase production medium containing 10 g inulin in a 250 ml flask, and cultivated on a rotary shaker (200 rpm) at 37°C for 24 h. This preculture broth was transferred with the inoculum size of 1.0% to an identical flask containing the production medium, and cultured at 37°C for appropriate periods of time. The culture broth was centrifugated at 15,000 g for 10 min at 4°C to remove the cells. The supernatant thus obtained was used as the crude enzyme solution.

Purification and Identification of Cyclofructan

The microorganism was cultured at 37°C for 48 h. Two hundred mililiters of 50 mM phosphate buffer (pH 7.0) containing 5% inulin was mixed to the same volume of crude enzyme solution. The mixture was incubated at 37°C for 5 h. The reaction was stopped by heating the mixture in boiling water for 10 min. The reaction mixture was concentrated under reduced pressure. The concentrate was purified by gel filtration using Bio-gel P2 column (20×1500 mm). The fractions containing inulooligosaccharides were detected by total sugar assay. The formation of cyclofructan was detected by HPLC. The purified cyclofructan was identified by ^{13}C-NMR as described under Analytical Methods.

Enzyme Assay

CFTase activity was measured using a reaction mixture consisting of 0.5 ml of 50 mM phosphate buffer (pH 7.0) containing 5% inulin and 0.5 ml of the crude enzyme solution. The mixture was incubated at 37°C for 1 h, and the reaction was stopped by heating the mixture in boiling water for 10 min. CF6 produced was deterimined by HPLC. One unit of the enzyme activity was defined as the amount of enzyme which produced 1 μmole of CF6 per min under this assay condition.

Analytical Methods

Paper chromatography. The paper was developed twice with a solvent system of *n*-butanol : pyridine : water (3:2:2, v/v). Sugars were detected by resorcinol·HCl (1).

Total sugar assay. Anthrone's method (11) was used. Four hundred microliter of saccharide sample was mixed to 1.6 ml of H_2SO_4 containing 0.2% orcinol. The mixture was incubated at 80°C for 10 min, and then the optical density at 420 nm was measured.

HPLC. Waters carbohydrate column (3.9×300 mm) and RI detector were used (mobile phase, acetonitrile : water [70:30]; flow rate, 1.2 ml/min).

NMR analysis. ^{13}C-NMR spectrum was recorded by Varian Unit 300 Spectrophotometer. 1,4-Dioxane was used as an internal standard in D_2O.

RESULTS AND DISCUSSION

Bacterial Isolation and Identification of the Isolated Strain

In the first stage of screening, we selected bacterial strains which had formed a clear zone around the colony after 7 days incubation on the selective medium. Next, the strains selected were tested by paper chromatography for their ability to produce inulooligosaccharides from inulin added to the medium as a sole carbon source. A bacterial strain capable of producing an inulooligosaccharide which was not hydrolyzed by β-fructofuranosidase was finally selected for further studies, and designated as the strain CFC1. The isolated strain CFC1 was a Gram-positive, rod-shape, and endospore-forming bacterium, which had a rhyzoid colony on the selective plate (Table 1, Fig. 1). The bacterial strain was identified as a strain of *Bacillus* sp. by its morphological, physiological, and biochemical characteristics.

Identification of Cyclofructan

Products of the inulin-decomposing reaction catalyzed by the CFTase was purified as described in Materials and Methods. Table 2 shows ^{13}C-NMR chemical shifts of the major product of the enzyme reaction. The values obtained from the ^{13}C-NMR spectrum agreed well with those of CF6 reported (7, 9). The main reaction product from inulin by this enzyme was thus confirmed to be CF6. CF7 as a minor product could also be detected in the reaction mixture (Fig. 2).

Culture Conditions for CFTase Production

In order to optimize conditions for the production of CFTase by the isolated strain, composition and pH of the CFTase production medium, and culture temperature were examined. Culture for the CFTase production was carried out using the CFTase production medium as a

Table 1. Morphological and biochemical characteristics of the isolated strain CFC1.

Factor	Characteristics
Type and size	rod (> 1 μm)
Gram staining	positive
Motility	negative
Endospore	forming
Mycelium	not forming
Colonies on agar plate	circular (rhyzoid colony)
Oxygen requirement	positive
Catalase production	weakly positive
Oxidase test	negative
H_2S production	negative
Gelatin liquifaction	weakly positive
Casein hydrolysis	positive
Nitrate reduction	positive
Indole production	negative
Gas on TSI agar	positive
Acetoin production	positive
Esclin hydrolysis	positive
β-Galactosidase	positive

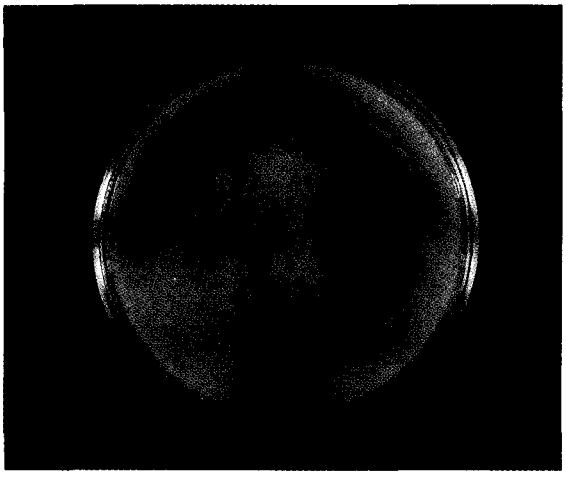

Fig. 1. Clear zone formation by the isolated strain CFC1 on the selective medium plate.

Table 2. ^{13}C-NMR chemical shift of cycloinulohexaose.

Assignment (Carbon atom number)	Chemical shift of ^{13}C-NMR of cycloinulohexaose
1	61.5 (61.4)
2	103.6 (103.7)
3	79.2 (79.1)
4	75.3 (75.2)
5	62.3 (62.3)
6	63.3 (63.3)

Data in parenthesis are from ref. (7).

basal medium as described in Materials and Methods.

Effect of carbon sources. Effect of carbon sources on the production of CFTase by the isolated *Bacillus* strain was examined. For the carbon source, inulin, starch, sucrose, fructose, glucose, maltose, lactose, α-cellulose, or glycerol was added to the basal medium at concentration of 20 g/l. CFTase production by the bacterium was induced only by inulin (data not shown).

Effect of inulin. To investigate the effect of inulin concentration on the CFTase production, inulin was added to the basal medium at the various concentrations indicated in the Table 3, and the CFTase activities were determined in the supernatant of the culture broths. As shown in Table 3, the CFTase production was the highest at the inulin concentration of 40 g/l. The cell growth and the level of CF6 formed in the culture broth were increased with increasing inulin concentrations added. Especially, the CF6 production in the culture broth was increased linearly up to the amount of inulin added in this work.

Effect of nitrogen sources. Production of CFTase by the *Bacillus* sp. CFC1 was examined in the presence of various organic nitrogen sources at concentration of 5 g/l in the basal medium supplemented with 4% inulin as a sole carbon source. As shown in Table 4, the CFTase activity showed the highest value when peptone was used, while the cells were grown best with corn steep solids and the most CF6 production was observed with yeast extract. The optimum concentration of peptone for the CFTase production was then determined to be 5 g/l.

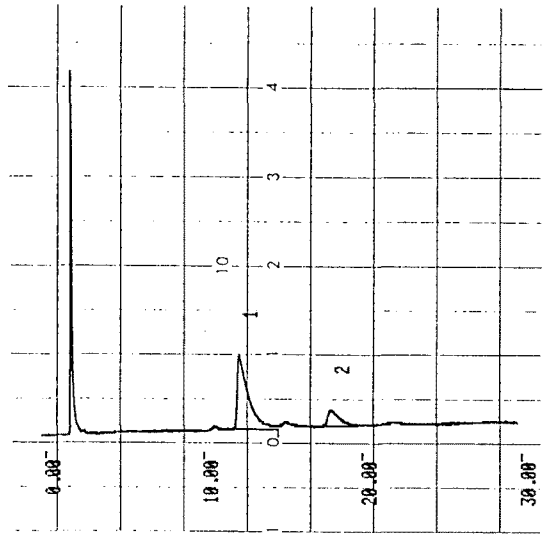

Fig. 2. HPLC chromatogram of the CF6 and CF7 in enzyme reaction mixture.
1, CF6; 2, CF7.

Table 3. Effect of inulin on production of CFTase.

Inulin (%)	Growth (A_{600})	CF6[a] (mg/ml)	CFTase activity (munits/ml)
0	0.21	0.00	0.0
2	0.93	4.81	11.0
4	1.05	10.83	60.0
6	1.25	16.65	58.0
8	2.36	20.80	55.0

Cultivation was carried out as described in Materials and Methods at 37°C for 72 h. [a]Amount of CF6 in the culture broth.

Table 4. Effect of orgarnic nitrogen sources on CFTase production.

Nitrogen source (0.5%, w/v)	Growth (A_{600})	CF6 (mg/ml)	CFTase activity (munits/ml)
None	0.13	1.50	0.0
Peptone	0.90	9.50	63.0
Tryptone	0.70	8.92	55.0
Yeast extract	1.50	12.10	38.0
C.S.S[a]	3.60	8.60	34.0
Malt extract	0.13	0.00	0.0

Cultivation was carried out as described in Materials and Methods at 37°C for 48 h except that the indicated nutrients replaced the organic nitrogen in the CFTase production medium containing 4% inulin. [a]Corn steep solids.

Table 5. Effect of $NaNO_3$ on CFTase production.

$NaNO_3$ (%)	Growth (A_{600})	CF6 (mg/ml)	CFTase activity (munits/ml)
0.0	1.08	8.48	47.0
0.2	1.19	5.90	30.0
0.4	0.98	4.66	29.0
0.8	0.77	4.56	2.0

Cultivation was carried out as described in Materials and Methods at 37°C for 48 h in the production medium containing 4% inulin and 0.5% peptone, respectively.

Table 6. Effect of culture pH on production of CFTase.

Initial pH	pH after autoclave	Final pH	Growth (A_{600})	CF6 (mg/ml)	CFTase activity (munits/ml)
5.5	5.58	5.21	0.16	0.60	0.0
6.5	6.49	5.64	0.70	0.86	0.0
7.0	6.94	5.74	0.96	1.20	3.0
7.5	7.35	6.74	0.93	7.96	58.0
8.0	7.61	6.94	0.83	7.28	55.0
8.5	7.84	7.00	0.90	7.10	14.0
9.5	8.10	7.04	0.72	6.82	9.0

Cultivation was carried out as described in Materials and Methods at 37°C for 48 h in the optimal production medium. pH was adjusted with NaOH or HCl solution.

At the concentrations higher than 5 g/l, the synthesis of CFTase by the *Bacillus* sp. CFC1 was reduced markedly (data not shown). Sodium nitrate was reported to be an effective inorganic nitrogen source for the production of the CFTase by *Bacillus circulans* MCI-2554 (9). Therefore, we examined the effect of $NaNO_3$ on the production of CFTase (Table 5). The CFTase activity was found rather to be decreased in the presence of sodium nitrate as a nitrogen source in our strain. It was concluded that the nitrogen source was very important for production of CFTase by the isolated strain. From these results, the optimum medium for the production of the enzyme was determined to consist of 40 g inulin, 5 g peptone, 4 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.5 g KCl per liter, and this medium was employed in the following experiments.

Effect of culture pH and temperature. Table 6 shows the effects of culture pH on the CFTase production. The strain CFC1 could produce CFTase only under weak alkaline conditions. The optimum initial pH for the production of CFTase was pH 7.5. The optimum culture temperature was 37°C for the enzyme synthesis by the bacterium. At the temperatures higher than 42°C, the enzyme production as well as cell growth was inhibited markedly (data not shown).

Time Course of CFTase Production

The time course of the enzyme production was determined under the cultural conditions optimal for the

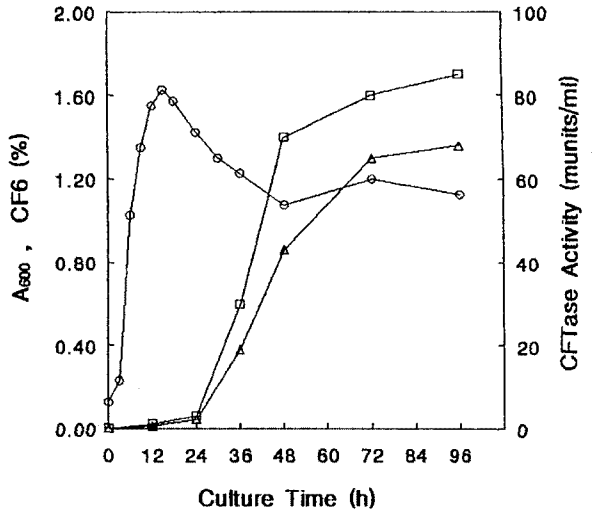

Fig. 3. Time course of CFTase production by *Bacillus* sp. CFC1. Cultivation was carried out as described in Materials and Methods at 37°C for 96 h in a 250 ml optimal production medium of a 2 liter flask. ○, growth; △, CF6; □, CFTase.

CFTase production by *Bacillus* sp. CFC1. As shown in Fig. 3, the maximum level of cell growth was observed after cultivation for 15 h. Whereas, the CFTase synthesis and CF6 production were detected initially in the culture broth obtained after 24 h cultivation. Thereafter the production of CFTase was enhanced with culture time, and reached the highest level of 85 munits/ml after 96 h culture, and kinetics of CF6 production during the culture showed a fairly similar pattern to that of the enzyme production.

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