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# Isolation and Identification of the Black Yeast Producing Fructosyl transferase

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# Fructosyl transferase를 생성하는 효모의 분리 및 동정

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**ABSTRACT: For the purpose of obtaining microorganisms producing high fructosyl transferase, the screening test was carried out. Among more than three hundred isolates, an isolate (C23-isolate) was selected for high fructosyl transferase producer from the dirts at the coffee vendig machine. The morphological and cultural characteristics of the isolate C23 on various culture media were studied and identified as *Aureobasidium pullulans* var. *melanigenum*.**

**KEYWORDS: *Aureobasidium pullulans* var. *melanigenum*, Fructosyl transferase, Fructo-oligosaccharide**

Fructosyl transferase (EC 2.4.1.9) was an enzyme to convert sucrose (disaccharide) into fructo-oligosaccharides. This enzyme was reported to be investigated at the metabolism of cell wall in the plants (Shiomi and Yamada, 1979; Robert *et al.*, 1980) and also mentioned in yeast (Edelman, 1954; Bacon, 1954) or fungi (Pazur, 1952; Dickerson, 1972; Gupta and Bhatia, 1980; 1982). It was at least considered to be involved in the carbohydrate metabolism in the plant or the fungal cells.

Recently, the production of fructo-oligosaccharides have been emphasized in food industry for a health food. Fructo-oligosaccharide represented 20-40% of sweetness as compared with that of sucrose and was reported not to be digested in the human intestine system (Oku *et al.*, 1984).

This experiment was to subject to screen the organism producing fructosyl transferase, to produce a health food. We isolated the black yeast from the coffee vending machine and identified it as *Aureobasidium pullulan* var. *melanigenum*. Also, the isolates was estimated, to produce fructosyl transferase (synthesizing fructo-oligosaccharide from sucrose).

## Materials and Methods

### Isolation

The organisms were isolated from the soil in Chungbuk National University and the dirts of coffee vending machines around Cheongju city. The isolation of them was employed by the dilution and directly streaking method in Czapek-Dox agar (sucrose 20-50%). The isolates were stored at 4°C in PD agar and selected as based on the high activities of fructosyl transferase after grown at 30°C for four days. For liquid culture, the 50 m*l* of Czapek-Dox broth (sucrose 30%) was placed in 250 m*l* elenmeyer flask with the cotton plug. The pH of Czapek-Dox broth was adjusted to 6.5 with 0.1 N HCl or 0.1 N NaOH before autoclaving and inoculated with

the 1 m*l* of $10^7$ colony forming units in PD agar after cooling.

**Growth measurement**

For cell growth, the cell growing broth was diluted and the optical density checked at 660 nm was converted into the dry cell weight with the standard curves made.

**Preparation of enzyme**

The broths cultured for four days were centrifuged and divided into two solutions; The supernatant solution was directly measured as the extracellular enzyme. The percipitated cells were harvested and washed again with 0.1 M (pH 5.5) sodium citrate buffer, and employed as the intracellular enzyme. Fructosyl transferase activity was determined by measuring the release of reducing sugar in the reaction mixture described below. One fructosyl transferase unit is defined as the amount of enzyme activity required to produce one $\mu$mol of glucose per minute under the following conditions: a) pH 5.5, b) temperature 55°C, c) reaction mixture consisted of the following composition: 3.75 m*l* of 80% (w/v) sucrose, 1.15 m*l* of 0.1 M citrate buffer (pH 5.5), and 0.1 m*l* enzyme solution. The enzyme reaction was stopped by heating at 100°C for 5 min.

**Quantitative determination of sugar**

Total sugars were determined by the method of phenol-sulphuric acid (Michel *et al.*, 1956) and reducing sugars were measured by the method of Somogyi-Nelson (Nelson, 1944).

**Paper chromatography**

The paper chromatography was employed for the determination of fructo-oligosaccharides synthesized by fructosyl transferase. The fructo-oligosaccharides were developed by the solvents mixtures (n-butanol: acetic acid: water = 4:2:1 (v/v)) for 12 hrs and, after then, colored with urea-hydrochloric acid alcohol (Wise *et al.*, 1955).

## Results and Discussions

**Screening**

More than 300 organisms were isolated from the soils around Chungbuk National University and from the dirts of coffee vending machines located at the city of Cheongju. Fructosyl transferase and utilization of sugar were measured after four days' shaking culture at 30°C. The five isolates shown in Table 1 were selected from more than 300 organisms as based on high activity of fructosyl transferase (see Table I). The five isolates were tested after three times of transfer in Czapek-Dox (sucrose 30%) agar and the total activity of fructosyl transferase of each isolate were estimated. The activities of fructosyl transferase by and utilization of sugar of five isolates were less than those by and of C23-isolate. The productivity of fructosyl transferase per sugar utilized by C23-isolate was high as compared with that by the others.

**Table I.** Utilization of sugar and Fructosyl transferase productions of various isolates.

| Isolate[a] | Utilization of sugar (mg/m*l*)[b] | Enzyme activities ($\mu$mole/m*l*)[c] |
|---|---|---|
| B53 | 112.73 | 22.0 |
| C12 | 118.34 | 22.1 |
| C14 | 117.57 | 23.9 |
| C23 | 126.28 | 40.7 |
| D11 | 140.02 | 23.9 |

[a] Isolates isolated from the coffee vending machines located in Cheongju and each values on average of duplicate.

[b] Residual sugar determined by phenol sulphuric acid and four days' shaking incubation at 30°C.

[c] The activities of fructosyl transferase indicating $\mu$mole of glucose produced at 55°C.

Oligosaccharides synthesized by fructosyl transferase were detected in paper chromatography and the standard oligosaccharides were also employed in Fig. 1 (The left). The enzyme of fructosyl transferase produced by C23-isolate synthesizes several oligosaccharides (Kestose ($GF_2$), Nystose ($GF_3$), and Fructosyl nystose ($GF_4$)) from sucrose. Both glucose and fructose were also detected by this enzymes. The quantitative amounts of each spot appeared in paper chromatography were not estimated, but the time-course works for these spots conducted during the time that sucrose was incubated with the crude prepared fructosyl transferase enzyme. The spots indicating higher

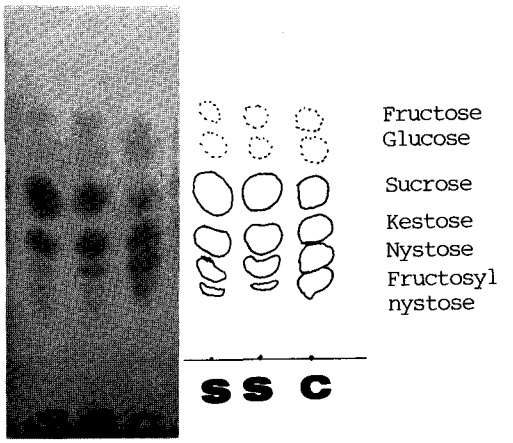


**Fig. 1.** The paper chromatography of various oligosaccharides synthesized by fructosyl transferase. The left arrawing the detail diagrams with or without the standard compounds (C) and samples (S).

number carbon compounds were increased in their diameters and appeared dark in the chromatographic color with the long incubation time. It was speculated with this result that sucrose was hydrolyzed into glucose and fructose and the fructose hydrolyzed was bound to sucrose or other higher molecules and converted to the higher carbon attached oligosaccharides.

**Cell growth**

By C23-isolate, the simple parameters involved in yeast physiology were conducted with daily periods (Fig. 2-5). The cell growth was sharply increased until seven days' shaking incubation, but slowly decreased after seven days (Fig. 2). The pH was fluctuated at the ranges of 7.5 to 6.5, and did not show any big change during ten days' incubation (Fig. 3). The utilization of sucrose in Czapek-Dox (sucrose 15%) broth was increased with the cell growths and

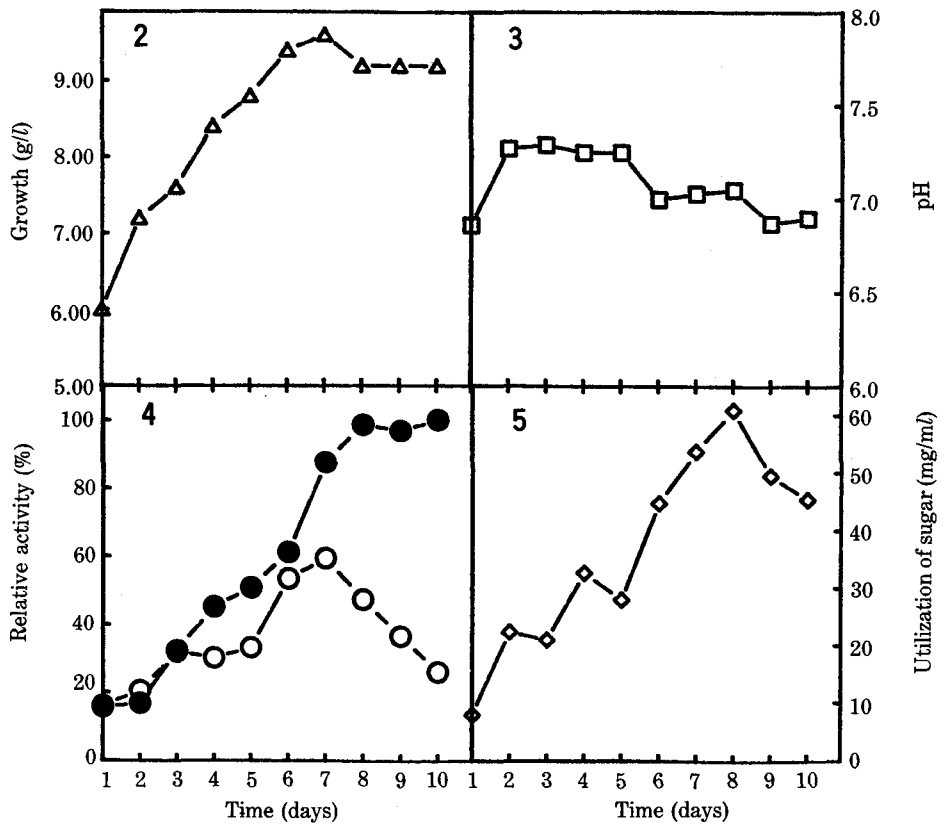


**Fig. 2-5.** The growth (△-△) and pH (□-□), the activities of fructosyl transferase (intracellular; ○-○ extracellular; ●-● ) and utilization of sucrose (◇-◇) of the isolate-C23 grown at 30°C.

after eight days' incubation, was stabilized at 9.2 g/*l* (Fig. 5). The total relative activity of fructosyl transferase (extracellular plus intracellular enzyme not indicated in Fig. 4) was increased with the shaking incubation days. The extracellular fructosyl transferase activity was increased throughout ten days' but in the different rates. The activity of intracellular fructosyl transferase was also increased until seven days' shaking incubation, but decreased after seven days' shaking incubation. The curve of cell growth was similar to that of the activity of extracellular fructosyl transferase (Fig. 2, 4). The cell growth of C23-isolate was considered to the related to utilization of sucrose in Czapek-Dox (sucrose 15%) broth. The ratio of activities of extracellular to intracellular fructosyl transferase was almostly one until three days' incubation, but increased after seven days' incubation. This was speculated to be related to the cell growth. At seven days' incubation, the cell growth was hibernated, but the extracellular fructosyl transferase activity was continuously increased. It was also speculated that the extracellular fructosyl transferase was originated from the intracellular fructosyl transferase.

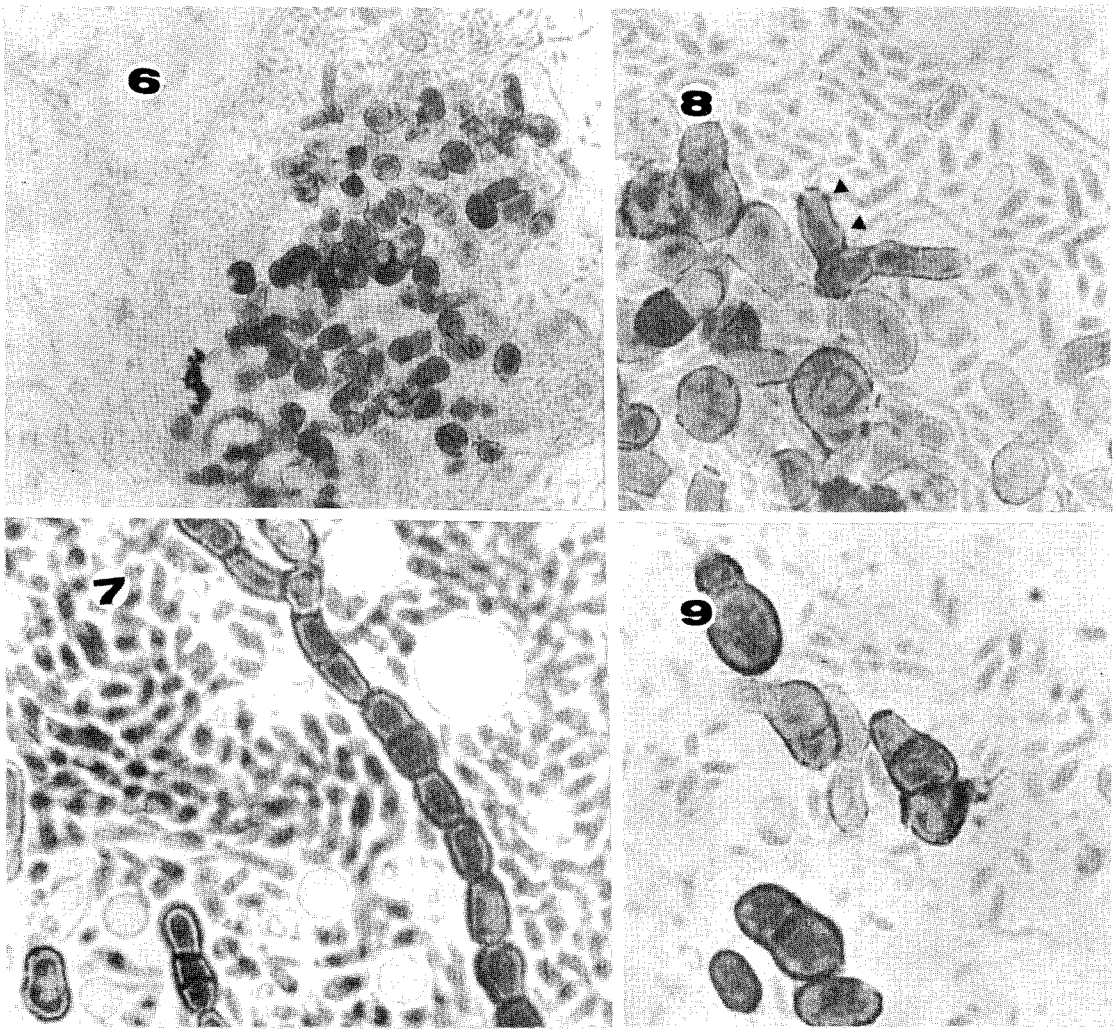


**Fig. 6-9.** The vegetative growth of *Aureobasidium pullulans* var. *melanigenum*.
6) ×320 white and black cells, 7) ×800 black hyphae with thick walls, 8) ×800 black cells with the denticles (▲), and 9) ×800 black and blastic cells.

In conclusion, C23-isolate obtained from the coffee vending machines was estimated as an organisms producing high fructosyl transferase activity among all isolates. The fructosyl transferase produced by this organism also synthesize several oligosaccharides from sucrose. Quantitative analysis of different oligosaccharides synthesized and industrial estimation for this isolate would be needed in the next experiment, again.

**Identification**

The C23-isolate selected in the above were identified; *Aureobasidium pullulans* (deBary) Arn. var. *melanigenum*: Hermanides-Nijhof, 1977. Fassatiova (1986): 164, 170; Baron (1968): 63, 95; Kendrick and Carmichael (1973).

Organisms isolated from the coffee vending machines in Chungbuk areas. The slides mounted with and without lactophenol blue stored at Herbarium in Korea National University of Education, Department of Biology Education. KNUE-D1 grown on Czapek-Dox agar, D2 and D3 on PDA, D4 and D6 on Sabouroud agar, D5 and D7 on YM agar, and D8 and D9 on Malt agars, respectively.

**Description:** Stromata absent on PD, Malt, and Sabouroud agars. Colonies grown on both Malt and PD agars at room temperature showing the white cream mass with several greenish black points. The colonies consisted of a mass of yeast like ovoid cells and black hyphae. The hyphae restricted, but present with the hyaline cylindric and black thick cell walled ovoid cells. The hyphae observed on Malt agar, consisted of the globose to ellipsoid cells (called "chlamydospores") and irregularly branched. Conidiospores blastospores singular hyphae, globose to ovoid (often cylindric), and sized $7.09 \times 2.79 \mu$ m long. The conidiospore produced from the unidifferentiated conidia (strictly not indicated "conidiospores" through the denticles (see Fig. 6-9).

The distinction between *Aureobasidium pullulans* var. *pullulan* and *melanigenum* was not clear. The colonies and any conidiospores not showed the color except for the black. The chlamydiospores (hyphal chain) showed the black and thick walled cells were considered to be different from those of *A. pullulans* var. *pullalans*.

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## 摘 要

Fructosyl transferase의 생산성이 높은 미생물을 얻기 위해 자연계에서 폭넓은 검색을 수행하였다. 300점 이상의 미생물을 분리하고 각각의 효소의 활성을 측정하였다. 그 결과 커피 자동판매기로부터 분리한 C23번 분리균은 다른 분리균들과 비교하여 높은 효소의 활성을 나타내어 fructosyl transferase 생산균으로 선발되었다. 다양한 배지에서 C23 분리균을 배양하여 형태, 그리고 배양특징 등을 연구하여 *Aureobasidium pullulans* var. *melanigenum* 으로 동정하였다.

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