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Screening and Isolation of Trehalase-producing Bacteria from a Dairy Farm Drain

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INTRODUCTION

Trehalose (*O*-α-D-glucopyranosyl-(1-1)-α-D-glucopyranoside) was separated and crystallized for the first time from rye malt by Wiggers in 1832, and named trehalose later by Berthlot in 1858[1,2]. Trehalose is a non-reducing disaccharide found in a wide variety of organisms such as yeasts, bacteria, fungi, algae, plants, insects and invertebrates[1,2]. Since it is widely distributed throughout the natural world, trehalose has been eaten from prehistoric times by humans. After eating trehalose, digestive absorption is carried out in the small intestine as with sucrose and maltose, but since digestive absorption speed is slow, the rate of increase in blood sugar is also slow compared with that of sucrose or maltose[2].

Trehalase (E.C. 3.2.1.28) is the enzyme which hydrolyzes the disaccharide trehalose into two α-D-glucose molecules. This enzyme was discovered as an extracellular enzyme from *Aspergillus niger*[3]. The substrate specificity of trehalase over trehalose was high, and it was clearly shown that there was no sugar transferase reaction. This enzyme was discovered in certain microbes, mold, yeast, insects, and so on[1]. Until now, trehalase has been refined from *Aspergillus niger* and *Saccharomyces cerevisiae*, and they have been reported to have an important biological role as in the release of spores by eumycetes[1,4,5]. Moreover, there are two kinds of trehalase enzymes; acid trehalase and neutral trehalase. The properties of these enzymes very dramatically; acid trehalase has an optimum pH as low as 3.7, whereas neutral trehalase is most active at a pH of 7-7.5[1]. It is supposed that this enzyme is now inducible; however, many points regarding the enzyme remain to be clarified, such as detailed character, structure, etc.

In recent years, trehalose has been produced more readily and in large quantities, and various characteristics have been clarified. However, most research on microbial trehalase has focused on cells that produce glucose as carbon source, and there have been very few reports examining trehalose as carbon source.

The drain on the dairy farm that we have been studying provides a unique environment in which cow excrement and milk are sometimes mixed, and we thought that microorganisms using trehalose might exist there. Therefore, in this research, we have screened bacteria that can use trehalose from our dairy farm drain. Then, when trehalose was used as carbon source, we investigated whether those bacteria would produce trehalase. Furthermore, a coliform bacterium which produces trehalase was identified.

MATERIALS AND METHODS

Isolation of bacterial strains. Bacterial strains were isolated using the pour plate method. Drain water samples were collected from our dairy farm (Research Farm, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan) and diluted 10-, 100- and 1,000-fold. The dilutions were added to Petri plates, and Chromocult Coliform Agar (MERCK, Darmstadt, Germany) was added. The agar plates were then incubated overnight at 37 ℃. Any red, purple or white colonies that appeared were harvested, and streaked on Chromocult Coliform Agar plates and incubated at 37 ℃. Well separated colonies from each plate were cultured on standard agar slants to obtain pure cultures. Isolates were identified

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using Gram stain, motility, gelatin hydrolysis, growth at 42 ℃, PHB (poly-β-hydroxybutyric acid, Wako Pure Chemical Industries Ltd., Osaka, Japan) accumulation, catalase test, oxidase test, OF test and the Bio Test 2 system (Eiken Chemical, Tokyo, Japan). All other strains were obtained from the culture collection of our laboratory.

Utilization of trehalose. Isolated bacteria were diluted to reach "McFarland No.1 turbidity" using a physiological salt solution. Fifty μl of the dilutions were added to 3 ml of the complex medium [polypepton (Nihon Seiyaku, Tokyo, Japan) at 10g liter^{-1}, trehalose (Hayashibara, Okayama, Japan) at 10g liter^{-1} and NaCl at 5g liter^{-1}] containing 0.004% BCP (Bromocresol purple, Wako Pure Chemical Industries Ltd., Osaka, Japan) in tubes. After culturing for 24 h at 37 ℃, the change in color (from purple to yellow), pH, and turbidity (O.D.$_{650}$) of the medium were measured.

Preparation of bacterial crude cell-free extract. Bacterial strains were routinely cultured in shake flasks (500-ml Sakaguchi flasks with a working volume of 250 ml) at 37 ℃ in the complex medium. For inoculation, 1% inocula of bacterial cultures (cultivated for 24 h) were used. Cells were collected by centrifugation at 5,800 *g* for 30 min at 4 ℃ and washed with 0.01 M sodium phosphate buffer (pH 7.0). The cells were suspended in 10 ml of the same buffer, frozen and stored at − 40 ℃ until use. The frozen cells were thawed with running water, and were disrupted by ultrasonication with a sonifier (Branson model 450) for 20 min at 15% output. The supernatant (cell-free extract) obtained by centrifugation at 27,700 *g* for 30 min at 4 ℃ was used for the measurement of trehalase activity.

Analytical methods. Protein was measured by the method of Lowry *et al*[6] using bovine serum albumin as a standard. To assay trehalase activity, the reaction mixture of 0.5 ml of 0.5% trehalose, 0.4 ml of 0.1 M sodium phosphate buffer (pH 7.0), and 0.1 ml of an appropriate concentration of cell-free extract were incubated at 37 ℃ for 10 min. The measurement of glucose was performed using the glucose-oxidase-peroxidase method (Glucose-ARII kit; Wako Pure Chemical Industries Ltd., Osaka, Japan). One unit of trehalase activity was defined as the amount of enzyme that catalyzed the hydrolysis of one μmol of trehalose per min.

RESULTS AND DISCUSSION

Utilization of trehalose.

When 76 bacteria isolated from our dairy farm drain were cultivated by trehalose as a carbon source, six strains (No. 09, 26, 31, 39, 40 and 55) were found to show good growth (turbidity, O. D.$_{650}$) and good acid production (Table 1). These six bacteria were selected as trehalose-utilizing bacteria, and their ability to produce trehalase was investigated.

Trehalase production.

Figure 1 shows the growth rates and trehalase activity curves of trehalose-utilizing bacteria on the complex medium containing trehalose. All bacteria were able to proliferate in the media, but only the cell-free extract of bacterium No. 55 was found to show trehalase activity. The reason the other cell-free extracts did not show trehalase activity is unclear. It is thought to be that bacterium can be grown using the ingredients excepted for trehalose in the complex medium, or that they have hydrolyzed trehalose with enzymes other than trehalase, and further study is needed to clarify the reason. No extracellular trehalase activity was detected in any bacterium (data not shown).

In bacterium No. 55, trehalase activity in-

Table 1 Utilization of trehalose by isolated bacteria.

Bacteria No.	Acidity*	pH	Growth (O.D.$_{650}$)
No.09	+	5.3	0.65
No.26	+	5.5	0.70
No.31	+	5.5	0.65
No.39	++	4.7	0.55
No.40	++	4.4	0.50
No.55	++	4.8	0.51

*Acidity was determined from the change in color of BCP from purple to yellow. +: yellow; ++: bright yellow.

creased according to growth, and peak activity was observed at 24 hours (Fig. 1). After growth had peaked, a reduction in activity was observed. The maximum trehalase activity of each bacterium is summarized in Table 2. The maximum trehalase activity in the cell-free extract of bacterium No. 55 was 0.298 U/ml, and specific trehalase activity was 0.03 U/mg of protein. The reported specific activity of *Escherichia coli* was 4.9 U/mg protein[7], and the value of *Bifidobacterium longum* was 0.003 U/mg protein[8]. Thus, it

Table 2 Trehalase activity, growth and medium pH of isolated bactera.

Bacteria No.	Culture hours	Trehalase activity (U/ml)	Growth ($O.D._{650}$)	pH
No. 09	48	nd	2.68	5.70
No. 26	36	nd	1.68	5.66
No. 31	48	nd	1.53	5.70
No. 39	24	nd	3.15	5.64
No. 40	24	nd	3.04	5.52
No. 55	24	0.298	3.07	5.00

nd: not detected.

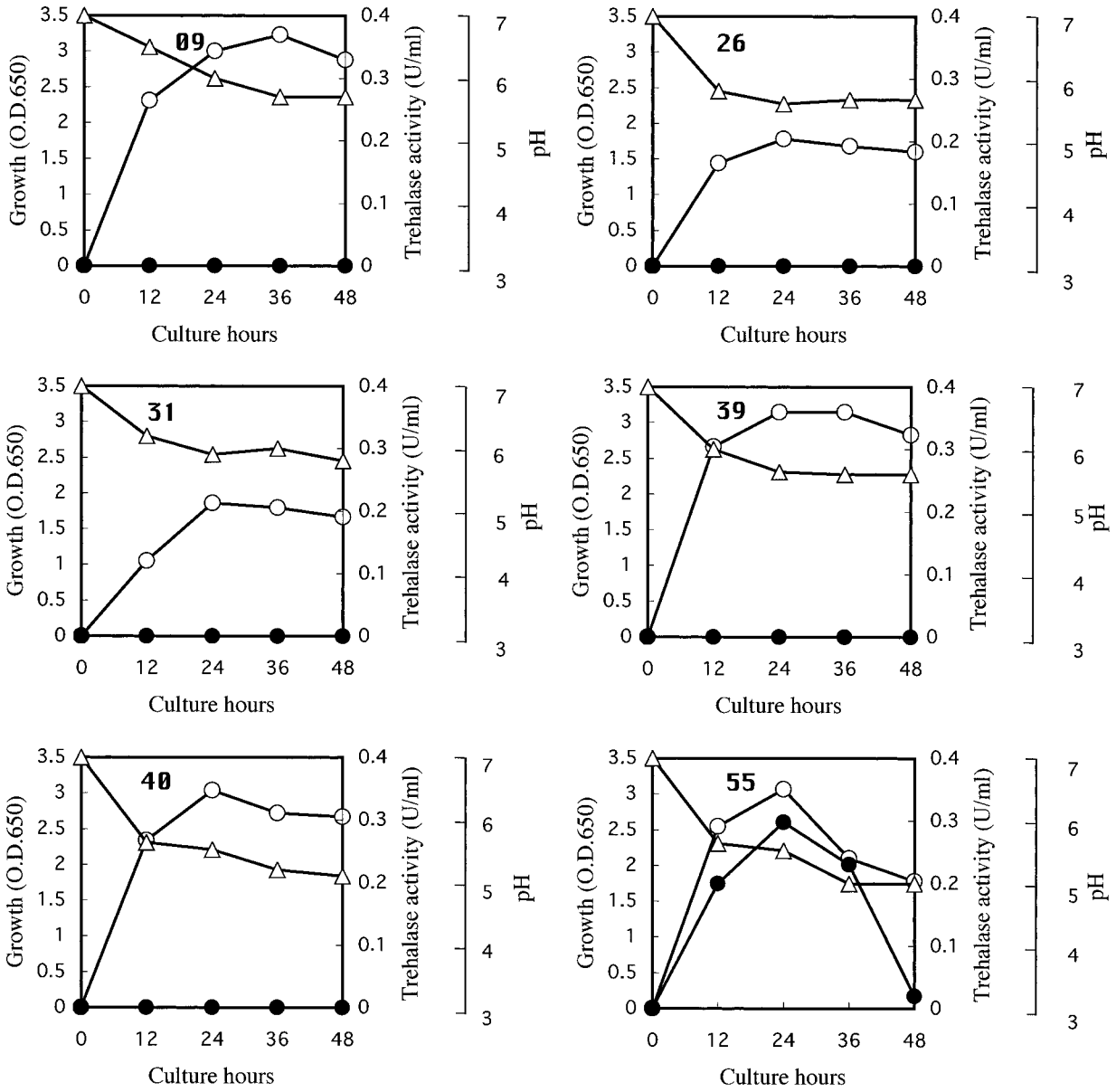

Fig. 1 Time course of growth, trehalase activity and pH in isolated bacteria.
Cultivation procedures and measurement of enzyme reaction are described in Materials and Methods.
The numbers 09, 26, 31, 39, 40 and 55 appearing on the graph represent the isolated bacteria strains.
○: growth as indicated by turbidity of 650 nm; ●: trehalase activity (U/ml); △: pH.

was shown that the activity in the cell-free extract from No. 55 strain was the different of those of the *E. coli* and *B. longum*.

Identification of bacterium No. 55.

Bacterium No. 55 was identified as *Moraxella osloensis* based on the following results (Table 3). It did not exhibit growth at 42 ℃. It was Gram-negative, non-motile, catalase-positive, oxidase-positive, OF test-positive, aerobic rods and cocci. It could not hydrolyse gelatin, and it could accumulate PHB. Further, its carbohydrate utilization pattern determined with the Bio Test 2 system showed that it could not utilize any of the carbohydrates examined. These results indicated that this bacterium did not use sugar at all. However, when it was cultivated under aerobic conditions, trehalose was used, and intracellular trehalase was produced. To clarify the nature of this microbe and enzyme, we are now engaged in the purification of this enzyme.

Table 3 Identification of isolated bacterium No. 55.

Test	Result
Gram stain	Rods/-
Catalase	+
Oxidase	+
OF	+
PHB accumulation	+
Motility	-
Gelatin hydrolysis	-
Nitrate reduce	-
Indol reaction	-
ONPG hydrolysis	-
H_2S production	-
Esculin hydrolysis	-
Citrate utilization	-
Malonate utilization	-
Arginine dihydrolase	-
Urease	-
Acetamide utilization	-
Glucose utilization	-
Xylose utilization	-
Mannose utilization	-
Arabinose utilization	-
Fructose utilization	-
Maltose utilization	-
Rhamnose utilization	-
Mannitol utilization	-
Sucrose utilization	-
Grown at 42℃	-

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ABSTRACT

When 76 bacteria isolated from our dairy farm drain were cultivated by trehalose as a carbon source, six bacteria were found to show good growth and acid production. These six bacteria were selected as trehalose-utilizing strains, and their capacity to produce trehalase was investigated. All bacteria were able to proliferate in the culture media used, but only the cell free extract of bacterium No. 55 showed trehalase activity. The maximum trehalase activity in No. 55 was 0.298 U/ml, and specific trehalose

activity was 0.03 U/mg protein. Strain No. 55 was identified as *Moraxella osloensis* on the basis of various identification tests and the Bio Test 2 system. The reported specific activity of *Escherichia coli* was 4.9 U/mg and the value of *Bifidobacterium longum* was 0.003 U/mg, and it was shown that the cell-free extract of *Moraxella osloensis* No. 55 strain was different from these two bacteria.

要　　約

酪農農場の側溝の排水から分離した76株の細菌を，炭素源をトレハロースとして培養したところ，6株が良好な増殖および酸生成を示した。この6株をトレハロース資化性菌として選抜し，トレハラーゼ生産能力について調べた。6株全てがトレハロースを含む培地中で良好な増殖を示したが，トレハラーゼ活性はNo. 55株の無細胞抽出液だけに確認された。No. 55株の無細胞抽出液の最大トレハラーゼ活性は0.298 U/ml，比活性は0.03 U/mg of proteinであった。No. 55株は種々の同定試験およびバイオテスト2システムの結果から*Moraxella osloensis*と分類された。すでに報告されている*Escherichia coli*の比活性は4.9 U/mg of protein，*Bifidobacterium longum*の比活性は0.003 U/mg of proteinであり，本菌はこれら二種類の細菌の値と大きく異なることが確認された。