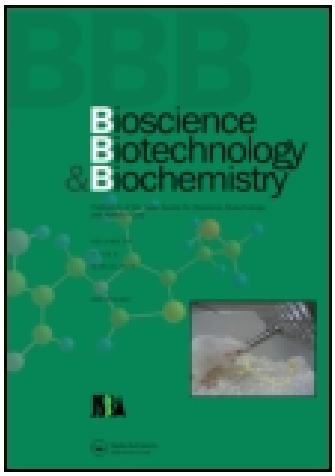

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
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Published online: 12 Jun 2014.

To cite this article: Hideki Kizawa, Jun-ichi Miyazaki, Akira Yokota, Yukihiro Kanegae, Ken-ichiro Miyagawa & Yoshio Sugiyama (1995) Trehalose Production by a Strain of Micrococcus varians, Bioscience, Biotechnology, and Biochemistry, 59:8, 1522-1527, DOI: 10.1271/bbb.59.1522

To link to this article: http://dx.doi.org/10.1271/bbb.59.1522

Biosci. Biotech. Biochem., **59** (8), 1522–1527, 1995

Trehalose Production by a Strain of *Micrococcus varians*

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Received February 15, 1995

A bacterium isolated from soil was found to accumulate abundant trehalose in its medium. The bacterium was identified as a strain of *Micrococcus varians* from its taxonomical characteristics and was designated *M. varians* strain No. 39. Several other strains of *Micrococcus* and *Deinococcus* also accumulated extracellular trehalose but *M. varians* strain No. 39 produced the largest amount. Addition of manganese ions, and excess thiamine and nicotinamide, stimulated trehalose accumulation. Addition of 20 g/liter CSL led to the maximum conversion yield of trehalose in the production phase. Keeping the pH of the culture broth at 6.0 facilitated the maximum trehalose production rate. Cultivation of strain No. 39 at 32°C during the growth phase and 34°C during the production phase resulted in maximal trehalose production. Trehalose non-assimilating mutants (strain No. 7 and 9) derived from strain No. 39 accumulated about 15% more trehalose than the parent strain. Strain No. 7 produced 40 mg/ml trehalose from 100 mg/ml glucose under optimized conditions in a 5-liter jar fermentor.

Trehalose (*O*-α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside), a non-reducing disaccharide, is widespread in nature; it occurs in microorganisms and various plant and animal species.[1] Trehalose was at first regarded only as a reserve metabolite, but recent research[2,3] has focused on the role of trehalose in protection against environmental stresses such as desiccation, high osmolarity, frost, and heat. Trehalose also preserves various unstable products such as enzymes, foods, pharmaceuticals, and cosmetics during dry storage,[4–9] and is becoming a preservative of practical importance. On an industrial scale, trehalose is prepared from yeast cells which store it, and the yield of trehalose extracted per unit amount of culture broth depends upon the quantity of yeast cells grown. If trehalose could be produced extracellularly, the yield might not depend on the number of cells, and increased production may be expected. A few microorganisms have so far been reported to accumulate trehalose extracellularly.[10–12]

During our search for oligosaccharide-producing bacteria, a bacterium isolated from soil was found to accumulate abundant extracellular trehalose. In this paper we describe the isolation and characterization of the product from the bacterium, the identification of the bacterium, the clarification of essential factors for the fermentative production of trehalose, the optimization of the culture conditions, and the derivation of a mutant strain with higher trehalose production.

Materials and Methods

Microorganisms. Strain No. 39, a trehalose producer isolated from soil, was the main organism used. The following strains were obtained from the Institute for Fermentation, Osaka (IFO): sixteen strains of *Micrococcus* (*M. agilis* IFO 15323, *M. aurantiacus* IFO 15364, *M. conglomeratus* IFO 15262, *M. conglomeratus* IFO 15263, *M. conglomeratus* IFO 15267, *M. halobius* IFO 15353, *M. kristinae* IFO 15354, *M. luteus* IFO 3067, *M. luteus* IFO 12708, *M. lylae* IFO 15355, *M. nishinomiyaensis* IFO 15356, *M. roseus* IFO 3768, *M. sedentarius* IFO 15357, *M. varians* IFO 3765, *M.* sp. IFO 15265, and *M.* sp. IFO 15385), five strains of *Deinococcus* (*D. erythromyxa* IFO 15344, *D. proteolyticus* IFO 15345, *D. radiodurans* IFO 15346, *D. radiophilus* IFO 15347, and *D. radiopugnans* IFO 15348), two strains of *Staphylococcus* (*Sta. aureus* IFO 13276 and *Sta. epidermidis* IFO 12993), two strains of *Streptococcus* (*Str. faecalis* IFO 12580 and *Str. lactis* IFO 12007), and a strain of *Pediococcus* (*P. parvulus* IFO 12234).

Media and culture conditions. The compositions of the media used are shown in Table I. Microorganisms were maintained on Trypticase soy broth (TSB) agar slants containing 10 g of glucose, 30 g of TSB, and 20 g of agar per liter.

(i) *Shake flask experiments.* Cells grown on the TSB agar slant were inoculated into a 200-ml conical flask containing 20 ml of seed medium A and cultured for 18 h at 32°C on a rotary shaker (200 rpm). One milliliter of this culture was then transferred to a 200-ml conical flask containing 30 ml of main medium A (or C) and was cultured for 5 d at 32°C on a rotary shaker (200 rpm). *M. agilis* IFO 15323 was cultured at 24°C. For *M. halobius* IFO 15353, the main medium A with NaCl added to a final concentration of 60 g/liter was used.

Main medium B was used as a minimum medium to examine the nutritional requirements for the growth of strain No. 39 and the factors affecting trehalose accumulation by this strain. Then the inoculum of the seed medium into the main medium was reduced to 0.1 ml/30 ml. The medium containing casamino acid, nucleic acid base mixture, and vitamin mixture besides main medium B was used as complete medium. The nucleic acid base mixture contained 10 mg each of adenine, guanine, thymine, cytosine, and uracil per liter. The vitamin mixture contained 400 μg each of thiamine·HCl, riboflavin, vitamin B_6, vitamin B_{12}, Ca-pantothenate, and nicotinamide, 10 μg each of biotin and folic acid, 200 μg of PABA, and 2 mg of inositol per liter.

(ii) *Jar fermentor experiments.* Seed culture was prepared as for shake flask experiments except that seed medium B was used instead of seed medium A. One hundred and twenty-five milliliters of the culture broth

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Abbreviations: TSB, Trypticase soy broth; KPB, KH_2PO_4–K_2HPO_4 buffer; PABA, *p*-aminobenzoic acid; PMSF, phenylmethylsulfonyl fluoride; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; CSL, corn steep liquor.

Table I. Composition of Media

Component	Seed		Main			
	A	B	A	B	C	D
Glucose	10 g/liter	20 g/liter	30 g/liter	30 g/liter	30 g/liter	60 g/liter
TSB	30	—	—	—	—	—
Yeast extract	—	—	6	—	—	—
CSL	—	8	—	—	8	20
Urea	—	5	—	—	—	—
$(NH_4)_2HPO_4$	—	—	—	2	1	—
$(NH_4)_2SO_4$	—	—	—	—	—	2.5
KH_2PO_4	—	2	4	4	2	1
$MgSO_4 \cdot 7H_2O$	—	4	4	4	4	0.5
$CaCl_2 \cdot 2H_2O$	—	—	0.01	0.01	—	—
$MnCl_2 \cdot 4H_2O$	—	—	0.01	0.01	0.01	0.01
$FeCl_3 \cdot 6H_2O$	—	—	0.01	0.01	—	—
Biotin	—	1 mg/liter	—	—	0.01 mg/liter	0.01 mg/liter
PABA	—	4	—	—	0.2	0.2
Thiamine·HCl	—	8	—	—	10	10
Nicotinamide	—	8	—	—	20	20

was transferred into a 5-liter jar containing 2.5 liters of main medium D. Cultivation was done at 32°C for 3 d under the following conditions: aeration, 1.25 liter/min; agitation, 600 rpm. During cultivation, the pH was kept at 6.0 with 10% NaOH.

Measurement of trehalose. Ten milliliters of culture broth were centrifuged at 5000 × *g* for 15 min. Trehalose present in the supernatant was measured as extracellular trehalose by high performance liquid chromatography (HPLC) under the following conditions: column, Shodex SUGAR SZ 5532 (Shodex); eluent, acetonitrile–water (80 : 20, v/v); flow rate, 1 ml/min; temperature, 55°C; detector, differential refractometer. Cells were harvested, washed once with water, and suspended in 6 ml of 70% ethanol. This suspension was heated at 100°C for 5 min, cooled in a water bath, and centrifuged at 10,000 × *g* for 5 min. Trehalose in the resultant supernatant was measured as intracellular trehalose. The amounts of both extracellular and intracellular trehalose were recorded as the weight per ml of culture broth.

Measurment of cell growth. Cell growth was measured by the optical density at 600 nm, or as mg dry cell weight per ml of culture broth (DCW). To measure DCW, cells harvested from 10 ml culture broth were washed once with water, desiccated at 80°C in a vacuum oven for 18 h, and weighed.

Purification of the product accumulated in the culture broth of strain No. 39. The culture (700 ml) of strain No. 39 with main medium A was centrifuged at 5000 × *g* for 15 min. The supernatant was concentrated to 180 ml under reduced pressure and put on a column (3 × 30 cm) packed with activated charcoal (LH2C carbon, Takeda Chemical Industries, Ltd., Japan). The column was washed with about 800 ml of distilled water and eluted with 300 ml of 10% (v/v) ethanol. The eluate was concentrated to 10 ml under reduced pressure, mixed gradually with 40 ml of 99.5% ethanol, and stood at 4°C overnight. The resulting crystals were collected by filtration, dissolved in 8 ml of distilled water, and then precipitated with appropriate amount of ethanol. The resultant crystals were washed with a small amount of ethanol and dried at 60°C for 5 h.

Analysis. The melting point was measured by differential thermal analysis with a SSC 5000 System (Seiko Denshi Kogyo, Tokyo). The specific rotation was measured with a Highly Sensitive Polarimeter SEPA-200 (HORIBA, Tokyo). The water content was measured with a Thermal Analysis Station TAS 100 (RIGAKU, Tokyo). The infrared (IR) spectrum was measured using the KBr method with a Fourier Transformation Infrared Spectrometer FT-530 (HORIBA, Tokyo).

Hydrolysis by trehalase. The reaction mixture (200 μl) containing 6.67 μmol of the product (calculated as the amount of trehalose), 40 μmol of citrate–sodium citrate buffer (pH 5.4), and 0.39 units trehalase was incubated at 37°C for 4 h. The mixture was heated in a boiling water bath for 10 min to stop the reaction, then 800 μl of ethanol was added and the mixture was centrifuged at 15,000 × *g* for 5 min. The supernatant was analysed by HPLC (column: Shodex SUGAR SP 0810, eluate: distilled water, flow rate: 0.5 ml/min, temperature: 80°C, detector: differential refractometer).

Isolation of trehalose non-assimilating mutants from strain No. 39. Cells of strain No. 39 were suspended (10^7 cells/ml) in 0.1 M KH_2PO_4–K_2HPO_4 buffer (KPB) (pH 7.0), irradiated with UV light from a distance of 30 cm to give a survival rate of 1%, and spread on an agar plate (G-plate) containing 5 g of glucose, 1 g of $(NH_4)_2HPO_4$, 4 g of $MgSO_4 \cdot 7H_2O$, 0.025 mg of biotin, 0.2 mg of *p*-aminobenzoic acid (PABA), 0.4 mg of thiamine·HCl, 20 mg of nicotinamide, 0.1 mol of KPB (pH 6.0), and 20 g of agar per liter. The plate was incubated at 32°C for 48 h. The colonies appeared were transferred by a conventional replication method to another G-plate and T-plate containing trehalose instead of glucose in the G-plate, and the plates were incubated at 32°C for 48 h.

Assay of trehalose phosphorylase. Cells were suspended (about 250 mg wet cell weight/ml) with 0.1 M KPB (pH 7.0) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated (190 W, 60 min) at 4°C. The cell debris was removed by centrifugation at 15,000 × *g* for 20 min. The resulting supernatant was used as the cell-free extract.

The reaction mixture contained 200 μmol trehalose, 50 μmol KPB (pH 7.0), 50 μmol *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–KOH buffer (pH 7.0), and cell-free extract (1 mg protein) in a total volume of 1.0 ml. The mixture was incubated at 30°C for 15 min and the glucose formed was measured by a glucose oxidase–peroxidase method. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose.

Materials. Trehalose dihydrate, yeast extract, and TSB were purchased from Sigma Chemical Co., Nihon Seiyaku Co., and Becton Dickinson and Company, respectively. Trehalase (prepared from porcine kidney) was purchased from Sigma Chemical Co. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd.

Results

Isolation and characterization of the product

After strain No. 39 had been cultivated in main medium A, the culture filtrate was analyzed by HPLC (Fig. 1). A peak at the same retention time (12.72 min) as authentic trehalose was observed.

Crystallized material (4.8 g) was isolated from the culture broth (700 ml) and characterized by various analyses. $[\alpha]_D$ and melting point (mp) were +175.4° and 90.4°C, respectively. These values almost coincided with those ($[\alpha]_D$ +179.5°, mp 87.1°C) of authentic trehalose (Sigma) and

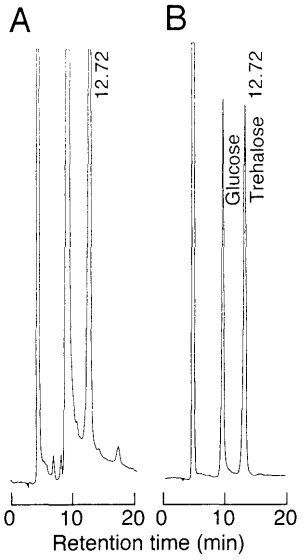

Fig. 1. HPLC Profiles of Culture Filtrate of Strain No. 39 (A) and Authentic Trehalose (B).

those ($[\alpha]_D$ +178°, mp 97°C) mentioned in the Biochemical Data Book.[13] The specific rotation of the crystals was clearly different from those (+70 ~ +95 and −42 ~ −38) of neotrehalose (*O*-α-D-glucopyranosyl-(1→1)-β-D-glucopyranoside) and isotrehalose (*O*-β-D-glucopyranosyl-(1→1)-β-D-glucopyranoside). Hydrolysis of the crystals by trehalase yielded only glucose. When the crystals were supposed to be trehalose, the molar ratio of the consumed to the formed glucose was 1 : 1.9. The water content of the crystals was found by thermogravimetric analysis to be 9.64%, which agreed with the theoretical value for trehalose dihydrate (9.52%). The IR spectrum of the crystals was consistent with that of authentic trehalose (data not shown). These results demonstrate that the product accumulated in the culture filtrate of strain No. 39 is trehalose (dihydrate).

Taxonomical studies on strain No. 39

Strain No. 39 was taxonomically characterized and identified according to Bergey's Manual of Systematic Bacteriology, Vol. 2[14] (Table II). This strain was a Gram-positive, nonmotile coccus (0.9–1.0 μm in diameter) and formed slightly yellowish colonies. The G+C content of the DNA was 69.9 (mol%). The cell wall peptidoglycan contained glutamic acid, lysine, and alanine with a molar ratio of about 1 : 1 : 6. The major menaquinone was MK-7 (H_2). These chemotaxonomical studies suggest that this strain belongs to the genus *Micrococcus* and is very similar to *M. varians*. Further cultural, physiological, and biochemical characteristics were examined. The strain grew on Simmons citrate agar[15] and produced acid from glucose. The cells reduced nitrate to nitrite. The strain had urease but not oxidase activity. From these results, strain No. 39 was identified as a type of *M. varians*.

Extracellular accumulation of trehalose by Micrococcus and Deinococcus species

Trehalose productivity was investigated in sixteen strains of *Micrococcus* spp and five strains of the related genus, *Deinococcus* spp, in flask culture with main medium A (Table III). Most strains tested accumulated extracellular trehalose. In *M. agilis* IFO 15323, *M. luteus* IFO 3067, *M. luteus* IFO 12708, *M. varians* IFO 3765, *D. erythromyxa* IFO 15344, *D. proteolyticus* IFO 15345, and *D. radiopugnans* IFO 15348, the amount of trehalose accumulated in the culture broth was over 2 mg/ml. Among these species, *D. proteolyticus* IFO 15345 produced the largest amount of trehalose (8.34 mg/ml). Under the same conditions, strain No. 39 produced 1.4 times as much extracellular trehalose as *D. proteolyticus* IFO 15345. These results indicate that many *Micrococcus* and *Deinococcus* strains are capable of accumulating extracellular trehalose. Further, to investigate whether trehalose producion was a general feature of Gram-positive cocci or not, trehalose productivity in several strains of *Staphylococcus*, *Streptococcus*, and *Pediococcus* spp was tested (Table III). All the strains tested, however,

Table II. Taxonomic Characteristics of Strain No. 39

Characteristic	Strain No. 39
Gram staining	+
Morphology	Cocci
Cell size	0.9–1.0 μm
Motility	−
Color of cells	Slightly yellowish
Catalase	+
Oxidase	−
Cellular fatty acids	anteiso-15 : 0 + anteiso-17 : 0 (No hydroxy acids)
Amino acids of peptidoglycan	Glu : Lys : Ala = 1.00 : 0.97 : 5.80
(Peptidoglycan type)	L-Lys-L-Ala_{3-4} (A3α, A11.7)
Menaquinone	MK-7 (H_2)
G+C content (mol%)	69.9
Urease	+
β-Galactosidase	+
Phosphatase	−
Arginine dihydrolase	−
Acetoin	−
Nitrate reduction	+
Hydrolysis of gelatin	+
Starch	−
Esculin	−
Polyoxyethylenesorbitan monooleate	−
Growth on	
Simmon's citrate agar	+
Inorganic nitrogen agar	−
Nutrient agar with	
10% NaCl	+
15% NaCl	−
Acid production from	
D-Glucose	+
D-Fructose	+
D-Galactose	+
D-Mannose	−
D-Xylose	+
L-Rhamnose	−
Maltose	+
Lactose	−
Sucrose	+
Trehalose	+
Glycerol	−
Sorbitol	−
Temp. range for growth (°C)	20–37°C
Optimum temp. (°C)	30°C
Strict aerobes	+
Lysozyme susceptibility	
(400 μg/ml)	Resistant
(800 μg/ml)	Sensitive

did not accumulate even intracellular trehalose. Therefore, the accumulation of trehalose was thought to be a characteristic of strains of *Micrococcus* and *Deinococcus* spp.

Factors affecting the extracellular accumulation of trehalose by strain No. 39

Strain No. 39 can grow in media containing yeast extract but not in synthetic media composed only of a carbon source, inorganic nitrogen source, and inorganic salts. We then examined the nutritional requirements of the strain, finding that simultaneous supply of biotin, PABA, thiamine, and nicotinamide was very important for the growth of strain No. 39 (data not shown).

So the effects of these four vitamins on trehalose accumulation in flask culture were tested (Fig. 2). The concentration of biotin and PABA necessary for maximum production of trehalose were the same levels as those necessary for growth (around 5 and 20 μg/liter, respectively), while in the case of thiamine and nicotinamide adding them in excess (around 8 mg/liter) was necessary for maximum production of trehalose. Further we found that only manganese ions markedly stimulated the accumulation of trehalose among various metal ions (data not shown). These results showed that thiamine, nicotinamide, and manganese ions were key factors for the accumulation of trehalose by strain No. 39.

Effect of corn steep liquor (CSL) on trehalose fermentation

The optimum conditions for trehalose production were investigated by experiments using a 5-liter jar fermentor. The effect of CSL on trehalose fermentation was tested (Table IV). With increasing CSL concentrations, the DCW increased and the culture period was shortened. The CSL concentration leading to the maximum trehalose production was 2 g/liter. The maximum conversion yield of trehalose (from glucose) during the production phase (from 24 h after cultivation to the end of fermentation) was obtained with 20 g/liter CSL, when the yield was as high as 70% (by weight). These results suggest that CSL promotes not only growth but also trehalose production in strain No. 39.

Effect of pH on trehalose fermentation

When the pH of the culture broth was kept at 6.0 during cultivation, the maximum production of trehalose was

Table III. Trehalose Accumulation by Species of *Micrococcus* and Related Genera

Species	IFO No.	DCW (mg/ml)	Trehalose (mg/ml)	
			Intracellular	Extracellular
M. agilis	15323	12.7	2.16	2.28
M. aurantiacus	15364	7.9	0.89	0.11
M. conglomeratus	15262	6.2	<0.01	0.83
M. conglomeratus	15263	5.2	0.05	0.53
M. conglomeratus	15267	6.1	<0.01	0.60
M. halobius	15353	4.5	0.87	0.05
M. kristinae	15354	9.2	0.44	<0.01
M. luteus	3067	12.4	0.21	2.31
M. luteus	12708	12.9	0.84	2.29
M. lylae	15355	3.1	0.04	0.65
M. nishinomiyaensis	15356	5.7	<0.01	0.05
M. roseus	3768	8.5	0.59	1.14
M. sedentarius	15357	6.7	0.32	0.37
M. varians	3765	9.0	0.83	2.23
M. sp.	15265	5.6	0.19	0.23
M. sp.	15385	2.2	0.19	0.43
D. erythromyxa	15344	8.2	0.09	2.05
D. proteolyticus	15345	6.4	0.43	8.34
D. radiodurans	15346	6.5	0.05	<0.01
D. radiophilus	15347	7.5	0.01	<0.01
D. radiopugnans	15348	6.1	0.92	2.84
Sta. aureus	13276	3.1	<0.01	<0.01
Sta. epidermidis	12993	2.9	<0.01	<0.01
Str. faecalis	12580	3.3	<0.01	<0.01
Str. lactis	12007	2.7	<0.01	<0.01
P. parvulus	12234	2.6	<0.01	<0.01
M. varians strain No. 39	–	5.8	0.72	11.90

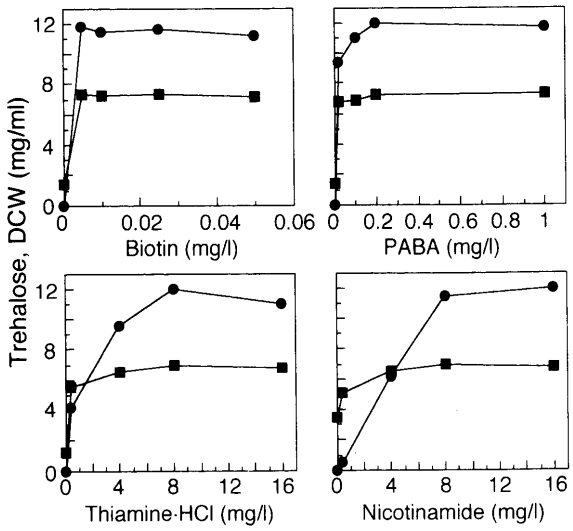

Fig. 2. Effects of Vitamins on Trehalose Production.

The concentration of one of the four essential vitamins was varied while the remaining three were added in constant amounts. The following are the constant concentrations per liter of basal medium for each vitamin: 0.01 mg biotin, 0.2 mg PABA, 10 mg thiamine·HCl, and 20 mg nicotinamide. ●, trehalose; ■, DCW.

Table IV. Effect of CSL Concentration on Trehalose Fermentation

CSL (g/liter)	DCW (mg/ml)	Trehalose (mg/ml)	Cultivation time (h)	Conversion yield[a] (%)
0	7.6	12.8	>120	22.7
2	8.0	25.0	120	61.2
4	8.3	23.1	120	62.2
8	10.2	22.5	96	65.5
20	15.0	21.7	72	70.0
30	18.9	17.0	72	48.6

[a] Calculated from (mg of trehalose produced extracellularly)/(mg of glucose consumed in the production phase) × 100.

Table V. Effect of pH on Trehalose Fermentation

pH	DCW (mg/ml)	Trehalose (mg/ml)	Cultivation time (h)
5.5	8.8	17.8	120
6.0	15.0	21.7	72
6.5	15.0	13.5	96
7.0	16.9	1.9	120

obtained (Table V). A deviation of only 0.5 from pH 6.0 markedly lowered the rate of trehalose production and delayed fermentation. These results show that keeping the pH of the culture broth at 6.0 is important for trehalose production.

Effect of temperature on trehalose fermentation

The effect of temperature on trehalose production by strain No. 39 was examined (Table VI). Maximum trehalose production was obtained with cultivation at 32°C during the growth phase (from initial cultivation to 24 h) and at 34°C during the production phase (from 24 h to the end of cultivation). Furthermore, cultivation at 34°C during the production phase shortened the culture time regardless of the incubation temperature during the growth phase.

Table VI. Effect of Temperature on Trehalose Fermentation

Temperature (°C)		DCW (mg/ml)	Trehalose (mg/ml)	Cultivation time (h)
Growth phase	Production phase			
32	32	15.2	32.7	168
32	34	15.0	35.6	125
32	36	15.4	32.9	144
30	34	14.9	32.6	125
34	34	14.8	34.7	125

After 48 h of cultivation, 40 g/liter glucose, calculated on the basis of the initial liquid volume, was added to the culture medium.

Table VII. Trehalose Productivity and Trehalose Phosphorylase Activity in Trehalose Non-assimilating Mutants

Strain No.	Growth (OD_{600})		Trehalose (mg/ml)	Trehalose phosphorylase activity (nmol/min·mg protein)
	Trehalose	Glucose		
1	0.11	3.23	11.2	0.0
2	0.39	2.36	11.3	0.0
3	0.09	1.80	11.6	0.0
5	0.20	2.24	10.7	0.0
7	0.28	4.04	12.8	0.0
9	0.68	3.04	12.6	26.6
Parent	4.68	3.48	11.1	32.6

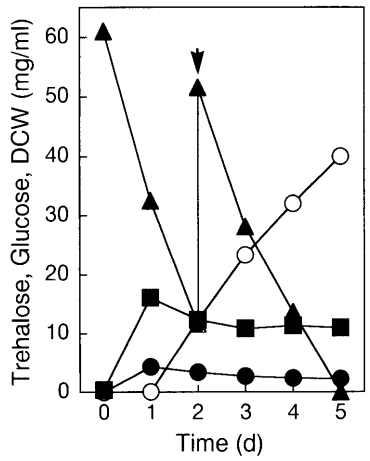

Fig. 3. Course of Trehalose Fermentation by *M. varians* Strain No. 7 in a 5-liter Jar Fermentor.
Strain No. 7 was cultivated at 32°C during the growth phase and 34°C during the production phase in main medium D. After 48 h of cultivation, 40 g/liter glucose, calculated on the basis of the initial liquid volume, was added to the culture medium. The arrow indicates the point at which glucose (40 g/liter) was added. ○, extracellular trehalose; ●, intracellular trehalose; ▲, glucose; ■, DCW.

Trehalose production by trehalose non-assimilating mutants

Strain No. 39 can grow well with trehalose as its sole carbon source. Trehalose productivity in trehalose non-assimilating mutants induced from strain No. 39 were also compared with that of strain No. 39 (Table VII). Strains No. 7 and No. 9 produced about 15% more trehalose than the parent on a flask scale. All the mutants except strain No. 9 were deficient in trehalose phosphorylase activity, but the parent strain had it.

Course of trehalose fermentation by a trehalose non-assimilating mutant, strain No. 7

Figure 3 shows the course of trehalose fermentation by strain No. 7 under optimized conditions. During fermentation, the pH was kept at 6.0 with 10% NaOH, while the temperature was kept at 32°C during the growth phase and 34°C during the production phase. During the growth phase, strain No. 7 produced only intracellular trehalose, while extracellular accumulation was observed after 24 h of cultivation. These results clearly show that the fermentation is of a non-growth associated type. Under the above conditions, strain No. 7 produced 40 mg/ml trehalose from 100 mg/ml glucose in 5 d: the trehalose yield was as high as 40% (by weight).

Discussion

We have found that *M. varians* strain No. 39, isolated from soil, accumulated abundant extracellular trehalose. Intracellular accumulation of trehalose by some species of the genus *Micrococcus* has already been reported[16] but extracellular accumulation has not. Most of the strains of *Micrococcus* and the related genus, *Deinococcus*, obtained from IFO also accumulated a small amount of extracellular trehalose: this is the first report of the accumulation of trehalose by *Deinococcus* bacteria.

Strain No. 39 can grow well with trehalose as its sole carbon source. We expected that some trehalose non-assimilating mutants induced from strain No. 39 would accumulate a larger amount of trehalose than the parent. Actually, as shown in Table VII, strains No. 7 and 9 accumulated about 15% more trehalose than the parent. Furthermore, we examined trehalose-degrading activity in the cell-free extracts of strain No. 39 and the mutants. The activity found in strain No. 39 proved to be that of a trehalose phosphorylase; however, all the mutants except for strain No. 9 were deficient in this activity. We also tested trehalase known to involve in degradation of trehalose among many organisms,[1] but could not detect it. If the trehalose phosphorylase in strain No. 39 was involved in trehalose synthesis, the amount of trehalose accumulated in trehalose phosphorylase-deficient mutants should be markedly reduced; yet no such mutants were observed. These results imply that trehalose phosphorylase in strain No. 39 is involved in the degradation (including the assimilation) of trehalose rather than its synthesis. It is presumed that strain No. 9 will be a mutant with alterations

somewhere in the steps involved in the assimilation of trehalose. The reason why the mutant strain No. 9 produced an elevated amount of trehalose is now under investigation. We have also purified and characterized the trehalose phosphorylase in strain No. 39, and will report on this in the near future.

Our results demonstrated that strain No. 7 produced the largest amount of trehalose in the strains we tested: that is, it produced 40 mg/ml trehalose from 100 mg/ml glucose in 5 d under optimized conditions. In conclusion, *M. varians* strain No. 39 and its trehalose non-assimilating mutants (especially strain No. 7) produced a superior conversion yield of carbon source into trehalose than other trehalose producing microorganisms such as yeasts or *Brevibacterium lactofermentum*,[12] and could be of potential use for manufacturing trehalose on an industrial scale.

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