MUTAGENESIS OF *LEUCONOSTOC MESENTEROIDES* AND SELECTION OF DEXTRANSUCRASE HYPERPRODUCING STRAINS

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ABSTRACT

Dextransucrase of *Leuconostoc mesenteroides* PTCC 1059 is an enzyme of industrial and medical interest that catalyzes the synthesis of a soluble dextran from sucrose. The mutant strains of *Leuconostoc mesenteroides* PTCC 1059 hyperproducing for dextransucrase were isolated after UV irradiation and treatment with ethyl methane sulfonate. The enzyme activity of one mutant strain, the 1059M5E4, was about 2.5-fold higher than that of the wild type, while its cell growth was relatively lower. The 1059M5E4 dextransucrase produced the same type of dextran as well as the wild type but showed higher thermal stability. These properties may be interesting for using this strain in enzymatic production of dextran.

Key words: Dextransucrase, Dextran, *Leuconostoc mesenteroides*, Mutation

INTRODUCTION

Dextrans are extracellular bacterial polymers of D-glucopyranose with predominantly α 1,6 linkages in the main chain and a variable amount of α 1,2, α 1,3 or α 1,4 branch linkages depending on the strain of the organism (1,2). The principal genera of bacteria that produce the enzymes (dextransucrases or glucansucrases) that catalyze the synthesis of dextrans from sucrose are *Leuconostoc*, *Streptococcus* and *Lactobacillus*. The majority of known dextrans are produced by strains of *Leuconostoc mesenteroides* (3,4). The *Leuconostoc mesenteroides* PTCC 1059 dextransucrase is an industrially important enzyme and has received wide attention. *Leuconostoc mesenteroides* PTCC 1059 elaborates a single dextransucrase that catalyzes the synthesis of a dextran with 95% α 1,6 linkages in the main chain and 5% α 1,3 branch linkages. This linear, water-soluble dextran is effective for a number of potential uses in industrial and pharmaceutical applications (5-9). Other strains of *Leuconostoc mesenteroides* elaborate more than one type of dextransucrase and form both soluble and insoluble dextrans. The synthesis of dextran from sucrose by a cell free bacterial culture was first demonstrated in 1940 (10).

The present study describes mutagenesis of *Leuconostoc mesenteroides* PTCC 1059 and isolation of dextransucrase hyperproducing strains and some aspects of the enzyme production by the wild type and the hyperproducer mutant.

MATERIALS AND METHODS

Materials:

Ethyl methane sulfonate (EMS), *Penicillium* dextranase and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma Chemical Co. MRS broth was purchased from Merck Co. All other chemicals were of analytical grade.

Strain and culture conditions:

Leuconostoc mesenteroides PTCC 1059 (NRRL B-512) was used as the wild strain and was obtained from the Persian Type Culture Collection. Stock cultures were maintained at 4°C by monthly transfers on MRS medium containing 1.5% (w/v) agar. The medium suggested by Jeanes (11), used for the enzyme production, was composed of 2% sucrose, 0.5% yeast extract, 2%KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.001% NaCl, 0.001% $FeSO_4.7H_2O$, 0.001%$MnSO_4.H_2O$ and supplemented with 0.005% $CaCl_2$ as recommended by Robyt & Walseth (12). The pH of the medium was adjusted to 7.2-7.4 after sterilization. The batch culture medium (50 ml) was inoculated with 2% (v/v) of a 15 h preculture (2×10^8 cells/ml) containing: 2%

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sucrose, 0.5% yeast extract, 0.25% tryptone and 0.5% K_2HPO_4. The initial pH was adjusted to 7.2-7.4. The cultures were then incubated at 25°C with shaking at 150 rpm. Incubation was terminated at the end of exponential growth phase, when the pH was 5.0-5.2.

Mutagenesis:
UV mutagenesis, *Leuconostoc mesenteroides* PTCC 1059 was grown at 25°C in 100 ml of liquid MRS medium to a cell density of about 2×10^8 cells/ml. The cells were harvested aseptically by centrifugation at 5,000 × g for 15 min and washed twice in 100 ml of cold, sterile 0.85% NaCl solution. 8 ml portions of cell suspension were transferred to sterile, glass Petri dishes 130 mm in diameter, and irradiated with UV light (253.7 nm) from a germicidal lamp (Philips, TUV 15 W) with a distance of 40 cm between lamp and cell suspensions. The irradiation dose was chosen to give 0.1% survivors as estimated from a UV survival curve. Each irradiated sample centrifuged and resuspended in 10 ml MRS broth and incubated at 25°C for 18 h. Then the cultures were diluted serially into sterile 0.85% NaCl. Dilutions were plated onto MRS agar and incubated in darkness at 25°C for 48h. EMS mutagenesis was carried out with a culture grown to the late logarithmic phase of growth in MRS broth. The cells were harvested and washed twice with sterile 50 mM sodium phosphate buffer; pH 6.2, and 40 µl (of 1.17g/l) of EMS was added to 2 ml cell suspension. The mixture was aerated on a shaker incubator at 30°C for 30 min. Then an equal volume of a freshly made 10% (w/v) filter-sterilized solution of sodium thiosulfate was added to cell suspension to quench the EMS (13). The treated cells were washed twice and resuspended with the same buffer and after serial dilution were spread on MRS agar plates and incubated at 25°C for 48 h.

Assay of enzyme activity:
Culture supernatant of selected mutants were prepared by centrifugation at 10,000×g for 15 min and used as the enzyme solution. Assay of dextransucrase activity employed a reaction mixture containing 50 µl of enzyme solution and 450 µl of sucrose buffer consisting of 10% (w/v) sucrose, 20mM $CaCl_2$ in 50 mM acetate buffer, pH 5.2 and incubated at 30°C for 20 min. The amount of reducing sugar (fructose), which released during dextran synthesis from sucrose, was estimated by the DNS method (14,15,16). One unit of dextransucrase activity was defined as the amount of enzyme that could release 1 µmole fructose from sucrose per minute at 30°C.

Polyacrylamide gel electrophoresis:
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 7.5% (w/v) acrylamide gel and the method of Laemmli (17). Protein was stained with silver nitrate.

Preparation and hydrolysis of dextran:
Dextran was prepared by addition of equal volume of culture supernatant to an equal volume of sucrose buffer. The reaction was carried out at 30°C for 8 h. Dextran was precipitated by addition of an equal volume of ethanol. Precipitates were dissolved in distilled water and reprecipitated with 50% ethanol and dried under vacuum. 5 milligram dextran (0.5% w/v in 50 mM citrate phosphate buffer, pH 5.5) incubated with 2 units of *Penicillium* dextranase at 30°C for 16 h and the amount of reducing sugar was measured. The carbohydrate composition of the digests was analyzed by thin layer chromatography, using the solvent system 1:2:3:4:5 (v/v) nitroethane/nitromethane/ethanol/water/1-propanol. The carbohydrates were visualized by dipping the plates into 5% (v/v) H_2SO_4 in ethanol containing 0.5% (w/v) α–naphthol, followed by drying, and heating for 10 min at 120°C.

RESULTS AND DISCUSSION

Cell suspensions of *Leuconostoc mesenteroides* PTCC 1059 were exposed to UV light from 5 sec to 2 min and irradiation time of 80 sec gave 0.1% survivors. This irradiation time was used for UV mutagenesis of cell suspensions as described in materials and methods. The mutant strains were selected at the basis of colony morphology (size and opacity) and their enzyme activities were determined in the cell free culture broth. Some of these strains are described in Table 1. The dextransucrase activities of these strains showed 20% to 40% increase, and the mutant 1059M5, which showed about 40% increase in enzyme activity (2.96 units/ml) was chosen for mutagenesis with EMS.

After EMS mutagenesis, about 100 colonies were isolated from the treated cells, and their enzyme activities were determined. About 10 mutants showed extremely poor activities, and the rest of the mutants showed higher activities from 2.96 to 5.2 units/ml.

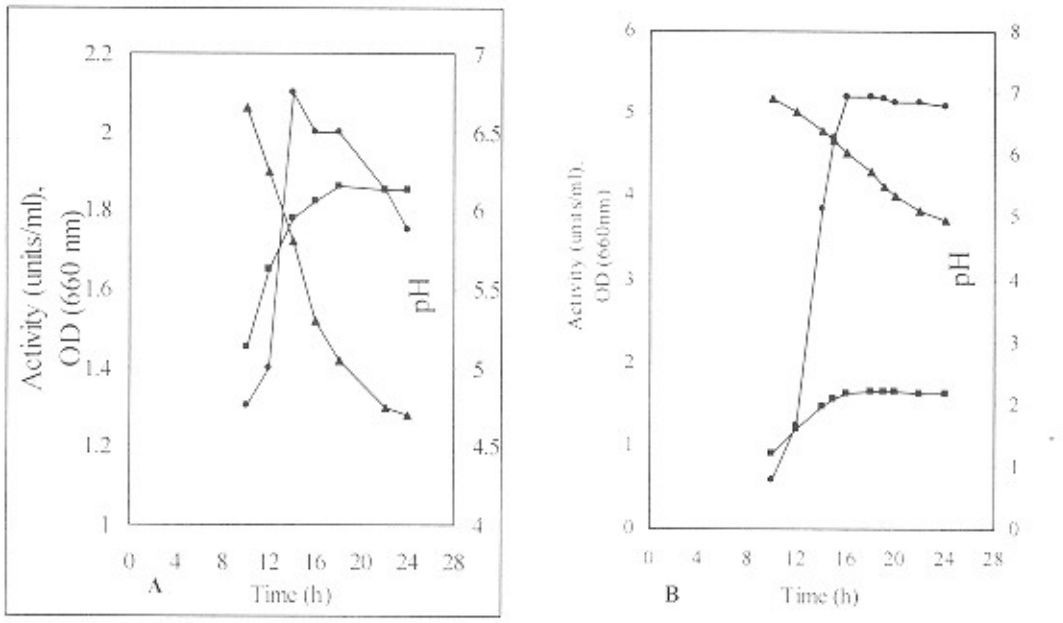

Fig. 1 Time course of dextransucrase production. A number of flask cultures were prepared, duplicate flasks were taken periodically and the contents used for measurement of •,Activity; ■, Cell growth; ▲, pH. A, *Leuconostoc mesenteroides* PTCC 1059; B, mutant strain 1059M5E4.

Table 2 shows enzyme activities of some mutant strains after EMS treatment. The mutant, 1059M5E4, which showed the highest enzyme activity (5.2 units/ml, about 2.5-fold increase) was selected for the subsequent experiments. Fig. 1 shows the time course of dextransucrase production in batch cultures of *Leuconostoc mesenteroides* PTCC 1059 and mutant 1059M5E4. Enzyme activity, pH and cell growth (OD at 660 nm) were estimated from 10 to 24 h. Dextransucrase production occurred during cell growth and maximum enzyme activity and cell growth were attained after 16 h for mutant, and after 14 h for wild type strain and showed that enzyme production was growth-associated. During culture, sucrose consumption by the cells led to dextransucrase induction and the conversion of the glucose moiety into lactic acid, acetic acid, ethanol and energy causes decrease in pH. For the mutant strain (Fig. 1B) sudden elaboration of the enzyme began when the pH was 6.9 and continued until it was 6.0 and it was similar to that of wild type strain (Fig. 1A) and previous reports (11,18).

Table 3 shows the effect of initial concentrations of sucrose on dextransucrase production. Increase of sucrose concentration from 0.5 to 2% caused higher enzyme production, but Increase of sucrose from 2 to 4% had not a significant effect on dextransucrase production and cell growth in both wild and mutant strains. At level of 4% sucrose the culture contained so much dextran and became viscous that separation of cells from such a culture were difficult. It was considered that the increase of viscosity of the culture medium interfered with the cell growth and enzyme production and formation of a complex of dextran with the enzyme, interfered with the isolation of dextransucrase (19). Accordingly, it was concluded that 2% sucrose was the optimum level for production of dextransucrase with wild type and mutant strains. The data showed that enzyme activities of the mutant grown on 2% and 4% sucrose were about 2.5-fold higher than that of the wild type, while the mutant exhibited relatively reduced growth rate and biomass yield.

SDS-PAGE electrophoresis (Fig. 2) of crude dextransucrases showed that there was no significant difference between protein profile of wild type and mutant strains, and it showed qualitatively that dextransucrase elaborated by the mutant 1059M5E4 was more than that of the wild-type.

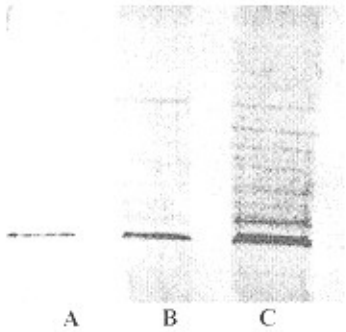

Fig. 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of crude dextransucrases. Samples containing equal volum of each enzyme solution (40-80 µg prot/ml), were loaded into each well. (A) *Leuconostoc mesenteroides* PTCC 1059; (B) 1059M5; (C) 1059M5E4.

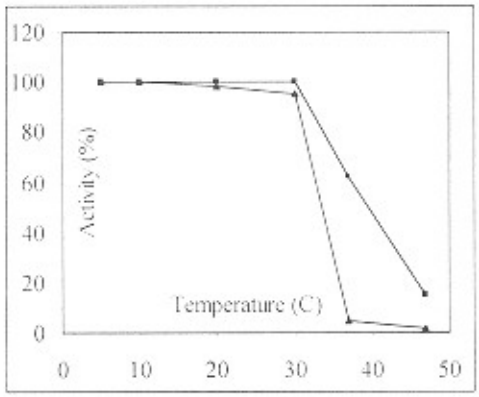

Fig. 3 Temperature stability of dextransucrases of▲, *Leuconostoc mesenteroides* PTCC 1059;■, mutant 1059M5E4.

The dextrans prepared from the wild and mutant strains were hydrolyzed with *Penicillium* dextranase, and their degree of hydrolysis and the hydrolyzate profiles were compared (table 4). The hydrolysis degree of dextran of 1059M5E4 was almost similar to that of the wild type and it was the same as the result of the hydrolysis of NRRL-B512 F dextran, which was 32.5% (20). The dextran produced by mutant and wild type dextransucrases also gave similar dextranase products including: glucose, isomaltose and a little branched saccharides. As judged by dextranase hydrolysis of dextrans, the 1059M5E4 produced a dextran with the same structural characteristics as the dextran produced by the dextransucrase of the wild type. The activity of crude 1059M5E4 enzyme showed less than 5% decrease at room temperature (21°C) over one month, where as the enzyme from the wild type showed a significant decrease in activity (35%) at the same condition. The wild type and mutant dextransucrases were held at various temperatures for 30 min and their activities were assayed. As shown in Fig. 3 the thermal stability of the 1059M5E4 dextransucrase was greater than that of the wild type dextransucrase. The 1059 dextransucrase activity decreased greatly above 30°C and at 37°C had 5% of maximum activity, while the mutant dextransucrase had 60% of maximum activity at 37°C. Experiments with this mutant strain have been repeated and carried out for 9 months, revealing no alterations in stability or performance. In this study, we report on *Leuconostoc mesenteroides* PTCC 1059 mutants that are hyperproducer for dextransucrase. Using physical and chemical mutagenesis we isolated several mutants and evaluated them for dextransucrase activity. Enzyme activity of the mutant, 1059M5E4 was about 2.5-fold higher than that of the wild type.

Although the fermentation method is used for the commercial production of dextran, the enzymatic synthesis have many advantageous over fermentation method, including greater size uniformity and simple purification of the product, and the hyperproducing strains may be considered useful in the production of the enzyme.

Table 1. Dextransucrase activity of wild type and mutant strains after UV mutagenesis

Strain	Dextransucrase activity (Units/ml)
1059 (wild)	2.10
1059M1	2.55
1059M2	2.73
1059M3	2.50
1059M4	2.85
1059M5	2.96
1059M6	2.80
1059M7	2.63

Table 2. Dextransucrase activity of mutant strains after EMS treatment

Strain	Dextransucrase activity (Units/ml)
1059M5E1	4.62
1059M5E2	4.84
1059M5E3	4.55
1059M5E4	5.20
1059M5E5	4.90
1059M5E6	4.15
1059M5E7	3.86
1059M5E8	5.05
1059M5E9	3.64

Table 3. Effects of initial concentrations of sucrose on dextransucrase production by *Leuconostoc mesenteroides* PTCC 1059 and its mutant 1059M5E4

Sucrose (%)	Cell growth (OD 660 nm)[a]		Activity (Units/ml)	
	1059	1059M5E4	1059[b]	1059M5E4[c]
0.5	0.26	0.22	0.18	0.70
1.0	0.45	0.38	0.44	1.60
2.0	1.80	1.60	2.15	5.15
4.0	1.78	1.60	2.20	5.20

[a]OD at 660 nm was determined using a Novaspec II Spectrophotometer, Pharmacia Biotech.
[b]Maximum enzyme activity for the wild type was attained after 14 h incubation.
[c]Maximum enzyme activity for the mutant was attained after 16 h incubation.

Table 4. Degree of dextranase hydrolysis of dextrans synthesized by *Leuconostoc mesenteroides* PTCC 1059 and mutant 1059M5E4 dextransucrases

Dextran	Degree of hydrolysis (%)
1059	33.8
1059M5E4	36.7

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