THE EFFECT OF CERTAIN CULTURAL FACTORS ON PRODUCTION OF DEXTRANSUCRASE BY LEUCONOSTOC MESENTEROIDES[1]

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Present knowledge on the characteristics of dextransucrase and its mode of action is based primarily on the important investigations of Hehre (1941, 1946, 1951) and Hehre and Sugg (1942). Hitherto, a serious impediment to studies of this interesting enzyme has been the difficulty of procuring dextransucrase. Development of further knowledge about it would be greatly facilitated by the availability of culture liquors rich in dextransucrase. The rapid formation of dextransucrase in high yields has been reported in a preliminary note (Koepsell and Tsuchiya, 1952). The present report deals in greater detail with our observations on factors affecting production of dextransucrase from *Leuconostoc mesenteroides*, strain NRRL B-512.[2] However, culture liquors high in activity have been obtained from a large number of the organisms tested.

The dextran produced by strain NRRL B-512 in the conventional whole culture procedure contains about 95 per cent α-1,6-glucopyranosidic linkage. Although the non-1,6 linkages have been assumed to be of the α-1,4 type, definite proof on this point is lacking (Jeanes and Wilham, 1950). *L. mesenteroides*, strain NRRL B-512, or its substrains, is the organism principally used in investigations of clinical dextran in the United States.

Although the term "dextransucrase" is used in the singular for convenience, the possibility that more than one enzyme may be involved in the synthesis of dextran is recognized.

EXPERIMENTAL METHODS

Materials and cultural methods. Corn steep liquor solids, as well as yeast extract, distillers' solubles, and soy meal preparations, were used to supply nitrogen and the accessory growth substances for the organism. Technical grade sucrose was used in these experiments.

Organisms of the *Leuconostoc* genus are generally considered to be microaerophilic. However, preliminary work showed that shaker cultures consistently gave higher yields than did still cultures. Accordingly, flask cultures were incubated on a reciprocating shaker. All cultures, except where noted, were incubated at 25 C for 24 hours.

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[2] This strain was isolated in 1943 by Dr. R. G. Benedict, Fermentation Division, Northern Regional Research Laboratory.

The rate of inoculation was 1 or 2 per cent. Preliminary tests showed that larger inocula accelerated the rate of fermentation, as measured by pH change in cultures, but decreased the final yield of dextransucrase.

Assay for dextransucrase activity. In the earlier experiments culture liquors which had first been centrifuged were rendered cell-free by passage through Seitz filters. Subsequently, it was found that centrifuged liquors which were substantially cell-free gave results comparable to Seitz-filtered liquors. Since the centrifugation treatment was more convenient, it was used in most of the experiments.

Dextransucrase activity of culture centrifugates was assayed by measuring the amount of fructose liberated, as determined by the copper-reduction method of Somogyi (1945), from sucrose in a given time under ideal reaction conditions.

TABLE 1

Effect of sucrose on production of dextransucrase by Leuconostoc mesenteroides

SUCROSE	DEXTRANSUCRASE
%	*units per ml*
0.5	6
1.0	17
2.0	86
3.0	99
4.0	110
5.0	120

Medium composition: sucrose, variable; corn steep liquor (dry basis), 2.0 per cent; KH_2PO_4, 2.0 per cent; R salts* (by vol), 0.5 per cent; initial pH, 7.2.

* R salts: $MgSO_4 \cdot 7H_2O$, 4 per cent; NaCl, 0.2 per cent; $FeSO_4 \cdot 7H_2O$, 0.2 per cent; $MnSO_4 \cdot H_2O$, 0.2 per cent.

Hehre (1946) had previously shown that increase of reducing sugar in reaction mixtures could be used as a measure of dextran formation and had proposed such a procedure for estimation of the enzyme. The assay reaction mixtures contained sucrose, culture centrifugate (or appropriately diluted sample), and acetate (pH 5.0) buffer, and were held at 30 C for 1 hour. In the reaction mixture, the concentration of the substrate was 10 per cent and that of the buffer 0.05 M. Blank determinations were conducted on both the substrate solution and culture centrifugate and the values used for correction. One dextransucrase unit is defined as that amount of enzyme which converts 1 mg of sucrose to dextran (liberation of 0.52 mg of reducing power, calculated as fructose) in 1 hour under these conditions.

Effect of sucrose concentration on production of dextransucrase. The concentration of sucrose, 10 per cent or higher, used conventionally in the medium in whole culture production of dextran results in a very viscous culture. Obviously, the separation of cells from such a culture would be difficult. Accordingly, the effect of sucrose concentration on the production of dextransucrase was tested. As will be noted from table 1, the higher sucrose levels induced better enzyme

production. However, even at levels of 3, 4, and 5 per cent sucrose, the cultures contained so much dextran as to render difficult the removal of cells. Accordingly, it was concluded that 2 per cent sucrose was the optimum level for production of dextransucrase with this strain of *Leuconostoc*. As has been noted by previous investigators, other sugars such as glucose, fructose, and maltose are incapable of inducing the formation of this adaptive enzyme. Where these sugars were used together with sucrose, the enzyme yield was proportional to the sucrose used.

Effect of nutrients and phosphate buffer on yield of dextransucrase. The concentrations of corn steep liquor solids, yeast extract, distillers' solubles, or soy meal preparations to satisfy the nitrogen and other nutrient requirements of the organism were considerably higher for maximal enzyme formation than for the

TABLE 2

Effect of corn steep liquor and KH_2PO_4 on production of dextransucrase by Leuconostoc mesenteroides

KH_2PO_4	DEXTRANSUCRASE, UNITS PER ML			
	0.5	1.0	2.0	3.5
%	*units per ml*	*units per ml*	*units per ml*	*units per ml*
0.5	6	6	15	
1.0	11	11	30	
2.0	29	37	45	46
3.5			55	55

Medium composition: sucrose, 2.0 per cent; corn steep liquor (dry basis), variable; KH_2PO_4, variable; R salts (by vol), 0.5 per cent; initial pH, 7.2.

conventional whole culture procedure for the formation of dextran. For example, 2 per cent corn steep liquor solids were required in the medium for dextransucrase production, while only 0.25 per cent sufficed for the whole culture procedure. However, the beneficial effect of high levels of these substances was realized only when high amounts of phosphate were present in the medium. This can be seen from the data in table 2 which show the results of a typical experiment in which corn steep liquor solids were used. Essentially similar results were obtained where yeast extract, distillers' solubles, or soy meal preparations were used in place of corn steep liquor.

Formation of dextransucrase. To follow the elaboration of dextransucrase and its accumulation in the menstruum, a number of replicate flask cultures were prepared. Duplicate flask cultures were taken periodically and the contents used for measurement of enzyme, pH, and reducing sugar, presumably fructose. The data are shown in figure 1. Despite the high phosphate buffer concentration, the pH dropped from 7.2 to 5.0 in 17 hours. Sudden and vigorous elaboration of the enzyme began when the pH was 6.9 and continued until the pH was 6.0. The level of fructose, liberated in the conversion of sucrose to dextran, reached a peak and then decreased as the rate of fructose metabolism exceeded the rate of

its formation. The high level of fructose and the sudden elaboration of dextransucrase did not always occur simultaneously as in this experiment. In such other instances the high sugar level occurred before maximal elaboration of the enzyme into the culture liquors. The enzyme activity in these cultures was such that 100 ml assaying 70 units per ml would have been capable of converting 7 g of sucrose to dextran in 1 hour under ideal reaction conditions.

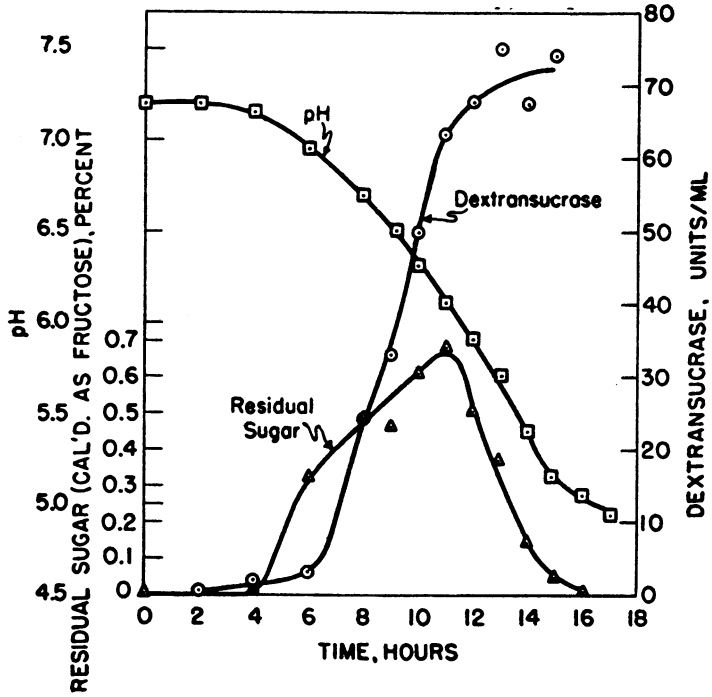

Figure 1. The formation of dextransucrase.

Effect of pH on production of dextransucrase. The effect of high levels of phosphate in the medium on enzyme production suggested a requirement for either phosphate or for a high pH in the culture. To determine which of the two possibilities was operative, fermentations were conducted in low phosphate medium in which the culture pH was controlled. Preliminary experiments were conducted with low phosphate medium in which samples were withdrawn every 10 minutes for pH measurements and alkali was added to the culture. However, the pH dropped so rapidly during the height of metabolic activity that it was impossible to maintain the pH at pre-set levels. Accordingly fermentors were fitted with glass electrodes and alkali reservoirs so that 5 N NaOH could be added continuously to control the pH. Results of a typical experiment in which the pH was controlled at 6.1, 6.3, 6.7, and 7.0, ± 0.1 pH unit, are shown in table 3. As will be noted, dextransucrase elaboration proceeded more rapidly during the early hours in cultures held at the lower pH values. However, the maximum yield

occurred in the culture held at pH 6.7. The decrease in enzymatic activity in all cultures at the end of the fermentation is attributable to the extreme sensitivity of dextransucrase to unfavorable pH conditions. Although enzymatic activity in culture filtrates is retained at pH 5.0 to 5.3 for at least 24 hours at 25 C, about 35 per cent of the activity is lost at pH 6.7 in 1 hour. The data show that maintenance of the pH close to 6.7 is the factor which stimulates high production of the enzyme rather than increased phosphate concentration in the medium.

TABLE 3

Effect of culture pH on production of dextransucrase by Leuconostoc mesenteroides

TIME	pH			
	6.1	6.4	6.7	7.0
	units per ml	*units per ml*	*units per ml*	*units per ml*
2	7	5	4	3
4	26	17	11	9
6	30	44	44	38
8	24	44	54	42
10	18	37	44	35

Medium composition: sucrose, 2.0 per cent; corn steep liquor (dry basis), 2.0 per cent; KH_2PO_4, 0.1 per cent; R salts (by vol), 0.5 per cent; pH, variable.

TABLE 4

Effect of ammonium ion on production of dextransucrase

KH_2PO_4	$(NH_4)_2HPO_4$	KCl	DEXTRANSUCRASE, UNITS PER ML
0.074 M	0	0	44
0.056 M	0.018 M	0.018 M	33
0.038 M	0.038 M	0.038 M	8
0.018 M	0.056 M	0.056 M	1
0	0.074 M	0.074 M	1

Medium composition: sucrose, 2.0 per cent; yeast extract, 1.0 per cent; KH_2PO_4, variable; $(NH_4)_2HPO_4$, variable; KCl, variable; initial pH, 7.6.

Either NaOH or KOH may be used to control the pH of cultures containing corn steep liquor. However, the ammonium ion exerts a detrimental effect on the organism. The results of an experiment demonstrating the adverse effect of ammonium ion on dextransucrase formation are shown in table 4. This may be the same effect which was noted also by Whiteside-Carlson and Rosano (1951). Accordingly, ammonium hydroxide should not be used to control the pH.

Effect of temperature on production of dextransucrase. As has been noted, the enzyme is rapidly inactivated at pH 6.7 even at 25 C. Higher temperature accentuates markedly the deleterious effect of pH on the enzyme. Thus, in an experiment where the sucrose, corn steep liquor cultures were maintained at pH 6.7 with alkali, but the incubation temperatures controlled at 20, 23, 26, and 29

C, the maximum unitages attained were 47, 51, 43, and 6.0 units per ml at 12, 9, 7, and 5 hours, respectively. Even if dextransucrase had been formed at 29 C, the destruction of enzyme would explain the low yield.

SUMMARY

Certain cultural factors affecting dextransucrase production by *Leuconostoc mesenteroides*, strain NRRL B-512, have been studied. The sucrose concentration in the medium should be lowered to a point such that viscosity of the culture is sufficiently low to permit the separation of bacterial cells. With *L. mesenteroides*, strain NRRL B-512, the optimal sugar level is 2 per cent. The sources of nitrogen and other nutrients are required in concentrations higher than those used in the whole culture production of dextran. The optimal pH for production of dextransucrase by *L. mesenteroides*, strain NRRL B-512, was 6.7. The pH of cultures could be controlled by the use of phosphate buffer or by continuous addition of alkali. The ease with which culture filtrates, highly potent in dextransucrase content, can be produced will facilitate fundamental studies of the polymerization process responsible for the formation of dextran.

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