DEXTRAN-DEGRADING ENZYMES FROM MOLDS[1]

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Enzymes capable of degrading dextran, the polymer of glucose produced from sucrose by the action of *Leuconostoc mesenteroides* and related organisms, are potentially valuable for fundamental research as well as for practical application. Although the α-1,6-glucopyranosidic linkage is present to the extent of 95 per cent or more in some easily obtainable dextrans, the only other known occurrence of this linkage is the 5 to 10 per cent found in starch, glycogen, and similar polysaccharides.

Thus, as has been pointed out previously by Jeanes *et al.* (1948), dextran provides a unique source of simpler carbohydrates containing the α-1,6-glucosidic linkage obtainable through enzymic degradation. Enzymes produced by organisms grown on dextran and capable of cleaving the 1,6 linkage may be useful in studies concerning starch structure. And lastly, as has been indicated already by Ingelman (1948) and by Whiteside-Carlson and Carlson (1952), other types of dextranases offer a means for degrading dextran to molecular size range suitable for use as synthetic blood volume expanders.

Although in this paper the term "dextranase" is used in the singular, we recognize the possibility of there being more than one enzyme that degrades dextran. Furthermore, a microorganism may produce more than one type of dextran-depolymerizing enzyme.

There are reported here our observations on the occurrence, production, and activity of certain mold enzymes that degrade dextran from *Leuconostoc mesenteroides*, strain NRRL B-512, to di- and higher oligosaccharides. This dextran is a linear type which contains approximately 95 per cent α-1,6-glucosidic linkages (Jeanes and Wilham, 1950).

In our search for dextran-hydrolyzing enzymes, begun in 1945, we have screened approximately 200 strains of bacteria, yeasts, and molds for their ability to produce extracellular dextranase. Like van Tieghem (1878), Colin and Belval (1940), and others, we were unable to find organisms producing large amounts of the enzyme among bacteria and yeasts. Ingelman (1948) has reported a low order of dextranase activity from *Cellvibrio fulva*. Recently, Hehre and Sery (1952) have isolated dextran-splitting anaerobic bacteria from the human intestine. Independently of Hultin and Nordström (1948, 1949), we found that certain strains of Penicillia produced extracellular dextranase, but in small amounts. However, when we tested the species of Penicillia used by Hultin and

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Nordström, we found high extracellular dextranase activity in contrast to their report of intracellular enzyme only.

EXPERIMENTAL MATERIALS AND METHODS

Materials and cultural procedure. Dextrans from *Leuconostoc mesenteroides*, strains NRRL B-512[2] and B-523, were prepared as previously described (Jeanes *et al.*, 1948), and dextran from strain NRRL B-742 was prepared similarly. The dextran used in cultures for the production of our dextranase preparations was derived from strain NRRL B-512. Unless otherwise stated, dextran from this organism was used in all assays and activity studies reported here.

The molds were usually grown in 100 ml of culture in 1 liter Erlenmeyer flasks at 30 C on a reciprocating shaker. The media used for the propagation of the molds and the production of the enzyme contained dextran and corn steep liquor solids. The latter was selected as the source of nitrogen and the necessary growth factors since preliminary experiments showed it to be equal to yeast extract and superior to malt extract and liver extract as a supplement for the production of the enzyme. Although preliminary work indicated that the enzymic activity in culture filtrates increased up to the ninth day, substantially all of the enzyme had been elaborated by the fifth. Therefore, an incubation period of 5 days was used throughout this investigation.

Assay procedure. After incubation, the mycelium was removed by either centrifugation or filtration and the culture liquors assayed. To determine dextranase activity, 5 ml of culture filtrates (or appropriately diluted samples) were adjusted to about pH 5.1 and added to 10 ml of an acetate buffered (pH 5.1) dextran solution. In the reaction mixtures the concentrations of dextran and buffer were 1.67 per cent and 0.1 M, respectively. The reaction mixtures were held at 40 C for 2 hours, at which time enzymic action was stopped by acidification with sulfuric acid. The reaction mixture was made 0.4 N with respect to the acid. Subsequently, it was adjusted to the phenolphthalein end point for the sugar determination. Reducing power was measured by the method of Somogyi (1945). Since paper chromatograms, as well as isolation of the products (Jeanes, *et al.*, 1952), showed that the disaccharide, isomaltose, was the principal reducing sugar formed, the activities were calculated in terms of mg equivalents of this sugar produced. One dextranase unit is defined as that amount of enzyme which produces 1 mg equivalent of isomaltose monohydrate in 1 hour at 40 C. Although total conversion of the dextran in the assay solution would have produced 278 mg of isomaltose monohydrate, the extent of reaction was controlled so that no more than 75 mg of this sugar were produced. Thus, the enzyme-substrate ratio was maintained in the region where it has been found that a linear relation existed between enzyme unitage and isomaltose produced.

RESULTS AND DISCUSSION

Survey of organisms. As previously noted, dextranase activities in the culture filtrate of certain Penicillia which we had tested were of low order. When Nord-

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

ström and Hultin (1948) reported the occurrence of intracellular dextranase in other species of Penicillia and Verticillia, we examined a strain of each of the *Penicillium* species from which they had obtained their intracellular enzyme, together with strains of *Aspergillus*, *Verticillium*, *Cephalosporium*, and *Spicaria* which were in the process of being screened. Results indicated that strains of *Penicillium lilacinum*, *P. funiculosum*, and *Spicaria* produced considerable amounts of extracellular dextranase. The survey medium contained 2 per cent dextran and 3 per cent corn steep liquor solids and had initial pH of 6.0. Therefore, the testing for extracellular dextranase was extended to 41 strains of Penicillia and *Spicaria*. Twenty strains of *P. lilacinum*, *P. funiculosum*, *P. verruculosum*, and *Spicaria violacea* produced from 7 to 310 units per ml of extracellular dextranase under our experimental conditions.

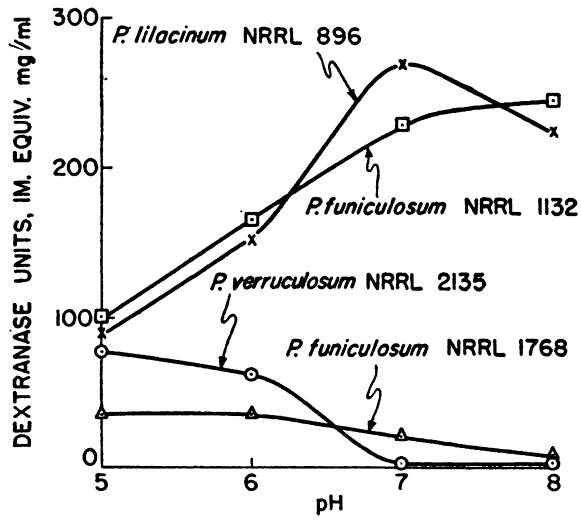

Figure 1. Effect of initial pH of culture on dextranase production.

Cultural factors affecting dextranase production. The organisms selected for further studies were *P. lilacinum*, strain NRRL 896, *P. verruculosum*, strain NRRL 2135, and *P. funiculosum*, strains NRRL 1132 and NRRL 1768. The effect of initial pH of culture on production of the enzyme was investigated. The composition of the medium was the same as that used in the survey. As shown in figure 1, enzyme yields increased with two of the organisms as the initial pH was raised. With the other two, the reverse effect was found.

The effect of varying the concentrations of corn steep liquor and dextran is shown in figure 2. The initial pH of the cultures was set at the optimum found for each organism. With *P. lilacinum*, strain NRRL 896, *P. verruculosum*, strain NRRL 2135, and *P. funiculosum*, strain NRRL 1132, 2 per cent dextran and 3 per cent corn steep liquor solids were the optimal nutrient levels. With *P. funiculosum*, strain NRRL 1768, 1 per cent dextran and 1 per cent corn steep liquor were optimal.

Optimal pH for dextranase activity and stability. The optimal pH for enzymic

activity of culture filtrates produced in the most suitable medium for each organism was in the range of 4.0 to 5.5. Enzymic activities in the pH range of 3.1 to 5.4 were measured in 0.2 M acetate, and in the pH range of 4.8 to 6.7 in citrate buffer. The activities in the citrate buffer were slightly lower than in the acetate buffer at the same pH values, possibly because some ion essential for dextranase activity may have been sequestered in complex form.

Stability of the dextranase activity in culture filtrates was measured at 30 C, the incubation temperature, and 40 C. Aliquots of culture filtrates were placed

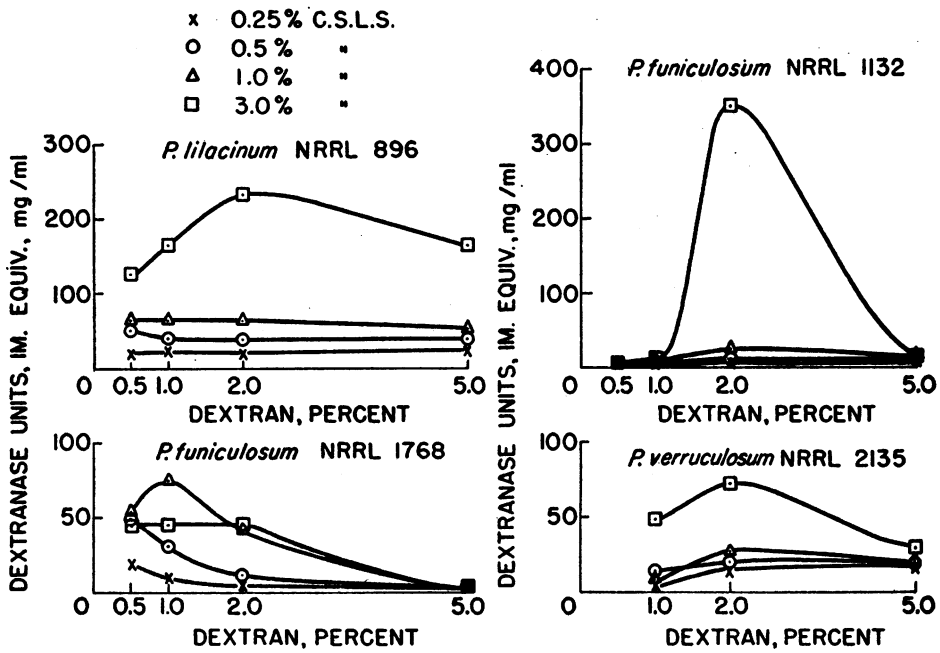

Figure 2. Effect of media composition on dextranase production.

in buffer solutions and held at the desired temperature for 18 hours. These crude enzyme preparations were stable over a wide range of pH from 4.3 to 7.9 at 30 C. Although purified enzyme preparations will doubtless show a narrower pH range for stability, these results suggest that the cultures need not be controlled as closely around pH 4 as recommended by Hultin and Nordström (1949). Even at 40 C, the crude enzymes were reasonably stable over a pH range of 4.1 to 7.25 for 18 hours.

Enzymatic activity on various substrates. When a culture filtrate of *P. funiculosum*, strain NRRL 1768, grown on medium containing dextran from L. *mesenteroides*, strain NRRL B-512, was allowed to act on dextran, not only from strain NRRL B-512 but also on dextrans from strains NRRL B-523 and NRRL B-742, differences in rates of polysaccharide degradation were found. The results are shown in figure 3. As might be anticipated, this dextranase from *P.*

funiculosum, strain NRRL 1768, readily degraded the "homologous"[3] dextran from strain NRRL B-512. Much more surprising was the observation that this dextranase produced on dextran from strain NRRL B-512 also depolymerized rapidly the "heterologous" dextran of strain NRRL B-523. These two dextrans differ markedly in their chemical structure. The water-soluble dextran of strain NRRL B-512 contains 95 per cent α-1,6-linked anhydroglucose units (Jeanes and Wilham, 1950), whereas the dextran of strain NRRL B-523 contains only 75 per cent 1,6-linked units (Jeanes and Wilham, 1950) and is water insoluble. Despite the

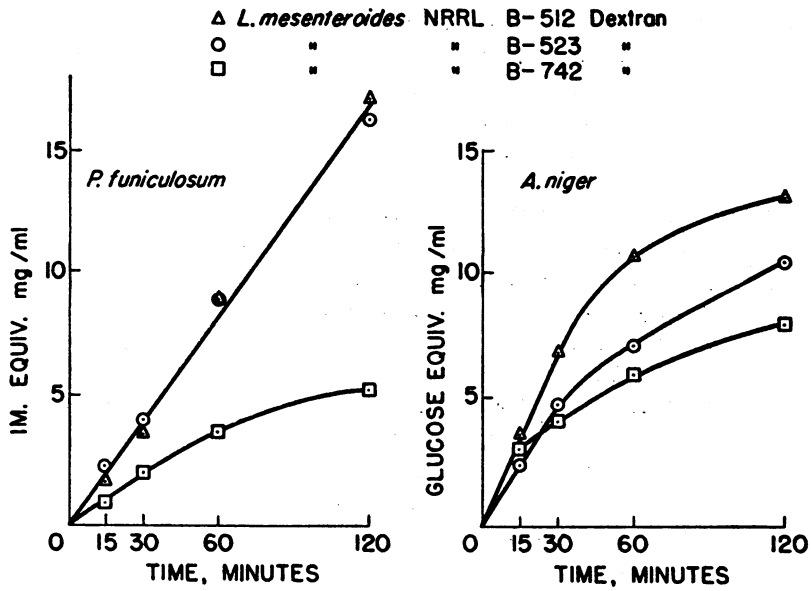

Figure 3. Degradation of dextrans by *Penicillium funiculosum*, strain NRRL 1768, and *Aspergillus niger*, strain NRRL 330.

marked difference in dextrans, the curves for the degradation of these two glucose polymers were superimposable. On the other hand, the heterologous dextran of *L. mesenteroides*, strain NRRL B-742, which also contains only 75 per cent α-1,6-linked anhydroglucose units (Jeanes and Wilham, 1950) and is water soluble, was considerably more refractory to the action of the dextranase produced by growing *P. funiculosum* on dextran from strain NRRL B-512. Martin (1945), who was interested in the utilization of dextran by various organisms as measured by their growth response, found differences in the availability of 3 dextran preparations for the organisms. Our observations on the susceptibility of dextrans to dextranases are in accordance with his findings.

Similar results were also obtained by the action of an amylase preparation of

[3] For the sake of brevity, the dextran used as substrate for enzymic action is termed "homologous" when it is the same as that on which the mold was grown to develop dextranase. The dextran used as substrate for enzymic action is termed "heterologous" when it is different from that on which dextranase was formed.

Aspergillus niger, strain NRRL 330, (Tsuchiya *et al.*, 1949) on these three dextran preparations. The activities were measured under standard assay conditions except that reaction mixtures contained 5 ml of a 1.5 per cent solution of a concentrate of *A. niger*, strain NRRL 330. The pH was adjusted to 4.4 since preliminary work showed this pH to be optimal for the dextranase activity of this enzyme concentrate. For estimating the action of this amylase preparation on dextran, the reducing sugar was calculated as glucose equivalents. Unlike the dextranase of *P. funiculosum*, strain NRRL 1768, this amylase preparation degraded the dextran of strain NRRL B-512 more readily than the dextran of strain NRRL B-523. Like the dextranase of strain NRRL 1768, this enzyme preparation was least able to degrade the dextran of strain NRRL B-742. These results suggest that dextranase preparations may prove useful supplementary tools in the differentiation and characterization of dextrans.

Since the action of culture filtrates from strain NRRL 1768 results primarily in the production of isomaltose from dextran, it was not surprising that these preparations demonstrated very limited activity toward this disaccharide. This is in contrast to the activity of the amylase preparation of *A. niger*, strain NRRL 330, which hydrolyzed isomaltose to glucose readily (Tsuchiya *et al.*, 1949) but attacked dextran very slowly. Thus the two enzyme preparations, both capable of cleaving the α-1,6-glucosidic linkage, demonstrated different activities toward substrates of different molecular size.

The culture filtrates from strain NRRL 1768 demonstrated very little, if any, activity on soluble starch. The preliminary tests made on the increase in reducing power of reaction mixtures containing starch were essentially negative. However, further tests are required since the methods used might not have been sensitive enough to detect the changes that occurred.

Our studies on the occurrence and production of dextranases, and on their action on dextrans and amylaceous polysaccharides, are being continued.

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SUMMARY

Twenty strains of *Penicillium lilacinum*, *P. funiculosum*, *P. verruculosum*, and *Spicaria violacea* were found to produce potent extracellular dextran-degrading enzymes in submerged culture. An amylase concentrate of *Aspergillus niger*, strain NRRL 330, also was found capable of degrading dextrans. Each organism displayed its own cultural requirements. The effect of pH on activity and stability

of crude enzyme preparations derived from certain strains of Penicillia was studied. The dextranase produced by *P. funiculosum*, strain NRRL 1768, when cultivated on one type of dextran, displayed varying degrees of activity toward dextrans of different microbial origin. The same was true of the dextran-degrading ability of an amylase preparation of *A. niger*, strain NRRL 330.

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