

Production of β -D-xylopyranosidases by fungi¹

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Two hundred and fifty microorganisms were tested for their ability to produce β -D-xylopyranosidase. Two of the best, *Aspergillus niger* and *Penicillium wortmanni*, were studied in greater detail for the effects of inducing compounds and of surfactants on yields. Under optimal conditions, culture filtrates were obtained which had specific activity values 10 to 60 times those of sources (sea snail, *Bacillus*, *Chaetomium*, hemicellulase) used by other investigators. In most instances, our unpurified filtrates had higher activities than did the extensively purified preparations from these other sources. The fungal β -D-xylopyranosidases hydrolyze aryl and alkyl β -D-xylopyranosides, oligomers of D-xylose, and L-serine β -D-xyloside. They do not hydrolyze β -D-xylosyl dextran, tomatin, or α -D-xylosides.

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Deux cent cinquante microorganismes ont été étudiés pour leur habilité à produire la β -D-xylopyranosidase. Deux des meilleurs organismes, *Aspergillus niger* et *Penicillium wortmanni*, furent étudiés en détails en regard de leur rendement affecté par des composés inductibles et des surfactants. Sous des conditions optimales avec des valeurs d'activités spécifiques de 10 à 60 fois plus fortes que les sources (escargot de mer, *Bacillus*, *Chaetomium*, hemicellulase) utilisées par d'autres chercheurs. Dans la plupart des cas, nos filtrats non purifiés ont des activités plus grandes que les préparations hautement purifiées de ces autres sources. La β -D-xylopyranosidase fongique hydrolyse l'aryl et l'alkyl β -D-xylopyranoside, les oligomères du D-xylose et le L-sérine β -D-xyloside. Ils n'hydrolysent pas le β -D-xylosyl dextran, la tomatine, ou les α -D-xylosides.

[Traduit par le journal]

This report is a continuation of our studies of the factors responsible for increasing the yields of various enzymes (14, 17, 18). The need for a β -D-xylosidase (EC 3.2.1.37) for investigating the carbohydrate to protein linkage in glycoproteins prompted our selection of this enzyme. Recently β -D-xylosidase has been isolated from the liver of *Charonia lampas* (5, 6) and from cells of *Bacillus pumilus* (1, 10). The former requires homogenization and the latter, disintegration of cells, processes which incorporate a great deal of foreign materials which must subsequently be removed during the purification process. From fungi, one obtains extracellular enzymes of much higher initial specific activity, thus simplifying subsequent purification. As far as the authors are aware, no one has investigated the factors favoring β -D-xylosidase production in fungi. Most investigators have either begun with crude commercial preparations containing xylosidase (2, 15), or have isolated the xylosidase which accompanies xylanase (8). This paper reports the production of β -D-xylosidase by fungi and the conditions favoring high enzyme yields. These sources are greatly superior to those previously reported.

Methods

Screening of Organisms for β -D-xylopyranosidases

Fungi and bacteria were grown in the *Trichoderma viride* (Tv) salts medium (13) containing a carbon source (0.5%) on a reciprocal shaker at 29°. The volume of medium was 50 ml/250-ml Erlenmeyer flask. The surfactant Tween 80 (0.1-0.2%) was added to all cultures. In part of the work, washed mycelium of 3-day cultures grown on starch or xylan (0.5%) was resuspended in Tv salts medium at pH 4.5. Methyl β -D-xylopyranoside (1 mg/ml of culture) was added, and incubation continued for 3 days at which time the enzyme content was determined. In most of the screening tests, however, the inducer was added directly to growing cultures and the enzyme assayed on the 7th and 11th days. The organisms used were obtained from the Culture Collection (QM) maintained at the Natick Laboratories.

Assay of Enzyme

The enzyme solution (0.5 ml) was added to the substrate solution (0.5 ml) and incubated at 50°. The substrate solution consisted of methyl- β -D-xylopyranoside (10 mg/ml) in M/20 citrate buffer pH 3.0. The incubation time was 4 h during the initial screening experiments, and reduced to 1 h for later work. Xylose was determined by the dinitrosalicylic acid method (18). The units (U) reported here are equivalent to μ mol xylose/min under the test conditions.

Protein was determined by the Lowry method (12).

Preparation of Inoculum

Several of the fungi (especially penicillia) which produce β -D-xylopyranosidase sporulate poorly on the usual agar media. Good sporulation resulted from the use of

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TABLE 1
 β -D-Xylopyranosidase-producing fungi

Organism		Methyl β -D-xylopyranosidase, $\times 10^{-1}$ U/ml
<i>Aspergillus miyakoensis</i>	QM3309	0.7
<i>A. niger</i>	QM877	0.8
<i>A. stromatoides</i>	QM8959	0.7
<i>Botryodiplodia</i> sp.	QM7092	0.8
<i>Penicillium chermisinum</i>	QM5120	0.9
<i>P. islandicum</i>	QM7571	0.6
<i>P. pusillum</i>	QM137g	0.8
<i>P. roseopurpureum</i>	QM3384A	0.8
<i>P. wortmanni</i>	QM7322	1.5
<i>Pestalotia virgatula</i>	QM479	0.2
<i>Trichoderma viride</i>	QM6a	0.4

formula M40Y (malt extract 2%, sucrose 40%, yeast extract 0.5%, agar 2%), as recommended by Miss B. Wiley.

Results

Screening

Over 250 organisms representing a wide variety of genera (Bacteria, Phycomycetes, Ascomycetes, Basidiomycetes, Fungi Imperfecti) were tested for extracellular β -D-xylopyranosidase production. None of the bacteria (21 strains of 13 genera) secreted β -D-xylopyranosidase, when grown on xylan plus methyl β -D-xylopyranoside. Other enzymes were secreted by some of these bacteria (β -D-xylanase; β -1,3-glucanase; amylase). Most fungi, similarly, produced no xylosidase detectable by the assay procedure. The most active enzyme producers are listed in Table 1. The black aspergilli produce appreciable amounts of enzyme, much of which, however, remain intracellular. Additional isolates (10-20) of the more active species were tested and also found to be active. Further investigation was limited largely to *A. niger* QM877 and *Penicillium wortmanni* QM7322.

Thirty-one commercial enzymes (mostly carbohydrases) were tested for their ability to hydrolyze methyl β -D-xylopyranoside. Four preparations (at the 1% w/v enzyme level) show activity: cellulase 20 000 and cellulase 9X (Miles Lab., Elkhart, Indiana); cellulase, Onozuka (All Japan Bioch. Co., Nishinomiya, Japan); and hemicellulase, NBC (Nutritional Bioch. Co., Cleveland, Ohio). They are much more active on *p*-nitrophenyl β -D-xylopyranoside than on methyl β -D-xylopyranoside. Takadiastase and almond emulsin have been reported (14) to have activity on aryl β -D-xylopyranosides. Our prepar-

ations of these have $\frac{1}{10}$ to $\frac{1}{50}$ as much aryl β -D-xylopyranosidase activity as have the four preparations noted above; and no detectable activity on methyl β -D-xylopyranoside.

The best commercial samples, cellulase 9X (from *A. niger*), and cellulase, Onozuka P500, have about 0.02 methyl β -D-xylopyranosidase U/mg. The only enzyme we have prepared exclusively by growth on solid bran is the nucleosidase of another black *Aspergillus* (*A. foetidus*), and, interestingly enough, the crude preparation of this also contains β -D-xylopyranosidase at about the same level as found in the commercial samples. The Flemish workers (2, 9, 13) used preparations of this type as the source of their fungal β -D-xylopyranosidase. "Hydrolasengemisch," almond emulsin, and *Helix* enzyme have extremely low levels of activity.

Location of Enzyme

The β -D-xylopyranosidase is located in the mycelium during the early stages of growth, and is secreted into the medium on further incubation. Little enzyme remains bound after 6 days. The *Botryodiplodia* results (Fig. 1) are typical of most of the fungi which produce β -xylosidase. In the black aspergilli, however, much more of the enzyme remains associated with the mycelium (Fig. 1). Since the assay procedure for bound enzyme involves incubating mycelium with substrate at 50°, it was possible that the mycelium was being killed, and enzyme liberated, i.e. that the observed activity might be of enzyme set free during the assay period. To test this, washed mycelium was incubated with buffer (pH 4.5 at 50° for 5 h), and the liberated, cell-free enzyme subsequently measured. Nearly all

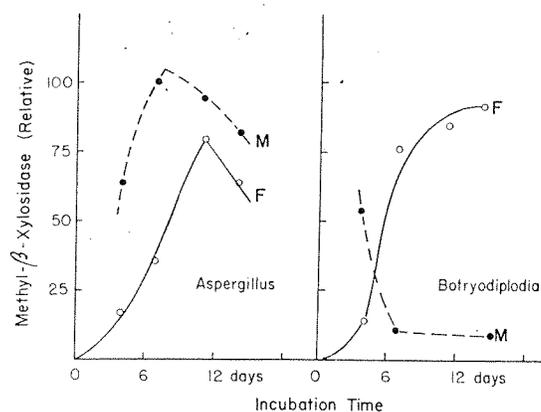


FIG. 1. Location of β -D-xylopyranosidase during growth of *Aspergillus niger* QM877; *Botryodiplodia* sp. QM7092. β -D-Xylopyranosidase activity (vs. methyl β -D-xylopyranoside) of mycelium (M, ●) and of extracellular culture fluid (F, ○).

of the mycelial "bound" enzyme (*A. niger*, *P. wortmanni*) was liberated under these assay conditions.

Induction, General

The β -D-xylopyranosidase is an inducible enzyme in the fungi tested. When the organisms were grown on starch, no β -D-xylopyranosidase was produced (Table 2). Addition of methyl β -D-xylopyranoside (0.1%) to the starch culture led to enzyme formation and secretion. Some xylanase was produced simultaneously. Xylan induces (not shown) probably by the oligosaccharides resulting from its hydrolysis. Xylose also induces in some organisms. Both are less effective than methyl β -D-xylopyranoside. Methyl α -D-xylopyranoside was a good inducer for some

fungi, and without effect on others. Methyl β -D-glucopyranoside did not induce. Black aspergilli and *Botryodiplodia* sp. react to these compounds in a similar manner to the organisms shown in Table 2. In induction experiments using washed mycelium, the optimal concentration of methyl β -D-xylopyranoside was 2 mg/ml.

Surfactant Effects, General

The effect of surfactants was tested in experiments with washed mycelium. Tween 80 was found to be most consistent in stimulating enzyme production in all organisms. Yields of enzyme increased as the Tween 80 concentration of the medium increased over the range used (Fig. 2). Digitonin, sodium oleate, and Tergitol NPX were effective in some cultures, but not in others. It is interesting that little or no enzyme was detectable in the absence of Tween 80 either within the mycelium (not shown) or in the extracellular fluid of the four organisms tested. However, in growth experiments, enzyme was produced in the absence of surfactants (Fig. 4), and the increases in yield obtained in its presence were less spectacular.

Conditions for Maximal Enzyme Production

Penicillium wortmanni QM7322 (best yield 0.55 U/ml)

Penicillium wortmanni has consistently given the highest yields of β -D-xylopyranosidase. To attain these values xylan was used as the major carbon source, Tween 80 was added, and a mixture of methyl β -D-xylopyranoside and methyl α -D-xylopyranoside was included (Table 3). The

TABLE 2
Induction of methyl β -D-xylopyranosidase

Fungi	QM	β -D-Xylopyranosidase (50° 3 h) as mg/ml xylose				
		No addition*	+ methyl β -D-xylopyranoside	+ methyl α -D-xylopyranoside	+ xylose	+ methyl β -D-glucopyranoside
<i>Aspergillus amstelodami</i>	8405	0	0.40	0.06	0.23	0
<i>Penicillium chermisinum</i>	5120	0	0.51	0.47	0.11	0
<i>Penicillium wortmanni</i>	7323	0	0.62	0.89	0.04	0
<i>Penicillium islandicum</i>	7571	0	0.44	0.20	0.11	0
<i>Trichoderma viride</i>	6a	0	0.31	0	0	0

*All cultures grown on soluble starch (0.5%) with additions of inducing compounds (0.1%) made at 48 h. Tween 80 (0.1%) present. Samples were assayed at 7, 11, and 14 days.

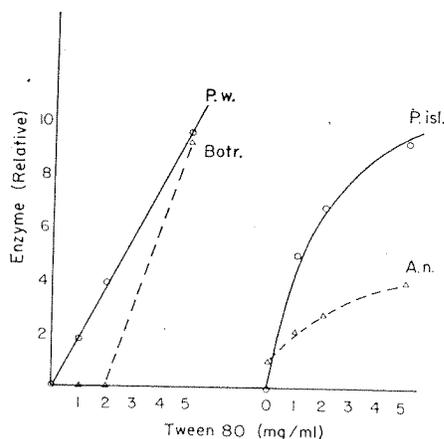


FIG. 2. Surfactant effect on enzyme production (methyl β -D-xylopyranosidase). Mycelium grown on starch in shake flasks for 3 days was washed and re-suspended in M/40 KH_2PO_4 (pH 4.5) containing methyl β -D-xylopyranoside (1 mg/ml). The cultures were incubated at 29° in shake flasks for 3 days and enzyme activity determined on the extracellular solution. Organisms: P.w. = *Penicillium wortmanni* (QM7323); Botr. = *Botryodiplodia* sp. (QM7092); P. isl. = *P. islandicum* (QM7571); A.n. = *Aspergillus niger* (QM9270).

incorporation of methyl α -D-xylopyranoside is an important factor. When used as the *only* C-source, however, methyl α -D-xylopyranoside did not induce.

Modified inducers have been prepared and tested as sole C-source (0.5%). On the monopalmitate of methyl β -D-xylopyranoside, this fungus gave about the same enzyme yield (0.16 U/ml) as did methyl β -D-xylopyranoside itself (0.18 U/ml). β -D-Xylopyranose tetraacetate was appreciably better (0.30 U/ml), and it was the best of all compounds when used as the only carbon source. D-Xylose and L-xylose were poor inducers (<0.02 U/ml). Tomatin, a com-

plex glycoside containing a terminal β -D-xylopyranose unit, was inhibitory to growth of the fungus.

Modified inducers were tested in washed mycelium experiments, with results similar to those of the growth tests (above). Again methyl α -D-xylopyranoside had no inducing effect when used alone. The palmitate of methyl β -D-xylopyranoside was also ineffective under conditions where the yields on methyl β -D-xylopyranoside were 0.20 U/ml. β -D-Xylopyranose tetraacetate, and the triacetate of methyl β -D-xylopyranoside, yielded 0.10 U of enzyme per milliliter. Similar data were obtained using *P. islandicum* as the test organism.

Penicillium wortmanni was also grown on bran by the koji method for 3 weeks, and the koji extracted with water. The aqueous extracts (about equivalent in volume to the bran culture) contained 0.34 U of enzyme/ml, yields that compare favorably with those obtained under shake-flask conditions. Bran suspended in culture medium in shake flasks yielded only 0.02 U/ml.

Aspergillus niger QM877 (best yield of extracellular enzyme 0.30 U/ml)

A comparison has been made of several of the black aspergilli, and QM877 was selected for more extensive tests. With this organism, xylan offers no advantage over starch as the major carbon source. It further differs from *P. wortmanni* in that the enzyme is retained in the mycelium for a longer time, and as a result yields in the extracellular solutions are somewhat lower. Incorporation of sodium chloride (1%), sucrose monopalmitate, and Tween 80 in the medium favored enzyme production (Fig. 3).

Yields on a medium containing starch (0.5%), methyl β -D-xylopyranoside (0.2%), and sucrose

TABLE 3
Production of methyl β -D-xylopyranosidase of *P. wortmanni* 7322

Supplement at 24 h	mg/ml	Methyl β -D-xylopyranoside μ /ml (max.) grown on:	
		starch 0.5%, $\times 10^{-1}$	xylan 0.5%*, $\times 10^{-1}$
Methyl β -D-xylopyranoside	1	2.3	2.0
	3	2.5	2.9
Methyl α -D-xylopyranoside	1	3.7	4.1
	3	4.1	4.1
Methyl β -D-xylopyranoside +	1		
Methyl α -D-xylopyranoside	1	3.5	5.5

*All cultures contain methyl β -D-xylopyranoside (1 mg/ml) and Tween 80 (1 mg/ml) in addition to the components shown above.

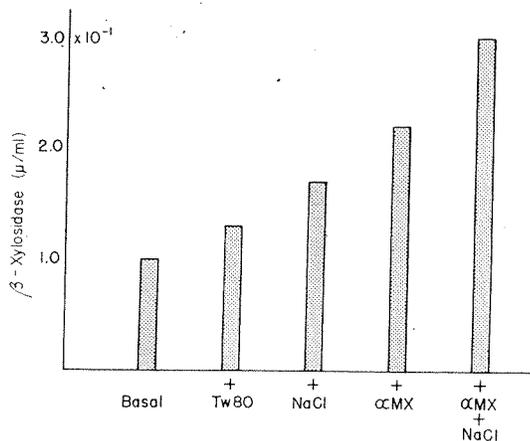


FIG. 3. Factors affecting yields of β -D-xylopyranosidase in *A. niger*. Basal medium contains starch (0.5%) + methyl β -D-xylopyranoside (0.2%) + sucrose monopalmitate (0.1%). Additives: Tween 80 (0.2%); NaCl (1%); methyl α -D-xylopyranoside (0.2%).

monopalmitate (0.1%) were about 0.10 U/ml. Further addition of Tween 80 (0.2%) gave 0.13 U/ml; of NaCl (1%) 0.17 U/ml; of methyl α -D-xylopyranoside (0.2%) 0.22 U/ml. Addition of both NaCl and methyl α -D-xylopyranoside gave 0.30 U/ml, the highest values obtained in shaken culture (Fig. 3).

Grown on wheat bran (100 g + 200 ml Tv salts solution) for 6 days, *A. niger* produced more β -D-xylopyranosidase than did *P. wortmanni*. Equivolume aqueous extracts contained 0.57 U/ml, nearly twice the concentrations reached in shake flasks.

Properties of the β -D-Xylopyranosidases

While it is the major purpose of this paper to report on the production of enzymes, a few studies have been made on the properties of β -D-xylopyranosidase, particularly with respect to factors affecting the measurement of activity. The optimum pH (Fig. 5) is rather low for glycosidases, about pH 3.0, and there is very little activity at or above pH 6.0. The rate of hydrolysis at 50° is 2.3 times that at 35° at pH 3.0; and 2.8 times that at 35° at pH 4.5 (*A. niger*; *Botryodiplodia* sp.). No detectable inactivation occurs during the incubation period used in the assay (1 h; 50°, pH 3.0–5.0). Some inactivation takes place at pH 6.2, and it is rapid at pH 8.7. The pH activity curves reflect this rapid inactivation at the higher pH values. At 70° both enzymes are more stable at pH 4.5 than at pH 3.0.

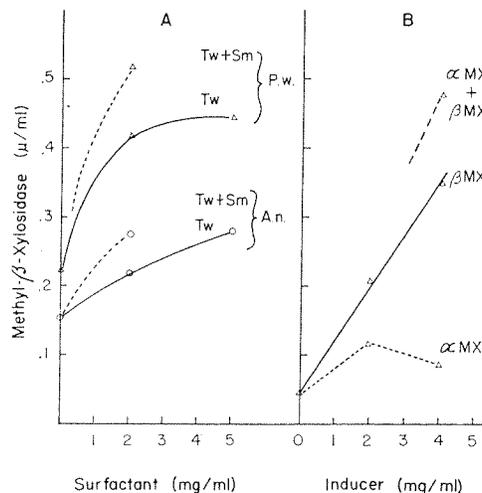


FIG. 4. Effect of surfactant and of inducer concentrations on yield of β -D-xylopyranosidase. A. Effect of surfactant. Cultures grown on C-source plus inducers methyl α -D-xylopyranoside (2 mg/ml) + methyl β -D-xylopyranoside (2 mg/ml). TW = Tween 80, Sm = sucrose monopalmitate. Pw = *P. wortmanni* QM7322, An = *A. niger* QM877. B. Effect of inducer. Cultures of *P. wortmanni* grown on C-source plus surfactant; Tween 80 0.2%. α MX = methyl α -D-xylopyranoside, β MX = methyl β -D-xylopyranoside.

Thus, at pH 3.0 *A. niger* enzyme was 68% inactivated in 5 min; while at pH 4.5 there was only 10% inactivation in 30 min. *Botryodiplodia* enzymes behaved in a similar way. Obviously the pH optima for stability and for activity are quite different (8).

Tests on inhibition of the activity of β -D-xylopyranosidase on methyl β -D-xylopyranoside (10 mM) indicate that xylonolactone is the best of the inhibitors tested. The concentration of xylonolactone to give 50% inhibition was found to be 4.8 mM (for *P. wortmanni* enzyme) and 5.9 mM (for *A. niger* enzyme). Δ -Gluconolactone at these concentrations had no effect.

Other inhibition experiments were carried out using *p*-nitrophenyl β -D-xylopyranoside (2 mM) as substrate. Xylonolactone was again the best inhibitor (1.5 mM). Measurable inhibition was observed with two other substances, but the concentrations of these required for 50% inhibition were much higher: methyl β -D-xylopyranoside 46 mM; D-xylose 33 mM (vs. *P. wortmanni* enzyme). The following compounds showed no inhibitory activity at the highest concentration tested: L-xylose (10 mM), D-arabinose (10 mM); L-arabinose (10 mM); D-glucose

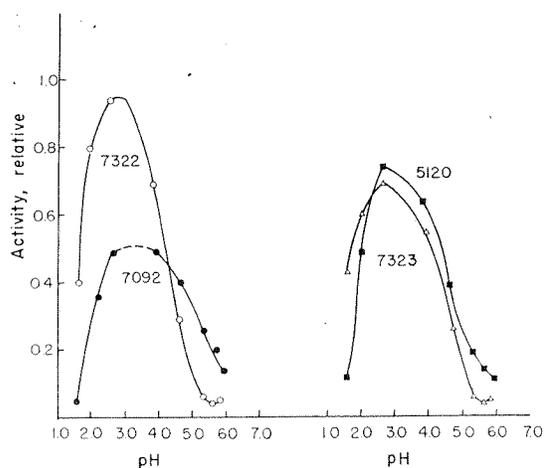


FIG. 5. Effect of pH on β -D-xylopyranosidase activity (vs. methyl β -D-xylopyranoside, 5 mg/ml). Activity in M/30 citrate buffer; 50°; 60 min. 7322 = *P. wortmanni* QM7322; 7092 = *Botryodiplodia* sp.; 7323 = *P. wortmanni* QM7323; and 5120 = *P. chermisinum*.

(10 mM); levoglucosan (10 mM); methyl 6-deoxy β -D-glucopyranoside (10 mM); serine β -D-xylopyranoside (14 mM); nojirimycin (2 mM), and 5-acetamido-5-deoxy-D-xylopiperidino-2,7 (2.7 mM, gift from Dr. H. Paulsen, Hamburg).

As salts have a stimulatory effect on the activity of the β -D-xylopyranosidases of *Charonia* (5), and of *B. pumilus* (10), the fungal enzymes were subjected to similar conditions. Neither NaCl (0.5 M) nor Na₂SO₄ (0.5 M) had any effect (see also ref. 2).

Specificity of β -D-Xylopyranosidases

To determine whether more than one xylosidase is produced by an organism a variety of filtrates (representing different conditions of growth) was tested for activity against methyl β -D-xylopyranoside and *p*-nitrophenyl β -D-xylopyranoside. For *A. niger*, a straight line runs through all points (Fig. 6), and presumably one enzyme is involved in hydrolysis of both substrates. For *Botryodiplodia* sp., however, two points are markedly out of line (growth in presence of digitonin, and of Tergitol NPX), and two enzymes are probably implicated. A sonicate of *Botryodiplodia* cells also had a high *R* value.

$R =$

$$\frac{\text{activity on } p\text{-nitrophenyl } \beta\text{-D-xylopyranoside}}{\text{activity on methyl } \beta\text{-D-xylopyranoside}}$$

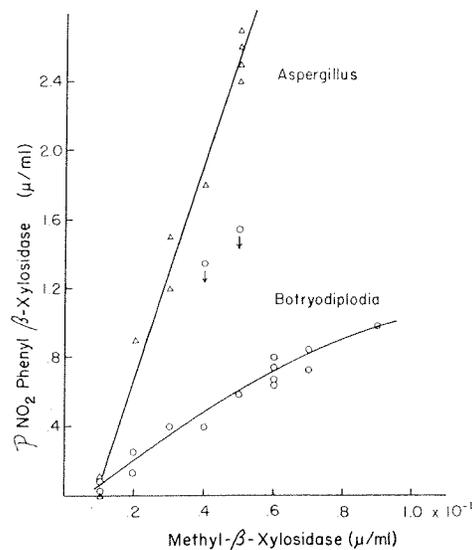


FIG. 6. Comparison of β -D-xylopyranosidase activity on two different substrates, *p*-nitrophenyl β -D-xylopyranoside, and methyl β -D-xylopyranoside. \circ = *Botryodiplodia*; \triangle = *A. niger*.

These data indicate that the intracellular enzymes(s) of this organism have a higher *R* value than those normally secreted; and that digitonin, NPX, and sonication aid in the release of these. *Botryodiplodia* filtrates have, in general, the lowest *R* value of any organism tested.

Our limited data on specificity confirm and extend the much more detailed studies made by the Flemish workers (2, 9, 10, 20). *O*- and *p*-nitrophenyl β -D-xylopyranosides are the most susceptible substrates, followed by phenyl β -D-xylopyranoside, reduced xylotriose, and L-serine β -D-xylopyranoside (Table 4). We find that the action on L-serine β -D-xylopyranoside can readily be detected in the fungal preparations, but that the action does not go to completion. Even after 24-h incubation, most of the glycoside remained (paper chromatography). Serine, glycoside, and xylose were detected on paper chromatograms. The *Charonia* xylosidase (5, 6) also acts slowly on this substrate, but more extensively than do the fungal preparations (88% hydrolysis occurred in 72 h). *B. pumilus* enzyme (from Kersters-Hilderson, Belgium) did not act on the L-serine β -D-xylopyranoside (pH 7.2, 24 h). β -D-Xylosyl dextran was not hydrolyzed (24 h) by either the fungal or the bacterial preparations.

The relative affinity of the fungal enzymes

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TABLE 4
Specificity of β -D-xylopyranosidases

Substrate	Enzyme	Susceptibility (relative)			
		<i>P. wortmanni</i>	<i>A. niger</i>		
			QM877	Hemi*	<i>B. pumilus</i> †
<i>p</i> -Nitrophenyl β -D-xylopyranoside	100‡	100‡	100	100	
<i>o</i> -Nitrophenyl β -D-xylopyranoside	112	87		182	
Phenyl β -D-xylopyranoside	33	43	9	7	
Xylotriose, reduced	8	14			
Methyl β -D-xylopyranoside	2	2	3	0	
L-Serine β -D-xylopyranoside	0.1	0.1			

* *A. niger* (2).

† *B. pumilus* (10).

‡ *P. wortmanni* 19 μ mol/min per milligram protein; *A. niger* 877 13 μ mol/min per milligram protein (pH 3.0; 40° and at *p*-nitrophenyl β -D-xylopyranoside concentration of 3 μ mol/ml).

(*A. niger*, *P. wortmanni*) for some of these substrates can be deduced from the K_m values found (Lineweaver-Burk plots).

<i>p</i> -Nitrophenyl β -D-xylopyranoside	0.3–0.5
	$\times 10^{-3}$ M
Methyl β -D-xylopyranoside	6.0–12.0
	$\times 10^{-3}$
Xylotriose, reduced	10.0
	$\times 10^{-3}$

Comparison of Sources of β -D-Xylopyranosidase

Through the use of surfactants and inducers, we have developed two good fungal sources of β -D-xylopyranosidase. Just how do these compare in activity with sources used by other investigators? Since *p*-nitrophenyl β -D-xylopyranoside has been used by most workers for the assay of activity, a comparison was made using this substrate. The two poorest sources of enzyme are snail (*Charonia*) and *Chaetomium* (Table 5), with only 0.03 U/mg protein of the starting material. After extensive purification procedures, the final products still had lower specific activities than did our untreated culture filtrates. Similarly, a *Bacillus* extract (0.15 U/mg) after 34-fold purification had a specific activity between those of our *A. niger* and *P. wortmanni* values. The best of the materials used before was hemicellulase (NBC) which had about one-sixth the activity of our *A. niger* solutions. The 100-fold purified enzyme from this is the only preparation having higher specific activity than that of our untreated *P. wortmanni* filtrates.

There is no doubt that the comparisons of this sort are open to criticism. However, the failure

to make such comparisons is almost universal, and, perhaps even more objectionable. In the absence of procedures common to all of the work, one never knows how to evaluate the reported activities. Our prime purpose here is to demonstrate the value of selecting and developing a very active enzyme system, before attempting any purification of enzyme.

Discussion

A. What is the inducer of β -D-xylopyranosidase? When used as the only carbon source, β -D-xylopyranoside tetraacetate gave the highest yield. Slow hydrolysis of this supplies xylose at low concentration over an extended time. Similarly, methyl α -D-xylopyranoside is a resistant material which achieves the same end result. The data showing that xylose is not an inducer may be interpreted as indicating a repressing effect at the concentration (0.5%) used, and it is possible that constant feeding at very low concentrations would result in induction (18). We must concede, at this stage, that we do not know whether it is xylose (the product), or methyl β -D-xylopyranoside (the substrate) that is the true inducer.

B. The question of identity of β -D-xylopyranosidase and β -D-glucopyranosidase is unresolved. All possibilities are reported to exist:

- (1) β -D-xylopyranosidase, free of β -D-glucopyranosidase (3, 7, 10, 11, 13, 15),
- (2) β -D-xylopyranosidase with some action on β -D-glucopyranosides (2, 5),
- (3) β -D-glucopyranosidases with some action on β -D-xylopyranosides (3, 4, 4a, 15, 16),

TABLE 5
Comparison of sources of β -D-xylopyranosidase

Organism	Activity vs. <i>p</i> -nitrophenyl β -D-xylopyranoside, units/mg protein	Purification (subseq. achieved)	Reference
<i>Charonia lampas</i>	0.03	89 ×	Fukuda <i>et al.</i> (5)
<i>Aspergillus niger</i> QM877	2.00	—	Reese <i>et al.</i>
<i>Aspergillus niger</i>	0.36	100 ×	Kerstens- Hilderson <i>et al.</i> (2)
<i>Chaetomium trilaterale</i>	0.03*	16 ×	Kawamimami and Iizuta (8)
<i>Penicillium wortmanni</i>	8.00	—	Reese <i>et al.</i>
<i>Bacillus pumilus</i> 12	0.15	34 ×	Kerstens-Hilderson <i>et al.</i> (10)

*Substrate = phenyl rather than *p* nitrophenyl β -D-xylopyranoside.

(4) β -D-glucopyranosidases, free of β -D-xylopyranosidase activity (4, 4a)

The problem is complicated by the fact that aryl β -D-glucopyranosides have been used as substrates, and these are relatively unstable. Data comparing enzyme activity on dimers of the sugars are relatively scarce. Therefore, the information developed is based on the use of unnatural materials. Other complications are (1) no distinctions are being made between β -D-xylopyranosidases, and *exo*- β -D-xylanases, (2) the degree of purity of the enzyme can never be firmly established, and (3) inhibition experiments have often required relatively high concentrations of inhibitor relative to the concentration of substrate. Indeed inhibition tests have indicated that there may be two types of β -D-xylopyranosidases (15). Whether these will coincide with the two types based on specificity differences (*Botrydiodia*, above) remains to be determined.

C. The specificity of the β -D-xylopyranosidases differs with the source. Unfortunately the most commonly used substrate, *p*-nitrophenyl β -D-xylopyranoside, is an unnatural, and somewhat unstable, substance and is much more rapidly hydrolyzed than the natural substrates. Most of the enzymes reported have, however, been shown to act on other β -D-xylopyranosides (7, 9, 20). The requirement is for a β -D-glycopyranoside having a pyranose ring with the arrangement about C₁, C₂, and C₃ as in xylose (2). Apart from their role in the decomposition of xylans, the β -D-xylopyranosidases are involved in the removal of single D-xylopyranose units from various polymers, the current interest being in their removal from glycoproteins (5, 6). Our fungal preparations and the *Charonia* enzyme (5, 6) have been found capable of hydrolyzing the D-xylose to L-serine linkage, while the *B. pumilus* and rat liver (4, 4a) enzymes lack this ability. Much of the specificity work has been directed to an understanding of substrate-enzyme relationship (9, 20), and the use of competitive inhibitors has served this same purpose. One of the most extensively investigated effects is the action of glyconolactones, where Conchie and Levvy (3) have shown that the action is usually but not always highly specific. Previously Morita (15), and now we, have found that xylonolactone is a good inhibitor of β -D-xylopyranosidase, while Δ -gluconolactone is without effect. Here,

again, one is faced with "what is a good inhibitor?" If 50% inhibition requires a concentration much greater than that of the substrate, the inhibitor may be interesting, but cannot be considered "highly active." In this category is the inhibition observed by D-xylose, interesting only because it is anticipated as product inhibition. The nature of the lactone inhibition is of value, not only to mechanistic studies, but to the specific inhibition of one glycosidase in the presence of others.

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