

## Some Products of the Metabolism of D-Xylose by *Pullularia pullulans*

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After the growth of *Pullularia pullulans* in nutrient media containing D-xylose as the sole carbon source, the following compounds were isolated from the cell- and polysaccharide-free culture solutions and characterised: D-xylulose, xylitol and D-xylonic acid. The presence of small amounts of glucose, gluconic and ribonic acids was detected, in addition, by paper chromatographic methods.

Moulds have the capacity to transform carbohydrate substrates into products, in many cases accumulating in large quantities in the culture media, which may reasonably be considered to be metabolic intermediates which, due to environmental conditions such as pH, or adverse competition from the original substrate, are only slowly transformed further or are not immediately required as sources of energy.

The metabolism of D-xylose by moulds has been studied but sparingly and relatively little is known of the mechanisms and pathways of its transformations to the small number of products which have so far been encountered. Two of the main types of end-product of the action of moulds on sugars are organic acids<sup>1</sup> and polysaccharides<sup>1</sup> and both types have been found to be formed from xylose. Although glucose is converted to gluconate by *Aspergillus* and *Penicillium*, as well as a few other species, including *Fusarium lini* and *Pullularia (Dematium) pullulans*, only the two latter have been observed to oxidise xylose to xylonate<sup>1</sup>. Xylose forms an excellent substrate for the formation of kojic acid by *Aspergillus flavus*<sup>2</sup> while it is converted to oxalic acid by *A. niger*<sup>3</sup>. This organism has also been found to incorporate <sup>14</sup>C from xylose into a mycelial glucan<sup>4</sup>.

More recently, another sequence of transformations of xylose by *Penicillium chrysogenum* has been observed which may help to cast some light on the pathways to the other fermentation products. Chiang and Knight<sup>5</sup> have found that D-xylose is reduced by the organism to xylitol and that this is subsequently dehydrogenated to D-xylulose. This compound, on conversion to the 5-phosphate, can enter the pentose shunt and thereby the main paths of carbohydrate metabolism.

During studies concerned mainly with the polysaccharides produced by *P. pullulans* when grown in solutions of D-xylose, a brief investigation has been made of the products of low molecular weight appearing synchronously with polysaccharide in the culture media, and the findings are now presented. Concentration of the cell- and polysaccharide-free culture solution yielded a syrup which was shown by paper chromatography to contain a variety of components other than xylose. Fractionation of this syrup by cellulose column chromatography yielded D-xylulose, D-xylose, xylitol, glucose, and a mixture of inorganic and organic acid salts. D-Xylulose and xylitol were converted to crystalline derivatives identical to the authentic compounds.

After freeing the salt mixture from phosphate and cations, the organic acids were purified by adsorption on ion-exchange resin. Examination of the mixture by paper chromatography and paper electrophoresis showed the principal component to behave as xylonic acid and to form a lactone with the same chromatographic mobility as xylonolactone. Partial reduction of the lactone components with sodium borohydride yielded xylose, along with small amounts of ribose and glucose, as the only reducing sugars. Distillation of a small sample of the mixture under high vacuum afforded a small amount of a crystalline compound, mp. 90°,  $[\alpha]_D^{23} + 95^\circ$ , behaving as xylonolactone on paper chromatograms. A further sample of the acidic components was isolated by ion-exchange methods and from the crude mixture D-xylonic acid was isolated as its brucine salt and as its double salt with cadmium bromide, both identical to the authentic compounds.

From paper chromatograms it was clear that several other acids were present, other than xylonic, ribonic, and gluconic acids, some of which formed lactones distinct from the lactones of the acids named. The characteristic colour reaction given by kojic acid with ferric chloride was not obtained. Traces of organic phosphates and aminoacids could be detected but no evidence for the presence of ketoacids could be obtained.

*P. pullulans* would appear from these observations to have more than one route for the metabolism of D-xylose. One reaction sequence is obviously the transformation to D-xylulose via xylitol as observed in *P. chrysogenum* but there would seem in addition to be a route involving the oxidation of C1. A similar duality has been observed in *in vitro* experiments with the action of enzymes from rat lens on D-xylose<sup>6</sup>. It should be stated, however, that although D-xylulose and not the racemic mixture of D- and L- forms was identified, the presence of L-xylulose in the system in minor quantity cannot be excluded. No evidence was obtained for the formation of kojic acid or ketoacids. The fact that glucose and gluconic acid were detected cannot be considered significant as traces of glucose are to be found in commercially available xylose preparations.

#### EXPERIMENTAL

All evaporations were carried out under reduced pressure at temperatures not exceeding 50°C.

Paper chromatography was carried out on Whatman No. 1 paper with the following solvent systems:

- (A) ethyl acetate, pyridine, water (8:2:1)  
 (B) ethyl acetate, acetic acid, water (3:1:3)  
 (C) water saturated methyl ethyl ketone

Paper electrophoresis was conducted on Whatman No. 3 paper in acetate buffer (pH 4) with 1 kV potential difference.

Components were detected on papers by the use of the following reagents:

- (1) silver nitrate-sodium hydroxide for reducing sugars, aldonic acids, and alditols
- (2) anisidine hydrochloride for reducing sugars
- (3) resorcinol — hydrochloric acid for ketoses
- (4) hydroxylamine—ferric chloride for lactones
- (5) thymol blue indicator for acids
- (6) dinitrophenyl hydrazine—hydrochloric acid for ketones
- (7) ninhydrin for amino compounds and
- (8) ammonium molybdate—hydrochloric acid for phosphates.

*Culture of the mould.* The general conditions of culture have been described previously<sup>7</sup>. The composition of the culture solution was 1.0 g  $\text{KH}_2\text{PO}_4$ , 0.2 g  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.3 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g NaCl, 0.02 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 2.5 g  $\text{NaNO}_3$  and 50 g D-xylose per litre. The xylose cultures were made in batches of 9 litres with continuous aeration and stirring.

*Isolation of the components of low molecular weight.* The cells were removed from the cultures by super-centrifugation and the polysaccharides precipitated from the solutions by the addition of ethanol. The ethanolic supernatant solutions obtained after the centrifugation of the precipitated polysaccharides were evaporated to syrups. These syrups, on examination by paper chromatography in solvent (A), showed a mixture of components with  $R_{\text{xylose}}$  values 1.58, 1.50, 1.00, 0.71, 0.44 and 0.00.

*Fractionation of the mixture.* A portion of the syrup (4.4 g) was slurried with water and cellulose powder and the slurry was freeze-dried. The powder thus obtained was packed on the top of a cellulose column (45 × 4.5 cm) and elution was performed with solvent (A). The effluent was collected in 50 ml portions and the fractions obtained are listed below. The final fraction was obtained by elution with water.

Fraction	Tube Nos.	$R_{\text{xylose}}$ of content	Weight, g
1	21—23	1.58	0.028
2	34—45	1.50	0.040
3	46—50	1.50, 1.00	0.050
4	51—60	1.00	0.542
5	61—70	1.00, 0.71	0.369
6	71—79	0.71, trace 1.00	0.514
7	80—98	0.71	0.800
8	99—120	0.71, 0.44	0.162
9	121—150	0.44	0.124
10	164—174	0.00	1.820

All fractions, but especially the minor ones, were contaminated by non-volatile products which could not be removed completely by treatment with charcoal.

*Examination of the fractions. Fraction 1.* A very small amount of a substance with  $R_x$  1.58 and reacting on paper chromatograms with reagents (1) and (2) was present. The fraction was not examined further.

*Fraction 2.* The syrup had  $R_x$  1.50 and reacted with reagents (1)–(3), giving a grey colour with the latter. It was identical in colour reaction and chromatographic mobility to xylulose and had  $[\alpha]_{\text{D}}^{25} -15^\circ$  (c, 0.4 in water); D-xylulose has  $[\alpha]_{\text{D}} -34^\circ$ . Reaction with phenylhydrazine gave a product which, after several recrystallisations from aqueous ethanol, melted at 162–163° and showed mutarotation in ethanol-pyridine solution (3:2) from  $[\alpha]_{\text{D}}^{23} -14^\circ$  to  $-31^\circ$  (c, 0.8). The melting-point was unchanged by admixture with authentic D-xylosazone and the infra-red spectra of the two compounds were identical.

*Fraction 4.* This material was identical in chromatographic mobility, colour reaction, and optical rotation to D-xylose.

*Fraction 7.* The syrup reacted on paper chromatograms only with reagent (1) and showed identical chromatographic mobility to xylitol, but was distinct from ribitol and arabinitol in solvent (A). No optical activity could be detected. Reaction with formaldehyde and hydrochloric acid<sup>9</sup> yielded a compound which, after several recrystallisations from aqueous ethanol, had mp. 199–201°, undepressed on admixture with authentic 2,4:3,5-di-O-methylidene-DL-xylitol. The infra-red spectra of the two compounds were identical. Reaction with benzaldehyde<sup>10</sup> afforded a crystalline derivative mp. 184–186° (lit. 188°).

*Fraction 9.* This material was identical to glucose on chromatograms in both mobility and colour reaction.

*Fraction 10.* The syrup had  $[\alpha]_D^{22} + 10^\circ$  (c, 3.63 in water) and  $R_x$  0.00 in solvent (A), 1.27 in solvent (B). Reaction was observed mainly with reagent (1) although the presence of phosphate and to a lesser extent aminoacid could be detected with reagents (7) and (8), respectively. Paper electrophoresis showed the main component detectable with reagent (1) to have the same mobility as xylonate. The mixture was dissolved in water, freed from cations by passage through Dowex 50 resin and the effluent neutralised with barium carbonate. The filtered solution was freed from barium ions by a repetition of the resin treatment. The acid, phosphate-free components were adsorbed on Dowex 2 (carbonate form). After washing away the neutral components with water, the acids were displaced with 10% formic acid and the eluate concentrated to a syrup (520 mg). Chromatographic examination of the syrup revealed a mixture of acids and lactones with the principal components of the two groups being identical in chromatographic mobility to xylonic acid – solvent (B) – and xylonolactone – solvent (A) – respectively.

Distillation of a portion of the mixture (100 mg) under high vacuum allowed the isolation of a crystalline sublimate (19 mg) with the same chromatographic mobility in solvent (A) as xylonolactone, m.p. 90°, and  $[\alpha]_D^{25} + 95^\circ$  (c, 0.19 in water), cf. lit.<sup>11</sup> mp. 99–103°,  $[\alpha]_D + 89.6^\circ$ . Partial reduction with sodium borohydride in pyridine<sup>12</sup> of a portion of the mixture and paper chromatographic examination of the products in solvent (A) showed the presence of xylose and traces of ribose and glucose as the only reducing sugars. Unchanged lactone, free acid, and pentitol could be detected by reagent (1).

*Further examination of the acidic components.* By the use of ion-exchange resins as described above, an acid fraction (1.3 g) was obtained from a portion of the original syrup (ca. 13 g). Treatment of a portion of the mixture (1 g) with brucine afforded a brucine salt (1.3 g) which had mp. 152–154° after repeated recrystallisation from ethanol. The melting-point was undepressed by the admixture of authentic brucine D-xylonate and the two compounds gave identical infra-red spectra. The melting-points of both compounds were lower than the values given in the literature viz. 176°<sup>11</sup>, 172–174°.<sup>19</sup>

A further portion (200 mg) was treated with cadmium carbonate and hydrobromic acid. From the reaction mixture a salt (90 mg) was obtained which on recrystallisation from aqueous ethanol gave  $[\alpha]_D^{24} + 6^\circ$  (c, 0.40 in water), cf. lit. + 8.8°<sup>14</sup>, an infra-red spectrum and an X-ray interference pattern identical to those obtained from authentic cadmium bromide xylonate.

Examination of the acid fraction on paper chromatograms run in solvents (B) and (C) using reagents (4), (5) and (6) showed that several acids other than xylonic, ribonic and gluconic acids, and several lactones other than xylono-, ribono-, and glucono-lactones were present and that no keto-acid could be detected. The characteristic reaction of kojic acid with ferric chloride was not observed. The complex mixture was not examined further.

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