PHYLOGENY OF PHOSPHOMANNAN-PRODUCING YEASTS

II. Phosphomannan Properties and Taxonomic Relationships

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ABSTRACT

Sloaki, M. E. (U. S. Department of Agriculture, Peoria, Ill.), Lynferd J. Wickerham, and M. C. Cadmus. Phylogeny of phosphomannan-producing yeasts. II. Phosphomannan properties and taxonomic relationships. J. Bacteriol. 82:269–274. 1961.—Primitive species of yeasts belonging to the genus Hansenula and closely related genera produce extracellular phosphorylated mannans from glucose. These polysaccharides, called phosphomannans, contain mannose and mannose 6-phosphate as the only carbohydrate constituents. A comparative study of these phosphomannans yielded valuable phylogenetic information bearing on the yeasts that produce them. Strains of the same species elaborate similar phosphomannans. An inverse relationship was found between the evolutionary status of the organism and the degree of phosphorylation of its phosphomannan.

Research on the production of extracellular microbial gums has been undertaken at the Northern Laboratory as part of a continuing program to increase industrial uses for cereal grains. The first such product to be intensively investigated is a mannan containing phosphorus which has been named “phosphomannan.” This polysaccharide is elaborated in high yields from glucose by a diploid strain of Hansenula holstii NRRL Y-2448 (Anderson et al., 1960; Jeanes et al., 1961; Rogovin, Sohns, and Griffin, 1961). A survey of other primitive Hansenula species—as well as primitive species in related genera—has revealed that the phosphomannans constitute a family of unique polysaccharides. This report presents results of analytical studies which have provided useful information concerning the phylogeny of yeasts that produce the various phosphomannan gums.

MATERIALS AND METHODS

Production and isolation of phosphomannans. The following medium was employed for the production of phosphomannans: glucose monohydrate, 6.0%; corn steep liquor, 0.1%; tryptone (Difco), 0.1%; KH₂PO₄, 0.5%; Speakman’s salt solution B (Snell and Strong, 1939), 0.5% (v/v); initial pH 5.0. The yeasts were grown at 25 °C in reciprocally shaken flasks at oxygen absorption rates of 0.5 to 1.0 as measured by Corman et al. (1957). After 4 days, the cells were removed by centrifugation following dilution of the cultures with methanol (25% v/v) to decrease viscosity. The polymers were precipitated from the cell-free methanolic broth by increasing the methanol concentration to 50% (v/v) and addition of small amounts of a saturated solution of potassium acetate. The supernatant liquids were removed by decanting and discarded. The cohesive slimes were dissolved in amounts of water corresponding to the original culture volumes and again precipitated in the same manner. Essentially complete removal of protein and of extraneous ionic material was achieved in a third precipitation by the addition, while stirring, of methanol containing 2.5% potassium acetate. The volume of methanol required (36 to 50%) depended upon the degree of phosphorylation of the polysaccharide; less methanol was needed to precipitate the more heavily phosphorylated polymers. The precipitates obtained at this stage were dissolved in water. The solutions were then dialyzed and lyophilized to obtain the phosphomannans as potassium salts.

Analytical procedures. Measurements of optical rotation were made on 1.0% solutions of polymer in 0.1 M KCl. Following removal of potassium ions by treatment with a cation ex-
TABLE 1. Some taxonomic characteristics of phosphomannan-producing yeasts

<table>
<thead>
<tr>
<th>NRRL no.</th>
<th>Species and strains</th>
<th>Nitrate assimilation</th>
<th>Sex</th>
<th>Sugars fermented&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ester production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-2448</td>
<td><em>Hansenula holstii</em></td>
<td>+</td>
<td>Heterothallic</td>
<td>D or Dg</td>
<td></td>
</tr>
<tr>
<td>YB-3070</td>
<td><em>Hansenula</em> sp. n.</td>
<td>+</td>
<td>Heterothallic</td>
<td>Dm</td>
<td>+ (weak)</td>
</tr>
<tr>
<td>Y-1889</td>
<td><em>Hansenula capsulata</em></td>
<td>+</td>
<td>Homothallic</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Y-1842</td>
<td><em>H. capsulata</em></td>
<td>+</td>
<td>Homothallic</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Y-411</td>
<td><em>Hansenula minuta</em></td>
<td>+</td>
<td>Homothallic</td>
<td>D or d</td>
<td></td>
</tr>
<tr>
<td>YB-2203</td>
<td><em>H. minuta</em></td>
<td>+</td>
<td>Homothallic</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>YB-2194</td>
<td><em>Hansenula</em> sp. n.</td>
<td>+</td>
<td>Homothallic</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Y-2461</td>
<td><em>Pachysolen</em> tannophilus</td>
<td>+</td>
<td>Not determined</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>YB-2537</td>
<td><em>Near Torulopsis</em> pinus</td>
<td>-</td>
<td>Heterothallic (?)</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>Y-2023</td>
<td><em>T. pinus</em></td>
<td>-</td>
<td>Heterothallic (?)</td>
<td>d</td>
<td></td>
</tr>
<tr>
<td>Y-2579</td>
<td><em>Saccharomyces</em> pini</td>
<td>-</td>
<td>Homothallic</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>YB-2097</td>
<td><em>Pichia</em> sp.</td>
<td>-</td>
<td>Heterothallic (?)</td>
<td>D or Ds</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> D, G, M, S refer to dextrose, galactose, maltose, and sucrose, respectively. 0 = Absence of gaseous fermentation. YB-3070 and YB-2194 represent separate species. Capital letter denotes strong fermentation; lower case, weak.

change resin (Amberlite 1R-120, H<sup>+</sup>), the polysaccharides were hydrolyzed in 4 N H<sub>2</sub>SO<sub>4</sub> for 75 min at 100 C. Reducing power measurements were made by the Nelson-Somogyi procedure (Somogyi, 1952). Apparent degrees of polymerization were estimated by reducing power measurements on solutions of the polysaccharides and neutralized hydrolyzates. Inorganic phosphorus was determined by the method of Fiske and SubbaRow (1925). Organic phosphorus was assayed as described by Leloir and Cardini (1957).

Hydrolyzates were chromatographed on Whatman no. 1 paper in n-butyl alcohol-pyridine-water (6:4:3) for 16 hr (Jeanes, Wise, and Dimler, 1951). Sugars were detected by the mino-sodiophenyl spray reagent of Timell, Gladelevens, and Currie (1956). Phosphate esters were chromatographically identified after descending irrigation on Whatman no. 1 paper in the tert-butyl alcohol-picric acid-water solvent system of Hanes and Isherwood (1949) and detection with the acid-molybdate reagent of Bandurski and Axelrod (1951).

Kinematic viscosity measurements were made on 0.5% phosphomannan solutions at 20 C using calibrated size 200 Ostwald-Cannon-Fenske viscosimeters (Merrington, 1949).

Polymer yields were estimated by the procedure of Anderson et al. (1960).

RESULTS

In Table 1 it can be seen that phosphomannans are elaborated not only by heterothallic and homothallic members of the genus *Hansenula*, but also by yeasts belonging to other genera. As can be judged by the few sugars fermented and by the limited ester production, the yeasts represented are the more primitive species of their respective genera. They occupy but small taxonomic areas in these genera (Wickerham and Burton, 1961). The presumed phylogenetic relationships of these groups are illustrated in some detail in Fig. 1.

Although the data presented in Table 2 show that the various phosphomannans possess widely differing properties, chemical characterizations reveal that, in all cases, mannose and mannose 6-phosphate are the sole carbohydrate components. Less than 5% of the total phosphorus in phosphomannans is obtained as orthophosphate upon acid hydrolysis. This result is in accord with the known stability of mannose 6-phosphate to acid hydrolysis (Leloir and Cardini, 1957). The presence of mannose in hydrolyzates was confirmed by the specific carbazole (Seibert and

<sup>2</sup> Phosphomannans were not found in the culture filtrates of the more highly evolved nonmucoid species described by Wickerham and Burton (1961). The extraction of the cells of these species yielded, in most instances, unphosphorylated mannansidic polysaccharides.
Atno, 1946) and cysteine-sulfuric acid (Dische, Shettles, and Osnos, 1949) color reactions.

The polymers may be arranged into distinct categories based upon increasing mannose to phosphorus molar ratios. With the possible exception of H. minuta, phosphomannans from different strains of the same species have similar degrees of phosphorylation.

Generally, the phosphomannans display high positive optical rotations. Exceptions are the H. capsulata polymers which have low rotations, indicating a greater preponderance of glycosidic linkages of the β-configuration, and the phosphomannan produced by Pachysolen tannophilus NRRL Y-2461. Interestingly, the latter is distinguished from those produced by H. holstii strains by its much lower positive specific rotation.

The alkaline copper reagent used in this study to determine degrees of polymerization of chains of mannose units does not react quantitatively with all of the phosphomannans. A series of phosphomannans of degrees of polymerization 50 to 600, as measured by copper reduction, were selected. These were oxidized by hypolodite (Neish, 1952) and found to give degrees of polymerization values ranging from 48 to 91. Generally, alkaline reagents do not reliably measure degrees of polymerization of carbohydrates (Launer and Tomimatsu, 1961). The apparent degrees of polymerization values obtained by use of the copper reagent, however, serve a diagnostic purpose insofar as they tend to be characteristic for a particular species; e.g., H. holstii phosphomannans have higher apparent degrees of polymerization values than those exhibited by other Hansenula species. Furthermore, phosphomannans produced by the nitrate-positive species have markedly higher apparent degrees of polymerization values than do phosphomannans of the nitrate-negative species. A lack of appreciable reactivity with alkaline copper is characteristic of aldehyde end groups adjacent to 1,2-glycosidic linkages. The presence of such linkages in Y-2448 phosphomannan has been demonstrated by the periodate oxidation studies of Jeanes et al. (1958). These linkages have also been found to predominate in the di- and trisaccharide phosphomonoesters formed upon mild acidic hydrolysis of Y-1842 phosphomannan (Slodki, unpublished results).

**DISCUSSION**

Experience in this laboratory with the production of H. holstii strains NRRL Y-2448 and NRRL Y-2154 phosphomannans under a wide variety of conditions has shown that, although the over-all polymer yields may vary considerably, the same basic polymer is always produced (Anderson et al., 1960). This study shows that phosphomannans produced by different strains of a given species are relatively similar in their properties. Another relationship which emerges from examination of the data in Table 2 concerns...
the degree of phosphorylation of the polymers. The most primitive species, such as *H. capsulata* and *H. holstii*, produce the most heavily phosphorylated polysaccharides. As indicated in Fig. 1, such species as *H. minuta* in the phylogenetic line that evolved toward dependence on coniferous trees and loss of fermentative capacity produce more lightly phosphorylated polymers. Nitrate-negative species of the genus *Pichia*, like the tree-dependent species of *Hansenula*, produce mannans containing less phosphate (Tables 1 and 2, Fig. 1). This observation is in accord with the Wickerham and Burton (1961) finding that biochemically less versatile species comprise a much greater percentage of the total species in *Pichia* than in *Hansenula*. The mannose to phosphorus ratios would seem to suggest that the nitrate-negative genus originated predominantly from species of the tree-dependent line in *Hansenula*.

Strains of *Saccharomyces pini* frequently occur in the diploid form. This fact indicates that *S. pini* is more highly evolved than such primitive species of *Hansenula* as *H. capsulata* and *H. holstii*, for these species are isolated in nature exclusively as haploids.  

A diploid form of *H. holstii* strain Y-2448 has been produced in the laboratory and is used for production of phosphomannan (Anderson et al., 1960) because it has certain advantages over haploid strains.

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**Table 2: Physical and chemical properties of phosphomannans isolated from yeast cultures**

<table>
<thead>
<tr>
<th>NRRL no.</th>
<th>Organism name</th>
<th>M:P</th>
<th>$[\alpha]_D^b$</th>
<th>DP</th>
<th>Viscosity $c$</th>
<th>Yield $d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-1842</td>
<td><em>Hansenula capsulata</em></td>
<td>2.5</td>
<td>-2°</td>
<td>244</td>
<td>33</td>
<td>1.7</td>
</tr>
<tr>
<td>Y-1889</td>
<td><em>H. capsulata</em></td>
<td>2.5</td>
<td>+21°</td>
<td>101</td>
<td>29</td>
<td>1.4</td>
</tr>
<tr>
<td>YB-1978</td>
<td><em>H. capsulata</em></td>
<td>2.4</td>
<td>-13°</td>
<td>91</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>YB-4061</td>
<td><em>H. capsulata</em></td>
<td>2.6</td>
<td>+6°</td>
<td>81</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>YB-3070</td>
<td><em>Hansenula</em> sp. n.</td>
<td>3.6</td>
<td>+100°</td>
<td>121</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>YB-1443</td>
<td><em>Hansenula</em> sp. n.</td>
<td>3.7</td>
<td>+78°</td>
<td>121</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>YB-3234</td>
<td><em>Hansenula</em> sp. n.</td>
<td>3.8</td>
<td>+77°</td>
<td>115</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Y-2461</td>
<td><em>Pachysolen tannophilus</em></td>
<td>4.2</td>
<td>+46°</td>
<td>256</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>YB-2316</td>
<td><em>Hansenula holstii</em></td>
<td>4.4</td>
<td>+92°</td>
<td>415</td>
<td>18</td>
<td>2.4</td>
</tr>
<tr>
<td>YB-3066</td>
<td><em>H. holstii</em></td>
<td>4.5</td>
<td>+84°</td>
<td>274</td>
<td>18</td>
<td>2.0</td>
</tr>
<tr>
<td>YB-3341</td>
<td><em>H. holstii</em></td>
<td>4.7</td>
<td>+82°</td>
<td>462</td>
<td>28</td>
<td>2.1</td>
</tr>
<tr>
<td>YB-347</td>
<td><em>H. holstii</em></td>
<td>5.0</td>
<td>+102°</td>
<td>701</td>
<td>29</td>
<td>2.5</td>
</tr>
<tr>
<td>YB-2099</td>
<td><em>H. holstii</em></td>
<td>5.1</td>
<td>+74°</td>
<td>796</td>
<td>17</td>
<td>2.3</td>
</tr>
<tr>
<td>YB-889</td>
<td><em>H. holstii</em></td>
<td>5.1</td>
<td>+79°</td>
<td>434</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Y-2154</td>
<td><em>H. holstii</em></td>
<td>5.2</td>
<td>+93°</td>
<td>600</td>
<td>19</td>
<td>2.4</td>
</tr>
<tr>
<td>YB-689</td>
<td><em>H. holstii</em></td>
<td>5.4</td>
<td>+77°</td>
<td>784</td>
<td>34</td>
<td>1.8</td>
</tr>
<tr>
<td>Y-2448</td>
<td><em>H. holstii</em></td>
<td>5.7</td>
<td>+106°</td>
<td>588</td>
<td>46</td>
<td>2.4</td>
</tr>
<tr>
<td>YB-2007</td>
<td><em>Pichia</em> sp.</td>
<td>7.2</td>
<td>+101°</td>
<td>17</td>
<td>13</td>
<td>1.8</td>
</tr>
<tr>
<td>Y-2023</td>
<td><em>Tolypopsis pinus</em></td>
<td>8.4</td>
<td>+68°</td>
<td>51</td>
<td>71</td>
<td>2.4</td>
</tr>
<tr>
<td>YB-2537</td>
<td><em>Near T. pinus</em></td>
<td>8.5</td>
<td>+102°</td>
<td>28</td>
<td>31</td>
<td>1.8</td>
</tr>
<tr>
<td>YB-2258</td>
<td><em>Saccharomyces pini</em></td>
<td>9.2</td>
<td>+91°</td>
<td>24</td>
<td>24</td>
<td>1.0</td>
</tr>
<tr>
<td>YB-2903</td>
<td><em>Hansenula minuta</em></td>
<td>9.8</td>
<td>+89°</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YB-1307</td>
<td><em>S. pini</em></td>
<td>11.0</td>
<td>+102°</td>
<td>28</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>YB-3706</td>
<td><em>S. pini</em></td>
<td>11.4</td>
<td>+88°</td>
<td>40</td>
<td>24</td>
<td>2.4</td>
</tr>
<tr>
<td>YB-3339</td>
<td><em>S. pini</em></td>
<td>11.5</td>
<td>+87°</td>
<td>32</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>YB-2194</td>
<td><em>Hansenula</em> sp.</td>
<td>11.9</td>
<td>+100°</td>
<td>155</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>YB-2022</td>
<td><em>S. pini</em></td>
<td>12.2</td>
<td>+97°</td>
<td>51</td>
<td>11</td>
<td>2.3</td>
</tr>
<tr>
<td>Y-2579</td>
<td><em>S. pini</em></td>
<td>13.0</td>
<td>+91°</td>
<td>50</td>
<td>83</td>
<td>2.4</td>
</tr>
<tr>
<td>Y-411</td>
<td><em>H. minuta</em></td>
<td>27.5</td>
<td>+88°</td>
<td>144</td>
<td>11</td>
<td>1.4</td>
</tr>
</tbody>
</table>

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* M:P = mannose to phosphorus molar ratio.
* DP = apparent degree of polymerization.
* Viscosity expressed in centistokes.
* Yield given in grams of polymer produced (mannose basis) per 100 ml of culture. YB-3070, YB-1443, and YB-3234 are strains belonging to the same species. YB-2194 belongs to a separate species.
nans synthesized by species of *S. pini* agree with the higher ratios exhibited by the phosphomannans from the more highly evolved *Hansenula* species. *Torulopsis pinus* NRRL Y-2023 is near *S. pini* taxonomically although it produces no ascospores. Both synthesize quite similar phosphomannans. The yeast, NRRL YB-2537, considered by the usual taxonomic criteria to be phylogenetically close to *T. pinus*, also produces a phosphomannan similar to that of Y-2023.

The data presented in Table 2 show a lack of correlation between viscosity on one hand and either mannose to phosphorus ratio or apparent degrees of polymerization on the other. In addition, degrees of polymerization values of a similar magnitude were obtained by the hypoidite oxidation of phosphomannans of widely differing viscosities. It should be emphasized at this point that the viscosity relationships shown in Table 2 are fairly reproducible although slight variations in observed values are often obtained. Concurrent investigations have established that the acid-stable mannose 6-phosphate residues are all cross-linked through acid-labile hemiacetal phosphate bonds (*Slodki, unpublished results*). All phosphomannans examined so far, regardless of degree of phosphorylation, possess exclusively these cross-linkages and may be considered polyesters of mannosidic oligosaccharides and phosphoric acid. The high viscosities displayed by phosphomannans that have low degrees of polymerization values (notably those polymers produced by *T. pinus* NRRL Y-2023 and by *S. pini* NRRL Y-2579) cannot be accounted for on the basis of this type of phosphodiester cross-linkage. Investigations which have established the nature of the phosphodiester linkages in phosphomannans as well as the elucidation of the structure of Y-1842 phosphomannan will be published elsewhere.

Interest has been expressed in the use of Y-2448 phosphomannan as an industrial hydrocolloid. The work reported in this and the preceding paper (Wickerham and Burton, 1961) has related concurrent biochemical and phylogenetic investigations in a new and expanding taxonomic area; it has uncovered other phosphomannans, some of which give much higher viscosities than does the Y-2448 polymer and are produced from glucose in similarly high yields (Table 2).

**LITERATURE CITED**


Rogovin, S. P., V. E. Sohns, and E. L. Griffin,