THE DISTRIBUTION OF XYLOSE IN THE CELLS OF
BALLISTOSPOROUS YEASTS—APPLICATION OF
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITHOUT DERIVATIZATION TO
THE ANALYSIS OF XYLOSE IN
WHOLE CELL HYDROLYSATES

MOTOFUMI SUZUKI AND TAKASHI NAKASE

*Japan Collection of Microorganisms, The Institute of Physical
and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan*

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The presence or absence of xylose in the whole cell hydrolysates from all type strains of 41 known species of the genera *Bullera*, *Sporidiobolus*, and *Sporobolomyces*, and three strains of *Itersonilia perplexans* was examined using high performance liquid chromatography (HPLC) which makes it possible to analyze the carbohydrates without derivatization. Of 16 species of the genus *Bullera*, 14 had xylose in the cells. The other two species, *Bullera salicina* and *Bullera tsugae*, had no xylose in the cells. None of the 25 species of the genera *Sporidiobolus* and *Sporobolomyces* had xylose in the cells. The lack of xylose in the cells of seven *Sporobolomyces* species with Q-9 as the major ubiquinone proves that these species are not yeast phases of certain species of the genus *Itersonilia* with Q-9, since both the yeast and filamentous phases of *Itersonilia perplexans* had xylose in the cells. These results show that the presence or absence of xylose in the cells is very useful as one criterion for the generic differentiation of *Bullera* and *Sporobolomyces*. The lack of xylose in the cells of *B. salicina* and *B. tsugae* indicates that these two species may be closely related to the genus *Sporobolomyces*. Our HPLC method, by which xylose in the whole cell hydrolysates can be analyzed without derivatization, is a useful way to differentiate taxa by this criterion.

Cellular carbohydrate composition provides useful taxonomic information at the level of genus or higher taxa of yeasts and yeast-like fungi (1–7). WEIJMAN and RODRIGUES DE MIRANDA (5) pointed out the significance of the presence or absence

Address reprint requests to: Dr. M. Suzuki, Japan Collection of Microorganisms (RIKEN), Wako, Saitama 351-01, Japan.

of xylose in whole cell hydrolysates in the taxonomy of the genera *Bullera* and *Sporobolomyces*; xylose is present in the majority of the former species, but not in the latter ones. Recently, however, SUGIYAMA et al. (7) reported that the type strain of *Sporidiobolus salmonicolor*, teleomorph of *Sporobolomyces salmonicolor*, contained xylose in whole cell hydrolysates. As both authors analyzed derivatized whole cell hydrolysates by gas-liquid chromatography (GLC) under different hydrolytic conditions and with different derivatization methods, this contradiction has remained unresolved.

To solve this problem, the presence or absence of xylose in the whole cell hydrolysates must be determined without derivatization. For this purpose in the present study, we established a method for analysis of xylose in whole cell hydrolysates using high performance liquid chromatography (HPLC) which allows one to analyze the carbohydrates without derivatization. Using this method, we examined the occurrence of xylose in the whole cells of all of the type strains of 41 species of the genera *Bullera*, *Sporobolomyces*, and *Sporidiobolus*, including species that have been newly described since 1982. Also, three representative strains of *Itersonilia perplexans* were studied. The results reported here show that our HPLC method is useful for analyzing xylose in the whole cell hydrolysates without derivatization and that the presence or absence of xylose in the cells can be the most important criterion for the delimitation of the genera *Bullera* and *Sporobolomyces*.

MATERIALS AND METHODS

Strains examined. The strains examined in this study are listed in Tables 1, 2, and 3. We used 16 type strains of 16 species of the genus *Bullera*, four type strains of four species of the genus *Sporidiobolus*, 21 type strains of 21 species of the genus *Sporobolomyces*, and three strains of *Itersonilia perplexans*.

Cultivation. Yeasts and filamentous fungi were cultivated in a 500-ml Erlenmeyer flask containing 200 ml of YM broth supplemented by 2% glucose on a rotary shaker at 150 rpm. Cultivation was carried out at 17°C. Cells grown on a YM agar slant (ca. 7.5 cm²/8 ml) for 3–5 days were used as the inoculum. After 4–5 days, the cells were harvested by centrifugation (5,000 rpm) and washed twice with deionized water.

Acid hydrolysis of whole cells. Cells dried with acetone (50–100 mg) were suspended in 1 ml of 2 M trifluoroacetic acid (TFA) in a test tube (Pyrex) with a teflon-sealed screw cap (13 × 100 mm), and kept at 100°C for 3 h in a metal block bath. After cooling, the hydrolysate was filtered with paper and evaporated to dryness. The residue was dissolved in water (0.5 ml), neutralized immediately with small amounts of Amberlite IRA 410 (OH form), filtered with a disposable filter unit (Shodex DT ED-13, Showa Denko, K. K., Tokyo, Japan), and subjected to high performance liquid chromatography (HPLC).

HPLC analysis of whole cell hydrolysates. HPLC analysis was performed on an SSC 3000 flow system connected with a column oven (Senshu Scientific

Table 1. The presence or absence of xylose in acid hydrolysates of the whole cells of *Bullera* species.

Species	Strain	Other designation or source	Xylose in the cells
<i>B. alba</i>	JCM 2954 ^T	CBS 501	+
<i>B. armeniaca</i>	JCM 2955 ^T	CBS 7091	+
<i>B. aurantiaca</i>	JCM 2956 ^T	CBS 6980	+
<i>B. crocea</i>	JCM 2961 ^T	CBS 6714	+
<i>B. dendrophila</i>	JCM 5357 ^T	CBS 6074	+
<i>B. derxii</i>	JCM 5280 ^T	<i>Oryza sativa</i>	+
<i>B. globospora</i>	JCM 2957 ^T	CBS 6981	+
<i>B. oryzae</i>	JCM 5281 ^T	<i>O. sativa</i>	+
<i>B. pseudoalba</i>	JCM 5290 ^T	<i>O. sativa</i>	+
<i>B. sinensis</i>	JCM 6253 ^T	Bu5	+
<i>B. megalospora</i>	JCM 5269 ^T	<i>O. sativa</i>	+
<i>B. piricola</i>	JCM 2958 ^T	CBS 6754	+
<i>B. punicea</i>	JCM 1535 ^T	AJ 4764	+
<i>B. variabilis</i>	JCM 5275 ^T	<i>O. sativa</i>	+
<i>B. salicina</i>	JCM 2959 ^T	CBS 6983	—
<i>B. tsugae</i>	JCM 2960 ^T	CBS 5038	—

T: type strain.

Abbreviations of culture collections: AJ, Central Research Laboratories, Ajinomoto Co., Kawasaki, Japan; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands; IFO, Institute for Fermentation, Osaka, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Saitama, Japan; UCD, Department of Food Science and Technology, University of California, California, U.S.A.

Company, Tokyo, Japan) using two different column systems: A) a Shodex SP1010 column (Showa Denko, K. K., Tokyo, Japan) with water (HPLC grade) as the mobile phase at a flow rate of 0.8 ml/min and at 80°C. B) a Shodex RS pak DC-613 (Na form) column (Showa Denko, K. K., Tokyo, Japan) with acetonitrile–water (80:20, v/v, HPLC grade) as the mobile phase at a flow rate of 0.8 ml/min at 75°C. A refractive index detector (ERC-7520, Erma Optical Works, Ltd., Tokyo, Japan) was used to detect the carbohydrates. A chromatopac C-R3A (Shimadzu Corporation, Kyoto, Japan) was used as a recorder. Neutral sugars and sugar alcohols were identified by comparing their retention times with those of standard neutral sugars and sugar alcohols.

RESULTS AND DISCUSSION

Analysis of xylose in whole cell hydrolysates

In this study, we took account of the following two points in the analysis of xylose. Firstly, as an acid for hydrolysis, we used trifluoroacetic acid (TFA) which gives better recoveries of pentoses such as xylose than hydrochloric acid and sulfuric acid (8), because, during acid hydrolysis, liberated pentoses tend to be destroyed

Table 2. The presence or absence of xylose in acid hydrolysates of whole cells of *Sporidiobolus* and *Sporobolomyces* species.

Species	Strain	Other designation or source	Xylose in the cells
<i>Sporid. johnsonii</i>	JCM 1840 ^T	UCD 68-29	—
<i>Sporid. pararoseus</i>	JCM 5350 ^T	CBS 484	—
<i>Sporid. ruinenii</i>	JCM 1839 ^T	UCD 67-67	—
<i>Sporid. salmonicolor</i>	JCM 1841 ^T	UCD 68-371	—
<i>Sp. albo-rubescence</i>	JCM 5352 ^T	CBS 482	—
<i>Sp. foliicola</i>	JCM 5355 ^T	CBS 8075	—
<i>Sp. gracilis</i>	JCM 2963 ^T	CBS 71	—
<i>Sp. holsaticus</i>	JCM 5296 ^T	IFO 1034	—
<i>Sp. kluyveri-nielii</i>	JCM 6356 ^T	CBS 7168	—
<i>Sp. oryzzicola</i>	JCM 5299 ^T	<i>O. sativa</i>	—
<i>Sp. roseus</i>	JCM 5353 ^T	CBS 486	—
<i>Sp. sasicola</i>	JCM 5979 ^T	<i>Sasa</i> sp.	—
<i>Sp. subbrunneus</i>	JCM 5278 ^T	<i>O. sativa</i>	—
<i>Sp. elongatus</i>	JCM 5354 ^T	CBS 8080	—
<i>Sp. singularis</i>	JCM 5356 ^T	CBS 5109	—
<i>Sp. griseoflavus</i>	JCM 5653 ^T	<i>Sasa</i> sp.	—
<i>Sp. inositophilus</i>	JCM 5654 ^T	<i>Sasa</i> sp.	—
<i>Sp. falcatus</i>	JCM 6838 ^T	<i>Sasa</i> sp.	—
<i>Sp. intermedius</i>	JCM 5291 ^T	<i>O. sativa</i>	—
<i>Sp. miscanthi</i>	JCM 5733 ^T	<i>M. sinensis</i>	—
<i>Sp. naganoensis</i>	JCM 5978 ^T	<i>Sasa</i> sp.	—
<i>Sp. subroseus</i>	JCM 5735 ^T	<i>M. sinensis</i>	—
<i>Sp. weijmanii</i>	JCM 5651 ^T	<i>M. sinensis</i>	—
<i>Sp. yuccicola</i>	JCM 6251 ^T	<i>Yucca</i> sp.	—
<i>Sp. yamatoanus</i>	JCM 2896 ^T	<i>M. sinensis</i>	—

T: type strain.

Abbreviations of culture collections are shown in Table 1.

Table 3. The presence or absence of xylose in acid hydrolysates of the whole cells of *Itersonilia perplexans*.

Species	Strain	Other designation or source	Xylose in the cells
<i>I. perplexans</i>	Y-1377 ^a	CBS 363.85	+
	Y-1422 ^b	CBS 363.85	+
	Y-1421 ^b	CBS 144.68	+

^a Yeast form.^b Filamentous form.

Abbreviations of culture collections are shown in Table 1.

more than other sugars. Secondly, to determine without derivatization the kinds of sugar alcohols in the whole cell hydrolysates, we adopted HPLC columns which allow distinct separation of sugar alcohols as well as neutral sugars, while the GLC method using trimethylsilyl derivatization does not distinguish pentitols from each other (1, 4).

High performance liquid chromatographic elution profiles of whole cell hydrolysates from representative strains are shown in Figs. 1 and 2. On the Shodex SP1010 column, xylose can be distinctly separated from other sugars and sugar alcohols, although xylose can not be completely separated from glucose. Also, on the Shodex RS pak DC-613 column, xylose can be separated from the other sugars and sugar alcohols although the retention time of xylose is close to that of ribose (xylose, 7.63 min; ribose, 7.96 min). With the chromatograms of these two columns, the presence or absence of xylose can be determined.

In sugar alcohols, a small amount of xylitol, in addition to glycerol and mannitol, was detected in the whole cell hydrolysates of many of the strains used in the present study. WEIJMAN et al. (4) detected pentitol in the whole cell hydrolysates of *Sporidiobolus* and *Sporobolomyces* strains. They assumed it was arabitol, though the trimethylsilyl derivatives of pentitols could not be separated from each other by their GLC method. However, arabitol was not detected by our HPLC method. On the other hand, the presence of xylitol in the whole cell hydrolysates may affect the identification of xylose by the GLC method of SUGIYAMA et al. (7), because with their GLC method it is impossible to distinguish the xylitol present in the hydrolysates before derivatization from the xylitol converted from xylose by the reduction of the hydrolysates.

The distribution of xylose in strains of Bullera species

Table 1 shows the presence or absence of xylose in whole cell hydrolysates of the type strains of 16 species of the genus *Bullera*.

The type strains of *Bullera alba* (9), *Bullera armeniaca* (10), *Bullera aurantiaca* (11), *Bullera dendrophila* (9), and *Bullera globospora* (11) contained xylose in the cells, as reported by VON ARX and WEIJMAN (3) and by WEIJMAN and RODRIGUES DE MIRANDA (5) who used the GLC method. Type strains of *Bullera crocea* (10), *Bullera derxii* (12), *Bullera oryzae* (13), *Bullera pseudoalba* (12), and *Bullera sinensis* (14) also contained xylose in the cells. Only in *Bullera crocea*, was xylose not detected on the Shodex SP1010 column because of a large amount of glucose. But it was clearly detected on the Shodex RS pak DC-613 column. Type strains of *Bullera megalospora* (15) and *Bullera piricola* (9), which produce large ballistospores with rotationally symmetrical and bilaterally symmetrical shapes, had xylose in the cells. The type strain of *Bullera punicea* (15), with large bilaterally symmetrical ballistospores, also contained xylose in the cells. *Bullera punicea* was listed as *Sporobolomyces puniceus* in the 3rd edition of The Yeasts: A Taxonomic Study (16). Later, we transferred it to the genus *Bullera* (15) as a new combination based on the data of WEIJMAN and RODRIGUES DE MIRANDA (5). We also confirmed the presence

Fig. 1.

Fig. 1. High performance liquid chromatographic profiles of whole cell hydrolysates from *Bullera pseudoalba* JCM 5290 and *Sporobolomyces yamatoanus* JCM 2896, using a Shodex SP1010 column.

A, *Bullera pseudoalba* JCM 5290; B, *Sporobolomyces yamatoanus* JCM 2896. Peaks: 1, glucose; 2, xylose; 3, galactose; 4, mannose; 5, glycerol; 6, mannitol; 7, ribose and xylitol.

Fig. 2. High performance liquid chromatographic profiles of whole cell hydrolysates from *Bullera pseudoalba* JCM 5290 and *Sporobolomyces yamatoanus* JCM 2896, using a Shodex DC-613 (Na) column.

A, *Bullera pseudoalba* JCM 5290; B, *Sporobolomyces yamatoanus* JCM 2896. Peaks: 1, glycerol; 2, xylose; 3, ribose; 4, xylitol; 5, glucose, mannose, galactose, and mannitol; 6 and 7, unknown.

Fig. 2.

of xylose in the cells of *B. punicea* by the HPLC method used in this study. The type strain of *Bullera variabilis*, which produces uniquely-shaped ballistospores (17), contained xylose in the cells. Exceptionally, in *Bullera salicina* (11) and *Bullera tsugae* (9), xylose was not detected by the HPLC method in this study. WEIJMAN and

RODRIGUES DE MIRANDA (5) reported that the peak of xylose was insignificant because of the chromatographic noise level in these two species. Therefore, it is obvious that these two species do not contain xylose in the cells. Of the *Bullera* species listed in the 3rd edition of *The Yeasts: A Taxonomic Study* (9), *Bullera grandispora* could not be studied because no authentic cultures are available.

The distribution of xylose in strains of Sporidiobolus and Sporobolomyces species

Table 2 shows the presence or absence of xylose in whole cell hydrolysates of the type strains of 25 species of the genera *Sporidiobolus* and *Sporobolomyces*.

The type strains of *Sporidiobolus johnsonii* (18), *Sporidiobolus pararoseus* (18), *Sporidiobolus ruinenii* (18), and *Sporidiobolus salmonicolor* (18) did not have xylose in the cells. These results agree with the data of VON ARX and WEIJMAN (3) and WEIJMAN and RODRIGUES DE MIRANDA (5) obtained by the GLC method. On the contrary, by an alternative GLC method, SUGIYAMA et al. (7) found that the type strain of *Sporidiobolus salmonicolor* contained xylose in the cells. With the HPLC method, however, we found that xylitol was present in the whole cell hydrolysates of that strain (data not shown). This finding shows the possibility that SUGIYAMA et al. (7) detected the xylitol present in the hydrolysates but not the xylitol converted from xylose by the reduction of the hydrolysates in that strain.

The type strains of five *Sporobolomyces* species, *Sp. albo-rubescence* (16), *Sp. foliicola* (19), *Sp. gracilis* (16), *Sp. holsaticus* (16), and *Sp. roseus* (16), did not contain xylose in the cells as reported by WEIJMAN and RODRIGUES DE MIRANDA (5). Although they showed that the peak of xylose was insignificant in *Sp. gracilis* because of a chromatographic noise level, the lack of xylose in the cells of this species was clearly demonstrated by our HPLC method. The type strains of *Sporobolomyces oryzipicola*, *Sporobolomyces sasicola*, and *Sporobolomyces subbrunneus*, which were described by us (13, 20, 21), did not have xylose in their cells. The type strain of *Sporobolomyces kluyveri-nielii* described recently by VAN DER WALT et al. (22) did not have xylose in the cells. All of the above species except *Sp. sasicola* are typical species of the genus *Sporobolomyces*, whose strains produce bilaterally symmetrical ballistospores and pink to red colonies and have Q-10 as the major ubiquinone. Although the type strain of *Sp. sasicola* produces pale colored colonies, it is considered to be closely related to typical, deeply pigmented species equipped with Q-10 as the major ubiquinone in the genus *Sporobolomyces* (21).

The type strain of *Sporobolomyces elongatus* (19) equipped with Q-10 (H₂) as the major ubiquinone (23) did not contain xylose in the cells, in agreement with the report of WEIJMAN and RODRIGUES DE MIRANDA (5).

Type strains of four species, *Sporobolomyces singularis* (24), *Sporobolomyces griseoflavus* (21), *Sporobolomyces inositolophilus* (25), and *Sporobolomyces falcatus* (26), which we called the "singularis group" (21, 26), did not have xylose in the cells. This group is comprised of the atypical *Sporobolomyces* yeasts that produce bilaterally symmetrical ballistospores, have Q-10 as the major ubiquinone, produce pale-colored colonies, and can assimilate 2-ketogluconic acid, 5-ketogluconic acid,

D-glucuronic acid and D-galacturonic acid as the sole carbon source (26). The lack of xylose in the cells of *Sp. singularis* has been reported by WEIJMAN and RODRIGUES DE MIRANDA (5), and was reconfirmed by HPLC method in this study.

The type strains of seven species, *Sporobolomyces intermedius* (20, 27), *Sporobolomyces miscanthi* (27), *Sporobolomyces naganoensis* (28), *Sporobolomyces subroseus* (27), *Sporobolomyces weijmanii* (27), *Sporobolomyces yuccicola* (29), and *Sporobolomyces yamatoanus* (30), which we called the "intermedius group" in the genus *Sporobolomyces* (28), did not contain xylose in the cells. Strains of the species of this group have Q-9 as the major ubiquinone and produce pale-colored, pale pink or pale orange colonies. The lack of xylose in whole cell hydrolysates of three strains of *Sp. intermedius*, *Sp. weijmanii*, and *Sp. yamatoanus* was also confirmed by the GLC method (A. C. M. WEIJMAN, personal communication). It is obvious that these seven species are not yeast phases of certain species of the genus *Itersonilia* with Q-9 as the major ubiquinone (31), because xylose was present in the whole cells of both the yeast and filamentous forms of *Itersonilia perplexans* as shown in Table 3.

The delimitation between genera Bullera and Sporobolomyces

Since the establishment of the genus *Bullera* by DERX (32), the genera *Bullera* and *Sporobolomyces* have been discriminated from each other by the shape of ballistospore and the color of colonies: The former produces pale colored colonies and symmetrical ballistospores, the latter produces pink to red colonies and asymmetrical ballistospores. However, some modifications of the delimitation by these characteristics has been made as follows. PHAFF and DO CARMO-SOUSA (24) emended the definition of the genus *Sporobolomyces* by including *Sp. singularis* which produces pale colored colonies. STADELMANN (33) emended the definition of the genus *Bullera* by including *B. piricola* which produces asymmetrical ballistospores as well as symmetrical ones. In *The Yeasts, a Taxonomic Study*, 3rd edition, RODRIGUES DE MIRANDA (9) and FELL and STATZELL TALLMAN (16) stated that *Bullera* species usually produce rotationally symmetrical ballistospores and cream colored to slightly yellowish or brownish colonies whereas *Sporobolomyces* species usually produce bilaterally symmetrical ballistospores and pink, red and orange colonies. The former author placed *Sp. singularis* in the genus *Bullera* even though it produces bilaterally symmetrical ballistospores. Furthermore, BUHAGIAR et al. (10) and JOHRI and BANDONI (11) recently described several *Bullera* species producing deep-orange colored colonies. So the boundary between the genera *Bullera* and *Sporobolomyces* has become more ambiguous and new reliable criteria are needed. As shown above, the data of WEIJMAN and RODRIGUES DE MIRANDA (5) and our data obtained in this expanded study strongly suggest that the presence or absence of xylose in the cells may be an important criterion for generic differentiation between *Bullera* and *Sporobolomyces*. Since *Bullera salicina* and *Bullera tsugae* do not contain xylose in the cells, these species may be more closely related to the genus *Sporobolomyces* than *Bullera*.

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