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Isolation and Screening of Yeasts That Ferment D-Xylose Directly to Ethanol

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Natural habitats of yeasts were examined for the presence of strains able to produce ethanol from D-xylose. Black knots, insect frass, and tree exudates were screened by enrichment in liquid D-xylose–yeast extract medium. These and each D-xylose-assimilating yeast in a collection from cactus fruits and *Drosophila* spp. were tested for alcohol production from this sugar. Among the 412 isolates examined, 36 produced more than 1 g of ethanol liter^{-1} from 20 g of D-xylose liter^{-1}, all under aerated conditions. Closer examination of the strains indicated that their time courses of D-xylose fermentation followed different patterns. Some strains produced more biomass than ethanol, and among these, ethanol may or may not be assimilated rapidly after depletion of D-xylose. Others produced more ethanol than biomass, but all catabolized ethanol after carbohydrate exhaustion. Ethanol production appeared best at low pH values and under mild aeration. Possible correlations between the nutritional profiles of the yeasts and their ability to produce ethanol from D-xylose were explored by multivariate analysis. D-Xylose appeared slightly better utilized by yeasts which rate poorly in terms of fermentation. The fermentation of D-glucose had no bearing on D-xylose fermentation. No specific nutritional trait could discriminate well between better D-xylose fermentors and other yeasts.

Considerable interest in the ability of certain yeasts to ferment the pentose sugar D-xylose stems from the abundance of this sugar in waste materials of the wood and paper industries. Screenings of D-xylose–fermenting yeasts have been limited largely to culture collections (11), and little knowledge exists on the ecological significance of D-xylose fermentation. In addition, ethanol yields from D-xylose have been low. Xylose fermentation may be associated with poor tolerance to ethanol, although it is not known whether this is coincidental or biochemically significant. A small number of yeasts have been candidates as potential industrial strains (3, 9). Fein and co-workers (4) have examined many such strains for their ability to produce ethanol from D-xylose in wood hydrolysates, and they observed a significant reduction in ethanol yields compared to the fermentation of purified D-xylose. For these reasons, it is of interest to determine whether natural plant substrates harbor yeasts actually capable of fermenting D-xylose efficiently. In this paper, we report a study of the presence of D-xylose–fermenting yeasts in natural substrates likely to contain D-xylose.

MATERIALS AND METHODS

Isolation of yeasts. Before inoculation, black knots (fungal stromata found on branches of cherry trees) were surface sterilized by flame and sectioned to expose granular material. The black knots were collected at Pinery Provincial Park, Ontario, Canada, and on the banks of the Thames River in London, Ontario. Insect frass and tree exudates were collected in the same localities in sterile culture tubes. Several tree species were included, among which oaks were predominant. The samples were then inoculated in broth containing 2% D-xylose and 0.5% yeast extract (pH 3.7), and the tubes were incubated at 25°C with mild agitation in a tissue culture Rollordrum (New Brunswick Scientific). When growth was detected (turbidity), a loopful of yeast suspension was streak inoculated onto a plate of same medium solidified with 2% agar. Colonies of each type were then picked for characterization.

Yeasts from a collection made during ecological studies of cactus fruits and associated *Drosophila* flies in the Bahamas and Caribbean Islands (H. J. Phaff, W. T. Starmer, and M. A. Lachance, unpublished data) were spot inoculated onto plates of D-xylose–yeast extract agar. Isolates exhibiting weak to strong growth were selected for further study.

Yeast identification was done by the standard methods proposed by van der Walt (12). Nutritional responses were evaluated by replica plating.

Assessment of ethanol production from D-xylose. Pure cultures were inoculated into D-xylose–yeast extract broth and incubated as described above. Samples were withdrawn for analysis after 72 and 96 h. More detailed growth data were obtained with shake-flask cultures (200 ml of medium in 500-ml flasks) incubated on a gyratory shaker at 150 rpm.

Analytical methods. Cell density was measured turbidimetrically at 600 nm. Ethanol concentration was determined by gas-liquid chromatography (Perkin-Elmer F11, flame ionization detection) with a stainless steel column (60 by 0.2 cm) of Chromosorb 107 (Johns-Manville, 80 to 100 mesh) at 165°C with nitrogen as carrier gas. Peak integration (Hewlett-Packard 3392A) was calibrated with known ethanol solutions. The coefficient of variation (unbiased estimate) on replicate ethanol determinations was 4.0% or less. D-Xylose was measured by the 2-5 dinitrosalicylic acid reaction (10).

Data analysis. Centroid principal components analysis was performed with Fortran 77 program DA (M. A. Lachance, unpublished data). Eigenvectors of a correlation matrix of the data pooled by groups are multiplied with the data for each individual, producing coordinates which represent all individuals relative to their centroids. The method forces principal component axes to maximize intergroup discrimination. Otherwise, its algorithm is identical to that used for standard principal components analysis (2).

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TABLE 1. Fermentation of D-xylose by yeasts associated with tree injuries[a]

Source	Taxonomic designation	Ethanol produced	
		<1 g liter^{-1}	>1 g liter^{-1}
Black knots	Unidentified	70	0
	A. pullulans	2	6
	Candida sp. strain 83-17	0	1
	Candida sp. strain 83-19	0	1
Insect frass	Unidentified	26	0
	Candida sp. strain 83-3	0	1
Exudates	Unidentified	57	0

[a] The numbers of different isolates are shown for each category.

RESULTS AND DISCUSSION

Injured trees. Sites of tree injury caused by fungi or insects normally contain an indigenous yeast flora. Direct screening by enrichment in D-xylose broth yielded 164 isolates able to assimilate this pentose sugar (Table 1). Only nine isolates, most of which were representatives of the yeast-like fungus *Aureobasidium pullulans*, were found to produce significant amounts of ethanol under conditions of mild aeration. This form of metabolism must be considered of minor consequence in the survival of those yeasts in their natural habitats. None were found to produce gas anaerobically from D-xylose. Although the number of yeasts unable to utilize D-xylose for growth was not determined in this case, it is known that yeasts from black knots (7) and from insect frass (8) usually assimilate D-xylose. By contrast, yeasts from tree exudates show a lesser utilization of this sugar (1).

Cactus tissue. The flesh of cacti contains a number of carbohydrates (6) including D-xylose, although the majority

TABLE 2. Fermentation of D-xylose by yeasts associated with cactus fruit and *Drosophila* spp.[a]

Taxonomic designation	Ethanol produced	
	<1 g liter^{-1}	>1 g liter^{-1}
A. pullulans	0	2
Candida famata	1	2
C. guilliermondii	7	5
C. maltosa	0	2
C. parapsilosis	1	1
C. rugosa	1	0
C. sonorensis	28	2
Candida sp. strain "JAM"[b]	6	0
Candida sp. strain "NAG"[b]	21	0
Citeromyces matritensis	1	0
"*Clavispora opuntiae*" complex[b]	40	8
Cryptococcus curvatus	1	0
Geotrichum sp.	0	4
Issatchenkia terricola	10	0
Kluyveromyces marxianus	1	0
Kluyveromyces thermotolerans	2	0
Kloeckera apiculata	5	0
Kloeckera apis	44	0
"*Pichia amethionina* var. *fermentans*"[b]	8	0
"*P. barkeri*"[b]	13	0
P. cactophila	21	1
"*P. deserticola*"[b]	2	0
P. norvegensis	13	0
P. membranaefaciens	3	0
P. mexicana complex[b]	4	0
Sporopachydermia cereana complex[b]	6	2
Torulaspora delbrueckii	3	0

[a] The numbers of different isolates are shown for each category. Quotes indicate provisional designations.
[b] Uncertain taxonomic status

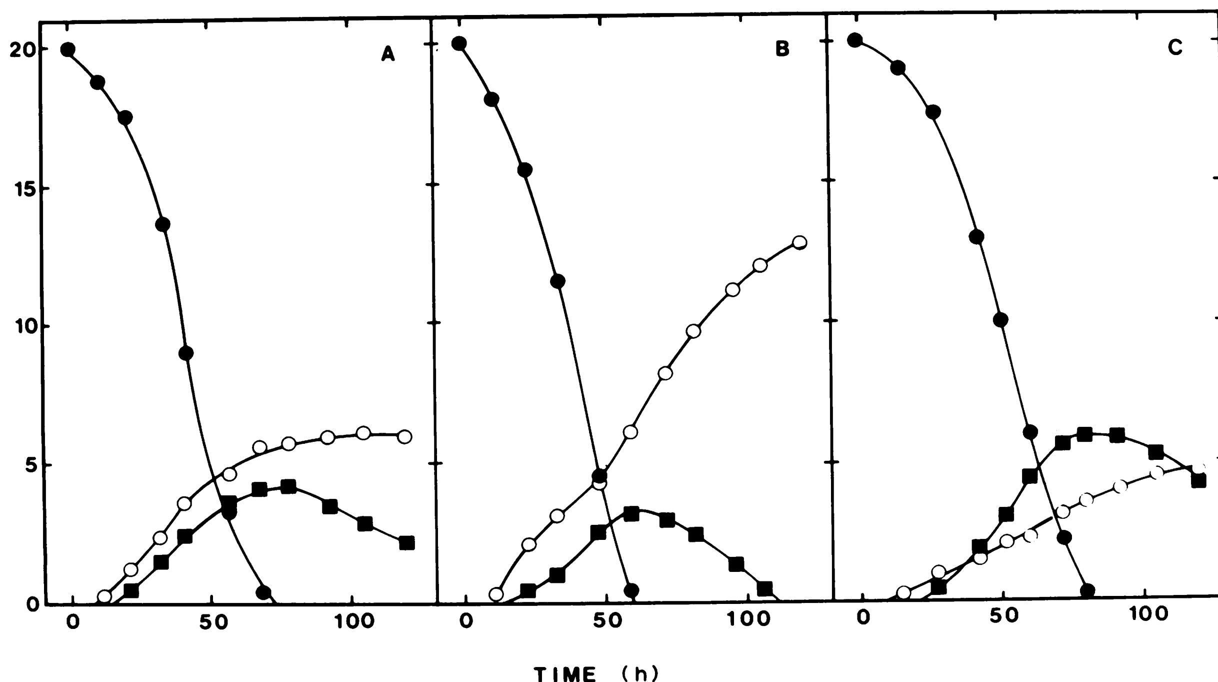

FIG. 1. Batch growth and ethanol production by *A. pullulans* UWO(PS)83-11 (A), *C. famata* UWO(PS)83-844-3 (B), and *Clavispora* sp. strain UWO(PS)83-877-1 (C). Symbols: ●, D-xylose concentration (g liter^{-1}); ○, biomass concentration (g [dry weight] liter^{-1}); ■, ethanol concentration (g liter^{-1}).

TABLE 3. Comparison of D-xylose fermentation by selected yeast strains[a]

Strain	Max ethanol concn (g liter^{-1})	Max ethanol yield coefficient (g g^{-1})	pH	Temp (°C)	% Theoretical yield[b]
Clavispora sp. strain 83-877-1[c]	5.9	0.29	3.0	25	57.5
Candida sp. strain 83-3[c]	5.8	0.29	3.5	25	56.8
Candida sp. strain 83-17[d]	4.6	0.23	4.5	26	45.1
A. pullulans[d,e]	4.2	0.21	4.5	27	41.6
A. pullulans 83-11[c,f]	4.1	0.20	4.5	27	39.9
C. famata 83-844-3[c]	3.9	0.20	4.5	25	39.0
Candida sp. strain 83-19[c]	2.4	0.12	4.5	27	23.5

[a] Initial D-xylose concentration was 20 g liter^{-1}
[b] Assumed theoretical value (ethanol/D-xylose) = 0.51 g g^{-1}.
[c] 100-ml culture in 250 shake flask, 100 rpm.
[d] 9-ml culture in 16-mm rolled tube, 100 rpm.
[e] Cactus isolate.
[f] Black knot isolate.

of yeasts isolated from cacti and associated habitats have a very low degree of nutritional breadth (5). Of the 262 yeasts listed in Table 2, many failed to utilize D-xylose when tested under standard conditions (12), but they exhibited a small amount of growth on D-xylose–yeast extract agar. Only yeasts clearly able to grow aerobically on D-xylose were able to produce ethanol from that sugar. Of these, 29 produced more than 1 g of ethanol liter^{-1} from 2% D-xylose.

Time course of D-xylose fermentation. The modalities of ethanol production differed from species to species. Three major types of time courses were observed (Fig. 1). In *Aureobasidium pullulans* (Fig. 1A) D-xylose utilization resulted more in biomass than in ethanol production, and on exhaustion of the carbohydrate, ethanol decreased slowly. Little additional cell growth occurred after D-xylose was depleted. It is not quite clear whether the decrease in ethanol concentration was due to its catabolism by the organism or to evaporation.

Biomass greatly exceeded ethanol in *Candida famata* (Fig. 1B), although in this case growth on ethanol proceeded, after a slight lag, at a rate comparable to the rate observed with D-xylose as the substrate. In addition, the growth of *C. famata* after D-xylose depletion exceeded the yield anticipated only from the consumption of ethanol. This pattern was observed in several other yeasts, including *Candida* strains 83-17 and 83-19 (Table 1).

At the other extreme, strain 83-877-1 of an undescribed species of *Clavispora* produced more ethanol than biomass (Fig. 1C). After depletion of D-xylose, ethanol was utilized for growth. *Candida* sp. strain 83-3 (tentatively designated as *Pichia vini* by Bowles and Lachance [1]) fell in the latter category as well.

Fermentation parameters of selected strains are shown in Table 3. In all cases, fermentation was studied in media adjusted to various initial pH values. Ethanol production appeared best at low pH values. None of the yeasts were able to ferment D-xylose anaerobically, and ethanol yields were highest under mild agitation. In all cases, a significant proportion of the carbohydrate consumed was not accounted for by biomass and ethanol. The microorganisms therefore must produce other metabolites, such as xylitol (4). Each of the yeasts above assimilated xylitol slowly but extensively under standard conditions (12). Strains 83-3 and 83-877-1 were the most promising in terms of potential applications, and they were retained for further studies.

Yeast nutrition and D-xylose fermentation. It was of interest to examine the physiological profiles of the yeasts in this study to determine whether any of their properties could be correlated with D-xylose fermentation abilities. This might provide some insight for designing methods of isolation based on enrichment procedures.

In the course of identification of the isolates listed in Tables 2 and 3, the responses to 68 nutritional characteristics were determined. The responses for each taxon were analyzed by centroid principal components analysis, a method which reveals combinations of correlated characters which best separate groups of individuals. The yeasts were partitioned into groups according to whether they rated "negative" (less than 1 g of ethanol liter^{-1}), "poor" (more than 1 g but less than 4 g of ethanol liter^{-1}), or "good" (4 g liter^{-1} or more of ethanol). The results are shown in Fig. 2. The first component, which summarizes 65% of the intergroup variation, polarizes the good and poor D-xylose-fermenting strains against those which gave less than 1 g of ethanol liter^{-1} from 20 g of D-xylose liter^{-1}, while the second axis (35% of variation) opposes yeast species rated poor to all others. Note that no more than two components may be generated when the yeasts are pooled into three groups.

The taxonomic growth responses correlated significantly (α = 0.01) with the first axis are listed in Table 4. Pentose utilization, not surprisingly, correlates well with the first component, but so do a large number of other growth

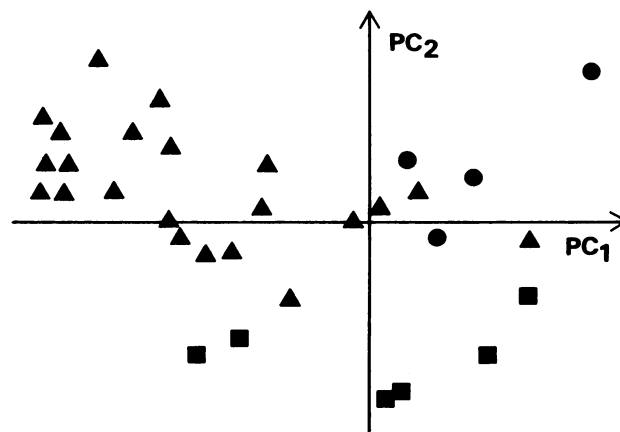

FIG. 2. Ordination of various yeast species by centroid principal components analysis. Symbols: ●, good; ■, poor; and ▲, negative xylose fermentation.

TABLE 4. Correlations between yeast growth responses and the first principal component in Fig. 2[a]

Growth test	Correlation
N-Acetyl glucosamine	0.79
Ribitol	0.75
Citric acid	0.73
D-Xylose	0.72
Trehalose	0.71
2-Keto-gluconate	0.71
Glucitol	0.68
Xylitol	0.67
L-Arabinose	0.65
Cellobiose	0.65
Salicin	0.64
Mannitol	0.63
Galactitol	0.62
L-Sorbose	0.61
Maltose	0.59
Sucrose	0.58
D-Galactose	0.57
Melezitose	0.57
DL-Malate	0.56

[a] Level of significance = 0.01.

responses. Resistance to cycloheximide was the only trait significantly (and negatively) correlated with the second axis. D-Xylose, although not significantly correlated with the second component, appeared better utilized by yeasts which rated poorly than by those which produced higher ethanol yields. The fermentation of D-glucose had no bearing on D-xylose fermentation. No specific set of nutritional traits could discriminate well between the better D-xylose fermentors and other yeasts. The identification of precise properties correlated with D-xylose fermentation would have been desirable in designing subsequent screening programs, but on the basis of the above discussion, such correlations are not yet at hand.

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