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Alcoholic Fermentation of D-Xylose by Yeasts

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Type strains of 200 species of yeasts able to ferment glucose and grow on xylose were screened for fermentation of D-xylose. In most of the strains tested, ethanol production was negligible. Nineteen were found to produce between 0.1 and 1.0 g of ethanol per liter. Strains of the following species produce more than 1 g of ethanol per liter in the fermentation test with 2% xylose: *Brettanomyces naardenensis*, *Candida shehatae*, *Candida tenuis*, *Pachysolen tannophilus*, *Pichia segobiensis*, and *Pichia stipitis*. Subsequent screening of these yeasts for their capacity to ferment D-cellobiose revealed that only *Candida tenuis* CBS 4435 was a good fermenter of both xylose and cellobiose under the test conditions used.

In view of the considerable amount of D-xylose present in hemicellulose and its potential as a substrate for the production of ethanol from biomass, many studies have been performed on the fermentation of this pentose sugar by yeasts (for recent reviews, see references 11, 12, 15, 16). So far, only a few yeasts have been found capable of xylose fermentation. Attention has been focused on *Pachysolen tannophilus* (3, 4, 6, 17, 18) and on a number of *Candida* species (7–10, 13, 14, 19). However, these yeasts appear to have limited value for ethanol production. Under anaerobic conditions, a large fraction of the xylose is converted to xylitol, and the yield of ethanol is correspondingly low. Oxygen is needed for optimal xylose fermentation (5, 7, 18, 19), and even under optimal conditions ethanol is produced at a relatively low rate.

In an attempt to extend and improve the possibilities for fermenting xylose with yeasts, we performed a systematic screening to determine whether there are more yeasts which can ferment xylose than those already reported. Of the 439 yeast species listed by Barnett et al. (1), 317 have some capacity to grow on D-xylose aerobically and 291 can ferment D-glucose. The enzyme systems for both xylose metabolism and alcoholic fermentation are required for the fermentation of D-xylose. We therefore restricted our screening program to those yeasts which can both grow on xylose and ferment glucose. We tested the type strains of this group, which comprises 193 species.

The standard fermentation test in yeast taxonomy is based upon the visual detection of CO_2 production in a Durham tube (20). However, many yeasts which, by this criterion, are "nonfermenting" nevertheless have been found to produce ethanol from glucose (van Dijken et al., Biotechnological research in The Netherlands. Abstracts of poster symposium, p. 169, 1983. Netherlands Biotechnological Society.). For this reason, we tested the yeasts for production of both gas and ethanol.

MATERIALS AND METHODS

Organisms. Type strains of 173 yeast species listed in Barnett et al. (1) as both glucose fermenting and xylose assimilating were examined, as well as those of the following 20 species not listed by Barnett et al.: *Candida auringiensis*, *Candida cariosilignicola*, *Candida hellenica*, *Candida loddderae*, *Candida methanosorba*, *Candida methylica*, *Candida quercitrusa*, *Candida succiphila*, *Clavispora lusitaniae*, *Debaryozyma yamadae*, *Hansenula misumaiensis*, *Mastigomyces philippovi*, *Pichia amylophila*, *Pichia kodamae*, *Pichia mexicana*, *Pichia mississippiensis*, *Pichia segobiensis*, *Pichia tannicola*, *Sporopachydermia quercuum*, and *Williopsis pratensis*. All strains were obtained from Centraalbureau voor Schimmelcultures (CBS), Yeast Division, Delft, The Netherlands.

Media and culture conditions. From slant cultures on 0.5% yeast extract agar with 2% glucose and 1% peptone, the yeasts were inoculated into culture tubes containing 5 ml of a filter-sterilized synthetic medium consisting of yeast nitrogen base (Difco Laboratories) with 2% D-xylose (E. Merck AG) (Wickerham medium [20]). All yeasts had grown after incubation for 7 days at 25°C with shaking. One drop of the culture was then transferred to a Durham tube (20) containing 5 ml of 1% yeast extract with 2% D-xylose (filter sterilized), and one drop was transferred to a Durham tube containing 5 ml of 1% yeast extract which served as a blank. Both Durham tubes were incubated for 10 days at 25°C without shaking and observed for gas formation after 4, 7, and 10 days. Gas formation was expressed as percent gas, indicating the extent to which the inverted vial of the Durham tube was filled with gas. Psychrophilic yeasts were incubated at 10°C for 10 days. At the end of the incubation period, all cultures were centrifuged, and the supernatant liquids were stored at −50°C in sealed vials until being analyzed (within 1 week).

In some cases, yeasts were tested for fermentation of cellobiose. The yeasts were inoculated into Durham tubes containing 5 ml of 1% yeast extract with 2% D-cellobiose (Merck; filter sterilized), which were incubated at 25°C and inspected for gas production for 4, 7, and 10 days, after which the ethanol content of the culture liquid was determined. Psychrophilic yeasts were incubated at 10°C for 10 days.

Determination of ethanol. The amount of ethanol in the medium was determined by gas chromatography with a Varian 3600, equipped with a CDS 111 integrator, on a Porapak (100-120 mesh) column (length, 6 ft [183 cm]; diameter, 2 mm). A temperature program between 170 and 245°C was completed in 5 min as follows. In the first 3 min the

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temperature was raised by 25°C min^{-1} and then was kept constant for the last 2 min. The injector temperature was 230°C, and the temperature of the flame ionization detector was 250°C. Nitrogen was used as the carrier gas at a flow rate of 30 ml min^{-1}. The detector received hydrogen (30 ml min^{-1}) and air (300 ml min^{-1}). Samples of 5 μl were introduced by automatic injection. Each sample was analyzed in duplicate.

RESULTS

Gas formation from xylose. Among more than 200 yeast strains tested, gas formation from D-xylose in the classic Durham tube test was observed after 10 days at 25°C with type strains of the yeasts *Brettanomyces naardenensis* 6042, *Candida shehatae* CBS 5813, *Pachysolen tannophilus* CBS 4044; *Pichia segobiensis* CBS 6857, and *Pichia stipitis* CBS 5773.

Ethanol formation from xylose. In Durham tube blanks containing yeast extract without added sugar, less than 0.05 g of ethanol per liter was detected after a 10-day incubation. In Durham tubes with yeast extract plus 2% D-xylose, as well as in the synthetic Wickerham medium, yeasts produced ethanol in widely varying amounts. A large group, comprising 175 type strains, had formed less than 0.1 g of ethanol per liter. A second group yielded between 0.1 and 1.0 g of ethanol per liter (Table 1).

The third group attained ethanol concentrations of between 1.0 and 6.6 g per liter. This group consisted of the yeasts *B. naardenensis*, *Candida shehatae*, *Pachysolen tannophilus*, *Pichia segobiensis*, and *Pichia stipitis*. Subsequently, all other strains of these species from the CBS culture collection were also tested for D-xylose fermentation. Since in separate studies we had found strong xylose fermentation by a strain of *Candida tenuis*, we included all available strains of this species in further tests. The maximum gas production and ethanol concentration attained in the test system are presented in Table 2.

Cellobiose fermentation. Strains of yeast species comprising good xylose-fermenting strains were subsequently tested for their capacity to ferment D-cellobiose. Of the strains in Table 2, only *Candida tenuis* CBS 2885, 4434, 4435, and 4604 produced more than 1 g of ethanol per liter.

DISCUSSION

From our screening program, a small number of yeasts have emerged as good fermenters of D-xylose (Table 2). Other than the well-known *Pachysolen tannophilus*, there are strains of *B. naardenensis*, *Candida shehatae*, *Candida tenuis*, *Pichia segobiensis*, and *Pichia stipitis*.

It is obvious from our results (Table 2) that gas production in the Durham tube is not a dependable criterion for assessing fermentative capacity in yeasts. This might partly explain why Kurtzman (12), using gas formation as the criterion, found no fermentation of D-xylose in *Pichia stipitis*.

Culturing of the yeasts in the synthetic Wickerham medium with xylose, followed by transfer to yeast extract-xylose medium, may have contributed to the sensitivity of our screening program. Yeasts thus already have developed the enzyme system for xylose metabolism before being transferred to the yeast extract medium. Moreover, since the yeast extract may serve as a substrate for growth, xylose may be largely used for energy production via xylose fermentation. Indeed, in most instances we found higher ethanol concentrations in the yeast extract-xylose medium than in the synthetic medium. Nevertheless, in repeated experiments large variations were found, and these may have resulted from, for instance, disappearance of ethanol by evaporation and metabolism during the 10-day incubation period.

The highest value found in our standard test system was 6.6 g of ethanol per liter, as compared with a maximum theoretical yield of 9 g per liter from 2% xylose. However, a high ethanol concentration obtained in a laboratory test does not necessarily correspond to high ethanol productivity under industrial conditions, and vice versa. But the ability of some yeasts to ferment xylose may have been overlooked by us because ethanol production was low, owing to suboptimal conditions in our tests.

Another possible source of error is that only type strains were included in the screening program. All strains of *Pichia stipitis* and its asporogenous state, *Candida shehatae*, can ferment D-xylose (Table 2). However, in *Candida tenuis* the type strain and seven other strains were found to be negative, whereas three strains of this species were good xylose fermenters. In *B. naardenensis* the type strain happened to give the best results.

In addition to good xylose fermentation by the yeasts presented in Table 2, slight production of ethanol from xylose was observed in a number of other yeasts (Table 1). It may well be that strains other than the type strain of these species, or even non-type strains of species not listed in Table 1, do ferment xylose.

It is striking that many of the yeasts mentioned in Tables 1 and 2 have been isolated from wood-inhabiting insects, decaying wood, or other wood-related sources, viz.: *Candida chilensis*, *Candida entomophila*, *Candida insectamans*, *Candida shehatae*, *Candida succiphila*, *Candida tenuis*, *Metschnikowia bicuspidata*, *Pichia naganishii*, *Pichia stipitis*, *S. quercuum*, and *Wingea robertsii*.

It is plausible that xylose-fermenting yeasts have an ecological niche in such habitats. Kurtzman (12), for similar reasons, tested a group of yeasts composed primarily of taxa from wood-related sources. In further searches for xylose-fermenting yeasts, it seems warranted to focus on wood-inhabiting insects and their larvae or on decaying wood.

Our results confirm the general conclusion that the ability to ferment xylose is not widespread among yeasts. Howev-

TABLE 1. Yeast species of which the type strain produced between 0.1 and 1.0 g of ethanol per liter after 10 days at 25°C in test medium with 2% D-xylose

Species	CBS no.
Candida albicans	562
Candida chilensis	5719
Candida entomophila	6160
Candida insectamans	6033
Candida intermedia	572
Candida lodderae	1924
Candida maltosa	5611
Candida steatolytica	5839
Candida succiphila	8003
Candida torresii	5152
Candida tropicalis	94
Candida viswanathii	4024
Metschnikowia bicuspidata	5575
Metschnikowia zobellii	4821
Pichia guilliermondii	2030
Pichia naganishii	6429
Pichia sargentensis	6342
Sporopachydermia quercuum	8070
Wingea robertsii	2934

TABLE 2. Xylose fermentation by yeast species including one or more strains that had produced over 1.0 g of ethanol per liter after 10 days at 25°C in test medium with 2% D-xylose

Species	CBS no.	% Gas in Durham vial	Ethanol (g/liter)
Brettanomyces naardenensis	6042	5	1.8
	6040	0	0.2
	6041	0	0.6
	6043	0	0.9
	6107	0	0.4
	6108	0	0.5
	6115	0	0.1
	6116	0	0.4
	6117	0	1.1
	6118	0	0.3
	6119	0	0.3
Candida shehatae	5813	100	6.6
	2779	10	3.4
	4286	0	2.6
	4287	0	2.4
	4705	100	6.5
	5712	100	3.7
Candida tenuis	615	0	0.3
	2226	0	0.1
	2308	0	0.3
	2309	0	0.3
	2885	0	0.1
	4113	100	5.2
	4238	0	0.4
	4285	100	5.2
	4434	0	0.1
	4435	100	6.4
	4604	0	0.2
Pachysolen tannophilus	4044	100	2.1
	4045	100	2.0
Pichia segobiensis	6857	100	5.0
Pichia stipitis	5773	100	5.9
	5774	100	3.4
	5775	100	2.2
	5776	100	3.7
	6054	100	4.5

er, apart from the intensively explored *Pachysolen tannophilus*, a few other yeasts emerge from our screenings as potential candidates for industrial fermentation of wood sugar. Indeed, *Candida shehatae* and *Pichia stipitis* have suitable characteristics (7; P. M. Bruinenberg, P. H. M. de Bot, J. P. van Dijken, and W. A. Scheffers, Appl. Microbiol. Biotechnol., in press).

The rarity of yeasts with the ability to ferment xylose must be ascribed to peculiarities in the initial steps involved in the conversion of this pentose. As shown by Bruinenberg et al. (2), anaerobic fermentation of D-xylose would only be possible in yeasts in which xylose reductase and xylitol dehydrogenase can function with the same coenzyme system. We have found that the good xylose fermenters listed in Table 2 fulfil this condition. In contrast to representatives of yeasts not able to metabolize xylose anaerobically, these organisms in addition to NADPH-linked xylose reductase also possess NADH-linked xylose reductase (Bruinenberg et al., in press).

The capacity of yeasts to ferment other sugars besides xylose present in wood and plant waste material might be of practical importance (14). We found *Candida tenuis* CBS 4435 to be a good fermenter of both D-xylose and D-cellobiose. Further studies should establish whether both sugars are utilized simultaneously or consecutively by *Candida tenuis* CBS 4435.

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