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COMPARATIVE EVALUATION OF ETHANOL PRODUCTION BY XYLOSE-FERMENTING YEASTS PRESENTED HIGH XYLOSE CONCENTRATIONS

P. J. Slininger[1]*, R. J. Bothast[1], M. R. Okos[2], and M. R. Ladisch[2]

[1]Northern Regional Research Center
Agricultural Research Service†
U.S. Department of Agriculture
Peoria, Illinois 61604

[2]Laboratory of Renewable Resources Engineering
Purdue University
West Lafayette, Indiana 47907

SUMMARY

Three strains of Pichia stipitis and three of Candida shehatae were compared with Pachysolen tannophilus in their abilities to ferment xylose at concentrations as high as 200 g/L when subjected to both aerobic and microaerophilic conditions. Evaluations based on accumulated ethanol concentrations, ethanol productivities, xylose consumption, and ethanol and xylitol yields were determined from batch culture time courses. Of the strains considered, P. stipitis NRRL Y-7124 seemed most promising since it was able to utilize all but 7 g/L of 150 g/L xylose supplied aerobically to produce 52 g/L ethanol at a yield of 0.39 g per gram xylose (76% of theoretical yield) and at a rate comparable to the fastest shown by C. shehatae NRRL Y-12878. For all strains tested, fermentation results from aerobic cultures were more favorable than those from microaerophilic cultures.

INTRODUCTION

Since initial reports of the unique ability of the yeast Pachysolen tannophilus to produce ethanol from xylose (Slininger et al., 1982; Schneider et al., 1981), a number of other yeast strains have been reported to undergo an ethanolic xylose fermentation. Based on preliminary tests at relatively low xylose concentrations, strains of Candida shehatae and of Pichia stipitis displayed particularly high potential for faster, more efficient conversions (du Preez et al., 1983, 1984; Toivola et al., 1984; Bruinenberg et al., 1984). The objective in this study was to supply representative strains with high xylose concentrations so a comparative evaluation could be made under standardized fermentation conditions. Recognizing a target value of 40 g/L ethanol as the minimum

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concentration required for an economically feasible product separation and recovery process (Ladisch *et al.*, 1979), the concentration of ethanol accumulated was a key point of interest.

MATERIALS AND METHODS

Organisms and growth medium. Stock cultures of *Pachysolen tannophilus* NRRL Y-2460 (CBS 4044), *Pichia stipitis* NRRL Y-7124, Y-11544, Y-11545 (CBS 5773, 5776, 6054, respectively) and *Candida shehatae* NRRL Y-12856, Y-12857, Y-12878 (CBS 4705, 5712, 2779, respectively) were obtained from the Northern Regional Research Center, ARS Culture Collection, Peoria, Illinois. All strains were maintained on the agar slants and cultivated in the growth medium described by Slininger *et al.*, (1982). *P. tannophilus* was kept at 32°C, and the others, at 25°C.

Experimental conditions. Inocula were prepared by transferring organisms by loop from 48-hour slants to 125-mL Erlenmeyer flasks containing 50 mL of the growth medium (50 g/L xylose, pH 4.5) and were incubated aerobically at 32°C (*P. tannophilus*) or 25°C (others) on a rotary shaker at 150 rpm for 48 h prior to use. All 400-mL experimental batch cultures were inoculated to an optical density of 0.1 at 620 nm and incubated in 500-mL flasks under the same conditions as inocula. Initial xylose concentration was varied in the range 50-200 g/L. Cultures referred to as "aerobic" were closed with Bellco silicon sponge caps while those referred to as "microaerophilic" had Bellco caps replaced after 72 h with Alwood valves containing concentrated sulfuric acid to block further air transfer.

Optical denisty measurements. Optical densities (A) were measured at 620 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer. Dry cell mass concentration (b) was calculated using the relationship $b = kA$ where k was assumed to be 0.136 g/L for all strains studied even though calibration was based on *P. tannophilus* cells.

Ethanol, xylitol, and xylose analyses. These were measured as described by Bolen and Slininger (1984). Ethanol concentrations were measured by the HPLC method for aerobic cultures but by the GC method for microaerophilic cultures.

On the average, HPLC and GC methods gave comparable values, but the precision was ± 4 g/L for HPLC data compared to ± 2 g/L for GC. The lower precision of aerobic data (HPLC) compared to microaerophilic data (GC) was compensated for by a four-fold increase in the number of samples taken during aerobic experiments.

RESULTS AND DISCUSSION

Time courses of optical density, ethanol, xylitol, and xylose concentrations were monitored in duplicate batch cultures representing each strain and test condition. Once ethanol production began, ethanol concentrations were linearly correlated with time at confidence levels exceeding 95% in all cases. Consequently average volumetric ethanol productivities (g/L/h) were calculated by linear regression analysis of ethanol concentration versus time data. The average dry cell mass concentration over the ethanol evolution period was taken to be the ratio of the area under the corresponding portion of the cell mass time course to the time interval and was computed by numerical integration of

the data. Volumetric productivities are proportional to the size of yeast population present and are likely to be higher for strains capable of rapid, abundant growth. Ethanol production rates normalized on a dry cell mass basis were referred to as "specific" productivities and were computed from the ratio of volumetric productivity to average dry cell mass concentration. Microaerophilic productivities were based only on data obtained after the Alwood valve was attached. Due to approximation of k, specific productivities calculated are not exact absolute values. Nevertheless, within each strain k is invariant, so comparisons of each strain's relative specific productivities at various sugar concentrations and aeration states are valid. Our volumetric rather than specific productivities should be relied upon for distinguishing between fermentation rates of different strains, however.

Given an abundance of oxygen, the yeasts screened consumed ethanol as a substrate for respiration. Consequently, ethanol concentration progressed through an optimum value in many of the batch time courses, and the ethanol yield was taken to be the ratio of the peak ethanol concentration to the xylose concentration consumed at the corresponding time. The residual xylose concentration was not necessarily zero when ethanol peaked. The xylitol concentration occurring with the peak ethanol concentration was similarly used in calculating xylitol yield. The byproduct xylitol accumulated to varying degrees depending on strain and aeration conditions. Details on xylitol's role in the metabolic scheme have been presented by Bruinenberg et al. (1984).

Table 1 summarizes the performance of each yeast tested aerobically at sugar concentrations from 50 to 200 g/L. None of the strains tested utilized all of the xylose present if supplied at initial concentrations ≥ 150 g/L; only Y-11545 and Y-12878 completely consumed initial concentrations of 100 g/L; but all strains except Y-12857 readily consumed 50 g/L xylose. For all strains tested, productivities declined with sugar concentrations in excess of 100 g/L. P. stipitis Y-7124 and C. shehatae Y-12878 showed superior ethanol volumetric and specific productivities, ca. 0.40 g/L/h and 0.22 g/g/h, respectively. Only P. tannophilus and C. shehatae Y-12878 had xylitol yields large enough to pose significant rivalry to ethanol yields, especially at initial xylose ≥ 100 g/L. Ethanol yields observed for all three strains of P. stipitis seemed insensitive to amount of xylose initially present, but those for strains of C. shehatae and P. tannophilus declined with increasing initial xylose. For example, Y-7124 yields ranged from 0.39 to 0.42, but those of Y-12878, from 0.26 to 0.37 g ethanol per gram xylose consumed. Falling ethanol concentrations that characterized the end of most aerobic batch time courses indicated product consumption to cause significant yield loses. Highest ethanol yields (lowest xylitol yields) were attained by C. shehatae Y-12856 though ethanol accumulation and xylose consumption were relatively poor at initial xylose concentrations ≥ 100 g/L. Product inhibition or a nutrient deficiency may limit its performance. P. stipitis Y-7124 accumulated the greatest quantity of ethanol, up to 57 g/L, which is in excess of the 40 g/L target based on the economy of traditional ethanol distillation methods.

TABLE 1. Aerobic fermentation performances

Yeast	Concentrations (g/L)			Ethanol Productivities		Yields (g/g xylose)	
NRRL Strain	Initial Xylose	Residual Xylose	Peak Ethanol	Average Volumetric (g/L/h)	Average Specific (g/g/h)	Xylitol	Ethanol
P. tannophilus							
Y-2460	50	0	16(146)[a]	0.16	0.076	0.14	0.32
	100	5	24(215)	0.13	0.058	0.24	0.25
	150	7	28(246)	0.10	0.029	0.24	0.20
	200	60	28(246)	0.09	0.036	0.27	0.18
P. stipitis							
Y-7124	50	0	20(97)	0.28	0.17	0	0.41
	100	7	39(139)	0.38	0.23	0.01	0.42
	150	15	52(289)	0.30	0.10	0.02	0.39
	200	55	57(311)	0.21	0.06	0.02	0.39
Y-11544	50	0	20(97)	0.22	0.094	0	0.39
	100	3	33(252)	0.24	0.075	0.05	0.34
	150	55	33(311)	0.081	0.020	0.02	0.34
	200	153	17(311)	0.041	0.013	0.04	0.36
Y-11545	50	0	19(97)	0.24	0.12	0	0.38
	100	0	35(192)	0.27	0.088	0	0.35
	150	30	49(311)	0.17	0.043	0.03	0.41
	200	110	38(311)	0.14	0.043	0.01	0.42
C. Shehatae							
Y-12856	50	0	24(118)	0.29	0.19	0.02	0.45
	100	25	33(125)	0.32	0.16	0.03	0.44
	150	77	30(174)	0.21	0.10	0	0.40
	200	130	26(174)	0.18	0.070	0	0.38
Y-12857	50	3	21(148)	0.14	0.065	0.02	0.44
	100	39	25(167)	0.16	0.069	0.02	0.40
	150	105	17(240)	0.068	0.020	0	0.37
	200	125	16(240)	0.046	0.016	0	0.21
Y-12878	50	0	19(139)	0.18	0.18	0.08	0.37
	100	0	29(145)	0.36	0.22	0.20	0.29
	150	14	37(147)	0.42	0.19	0.24	0.27
	200	46	40(174)	0.31	0.13	0.25	0.26

[a]The hour when the peak occurred following inoculation to 0.014 g/L yeast is in parentheses.

TABLE 2. Microaerophilic fermentation performances on 150 g/L initial xylose

NRRL Strain	Concentrations (g/L) Residual Xylose	Concentrations (g/L) Peak Ethanol	Ethanol Productivities Average Volumetric (g/L/h)	Ethanol Productivities Average Specific (g/g/h)	Yields (g/g xylose) Xylitol	Yields (g/g xylose) Ethanol
P. tannophilus						
Y-2460	47	22(297)[a]	0.086	0.059	0.57	0.21
P. stipitis						
Y-7124	30	45(240)	0.22	0.073	0.13	0.38
Y-11544	100	21(297)	0.055	0.025	0.15	0.43
Y-11545	87	26(264)	0.079	0.034	0.09	0.42
C. shehatae						
Y-12856	79	30(216)	0.12	0.062	0.08	0.42
Y-12857	100	21(240)	0.088	0.052	0.14	0.42
Y-12878	39	34(169)	0.19	0.094	0.27	0.31

[a]The hour when the peak occurred following inoculation to 0.014 g/L yeast is in parentheses.

Table 2 compares microaerophilic fermentations by each yeast initially supplied with 150 g/L xylose. Compared with aerobic fermentations, all strains except C. shehatae Y-12856 and Y-12857 had lower productivities. Xylitol yields increased for all strains, but C. shehatae Y-12856 produced the least. It is interesting that, despite higher xylitol yields, all strains showed equivalent or slightly higher ethanol yields under microaerophilic compared to aerobic conditions. As xylose was exhausted during microaerophilic time courses, ethanol concentration reached a stable plateau and did not pass through the unstable optimum that characterized aerobic runs. Respiration and associated ethanol consumption were minimized by blocking oxygen transfer, but losses of xylose to xylitol were amplified by this treatment. Though the performance of Y-12856 was least affected by the switch to microaerophilic conditions, the performance of strains relative to one another was about the same as under aerobic conditions with P. stipitis Y-7124 accumulating the highest ethanol concentration and exhibiting a superior overall performance.

According to data presented, economically recoverable ethanol concentrations can now be achieved from xylose fermentations though ethanol productivities are still low in comparison to those characteristic of traditional glucose processes. Immobilizing yeast cells in high concentration is a means of overcoming this limitation on continuous ethanol production schemes. Cell immobilization techniques and accompanying reactor designs compatible with the peculiarities of the xylose metabolism need to be developed.

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