Effects of Culture Conditions on the Fermentation of Xylose to Ethanol by *Candida shehatae*

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Summary

This research examined four factors on the fermentation of xylose by *Candida shehatae,* and the following conclusions were reached: (1) A minimal medium is effective for producing ethanol. (2) Peptone and casamino acids stimulate ethanol production. (3) Aeration is important in obtaining good ethanol production rates and yields. (4) The maximal rate of ethanol production is attained around pH 3.2–3.4. Under the best conditions employed, *C. shehatae* ATCC 22984 produced up to 4% (w/v) ethanol from 20% xylose in four days. With 9% xylose, ethanol yields approximating 0.4 g/g xylose were obtained. Keywords: nitrogen, pH, aeration, sugar concentration, peptone, casamino.

INTRODUCTION

Efficient utilization of D–xylose should be an integral part of any process for converting angiosperm lignocellulosic residues to ethanol. Even though it is not yet cheap or commercially abundant, xylose comprises a significant fraction of angiosperm hemicellulose,[1] and xylose is much more readily recovered from xylan than is D-glucose from cellulose.[2] Unfortunately, until recent years no yeasts were known that would ferment xylose to ethanol, and available ethanol-producing, xylose-fermenting bacteria exhibit poor ethanol production and tolerance.[3] Yeasts are generally better suited for ethanol production than most bacteria, and research in the last 4 years has shown that at least 41 yeast species are capable of fermenting xylose.[4-8] In most cases the rates and yields attained are appreciably lower than what might be considered commercially practicable.[9] In this regard, it is useful to compare the xylose fermentation with various glucose fermentations. *Candida shehatae* (=*Pichia stipitis*) has a specific xylose fermentation rate about 2–3 times that attained by the much-studied *Pachysolen tannophilus.*[10,11] The highest reported specific fermentation rate for xylose by *C. shehatae* is 0.28 g ethanol/g cells·h.[10] In

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comparison, *saccharomyces cerevisiae* ferments glucose at a specific rate of 1.78 g/g·h,[12] and *Zymomonas mobilis* is reponed to ferment glucose with a specific rate of as much as 2.5 g/g·h.[13] It is not obvious why such a difference should exist, but it is clear that there is much room for improvement in the xylose fermentation. Some improvement might be realized by optimizing the culture conditions.

Comparisons of culture techniques are less common than comparisons of yeast strains, possibly because of the difficulties involved in reproducing conditions in different laboratories. Even so, their systematic examination is desirable. Therefore, the objective of the research reponed here was to determine the effects of several nutritional and cultural factors of importance to ethanol production by *C. shehatae.* A defined minimal medium commonly used for testing carbon and nitrogen utilization and a defined rich medium commonly used for continuous culture studies were compared in order to test the effects of balanced mineral nutrients. Other factors examined included the effects of nitrogen sources, aeration, pH, and sugar concentration. The effect of nitrogen source and pH were examined because they are known to be important in determining metabolic activity. Aeration and sugar concentrations are also known to be important variables in regulating yeast fermentations. The basal medium and sugar concentration were found to be less important than nitrogen, pH, or aeration.

MATERIALS AND METHODS

Organisms

Candida shehatae ATCC 22984 was obtained from the American Type Culture Collection, Bethesda, Maryland. *C. shehatae* CSIR Y492 was obtained from J. P. van der Walt, Council for Scientific and Industrial Research, Pretoria, Republic of South Africa. Unless otherwise stated, ATCC 22984 was employed.

Media

Two basal media were employed. The defined minimal medium was yeast nitrogen base without ammonium sulfate or amino acids (Difco*). The defined rich medium was compounded after the formula of du Preez and van der Walt.[5] The compositions of these two media arc summarized in Table I.

Three out of the four nitrogen sources were employed at concentrations calculated to yield 1.06 g N/L [equivalent to 5.0 g $(NH_4)_2SO_4$/L)] for each single nitrogen source. These concentrations were as follows: U, 2.27 g/L; NH_4Cl, 4.05 g/L; P (Difco), 6.56 g/L. Vitamin-free casamino acids (Difco) was used at a concentration of 5.0 g/L (0.5 g N/L). The total nitrogen concentrations varied

*The use of a trade name is for convenience only and does not represent the endorsement or promotion of the product by this laboratory or the U.S. government.

TABLE I
Comparison of Yeast Base and Rich Medium[a]

Component	Yeast base (YB)	Rich medium (RM)
Salts (g/L)		
KH_2PO_4	1.0	2.5
$MgSO_4$	0.5	0.245
NaCl	0.1	–
$CaCl_2$	0.1	0.15
Trace elements (mg/L)		
$Al_2(SO_4)_3$	–	0.5
H_3BO_3	0.5	2.0
$CoCl_2$	–	1.09
$CuSO_4$	0.04	1.0
KI	0.1	0.35
$FeCl_3$	0.2	–
$FeSO_4$	–	26.6
$MnSO_4$	0.4	6.25
Na_2MoO_4	0.2	1.11
$ZnSO_4$	0.4	6.18
Vitamins (mg/L)		
d-Biotin	0.002	0.1
Ca pantothenate	0.4	20.0
Folic acid	0.002	–
Inositol	2.0	100.0
Niacin	0.4	5.0
p —— Aminobenzoicacid	0.2	1.0
Pyridoxine HCl	0.4	5.0
Riboflavin	0.2	–
Thiamine HCl	0.4	5.0

[a] All constituents reported as anhydrides

from one experiment to another, so the actual conditions employed for each experiment are indicated in the results section. Abbreviations used in the text are summarized in Table II.

Yeast malt P glucose agar (Difco) was employed for stock cultures and inoculum preparation. Yeast nitrogen base without ammonium sulfate or amino acids solidified with 2% agar and containing either 2% xylose or glucose plus U as a nitrogen source was also used for inoculum preparation.

Media were buffered with 0.05*M* or 0.075*M* citrate plus phosphate to yield the desired pH. Basal media and nitrogen sources were filter sterilized in 10- or 20-fold stock solutions and added to the various flasks in appropriate amounts. Buffer and sugar solutions were sterilized separately by autoclaving.

Cultivation

Cells were cultivated in 50 mL medium in a 125-mL Erlenmeyer flask shaken at 100 rpm and incubated at 32°C. These were the standard conditions used for

TABLE II
Abbreviations Used for Media and Constituents

Abbrerviation	Description
YB	Yeast base without ammonium sulfate or amino acids
RM	Rich medium (after the formulation of ref. 5)
YMPG	Yeast malt peptone glucose
U	Urea, 2.27 g/L
P	Peptone, 6.56 g/L
C	Vitamin-free casamino acids. 5.0 g/L

both inoculum preparation and fermentation tests. Unless otherwise stated, inocula were prepared either by streaking on a YB xylose agar plate for confluent growth and incubating for 24 h or by inoculating cells from a fresh YMPG plate and shaking for 72 h. With liquid-grown cells the same medium was used for both inoculum preparation and fermentation. Cells from a single 72-h-old broth culture were used to inoculate three 50-mL fermentation test flasks. For plate-grown inocula cells from one plate were used to inoculate one 50-mL test flask. When comparing inocula prepared under different conditions or with different strains, the cell suspensions were normalized by dilution to obtain the same initial optical densities in all fermentation test flasks. Each fermentation test condition was examined in triplicate flasks. For anaerobic studies cells were cultivated in 50-mL serum vials containing 33 mL medium that had been exhaustively flushed with N_2 prior to autoclaving.

Analytical

Samples (1.0 mL) were aseptically removed at 24-h intervals and analyzed for ethanol by gas chromatography and for xylose, xylitol, and glycerol by high-performance liquid chromatography.[14] Oxygen transport rates were measured by the sulfite oxidation method.[15] Cell growth was estimated by optical density at 525 nm after diluting samples to an OD range of 0.15–4.5 units. Within this range, 1.0 OD ≈ 0.19 mg dry wt cells/mL. Data for experiments in which pH was of interest were collected in the first 24–48 h to minimize the effects of pH shifts that occurred even in buffered media.

Experimental Design

A multiple variant factorial design was used to examine the effects of aeration and sugar concentration on the specific production rates and yields for ethanol, xylitol, and glycerol (Fig. 1). In this design 11 different aeration rates and three sugar concentrations were studied. For this experiment either 0–24-h data

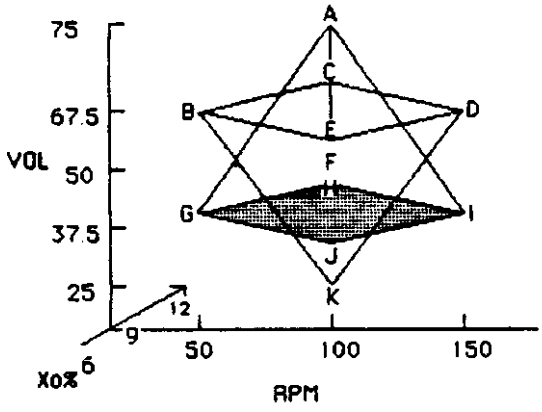

Fig. 1. Multiple variant partial factorial design to examine effects of aeration and sugar concentration.

(conditions *I, J, K*) or 24–48-h data (all other conditions) were used depending on when the sugar was exhausted and when the highest rates were attained. The effects of aeration and sugar were determined by comparing sets of conditions having a single variable–e.g., *A, F,* and *K*; *B* and *D.*

RESULTS

Effects of Nitrogen Sources

The nitrogen source employed affected the course of the medium pH during cultivation. Even in media buffered with 0.1*M* citrate–phosphate,the pH rose 0.7 units in cultures with U and fell 0.3 units in cultures with NH_4Cl as the sole nitrogen sources. Cultures with P, however, maintained a nearly constant pH.

The nitrogen source also affected the product ratios and formation rates. Generally, U and P were better nitrogen sources for ethanol production than was NH_4Cl. Phosphorus not only resulted in better ethanol production but also suppressed xylitol production (Fig. 2).

The effect of the nitrogen source on ethanol production also varied with the aeration rate for the culture. Phosphorus was a good nitrogen source at the standard 100-rpm aeration rate employed here (≈8.5 mmol O_2/L·h). However, at the moderately higher aeration rate provided by 150 rpm agitation, peptone-grown cells did not form as much ethanol as urea-grown cells (Fig. 3). Regardless of the aeration rate used for growth, P-grown cells formed ethanol better under anaerobic conditions than did U-grown cells, although much less ethanol was formed than when aeration was provided.

Results observed with RM and four nitrogen sources were similar to those shown with YB above. RM-P- and RM-U-grown cells produced the most ethanol followed by RM-NH_4Cl-grown cells and finally RM-C-grown cells (Fig. 4a). Cell densities followed patterns similar to those observed with ethanol produc-

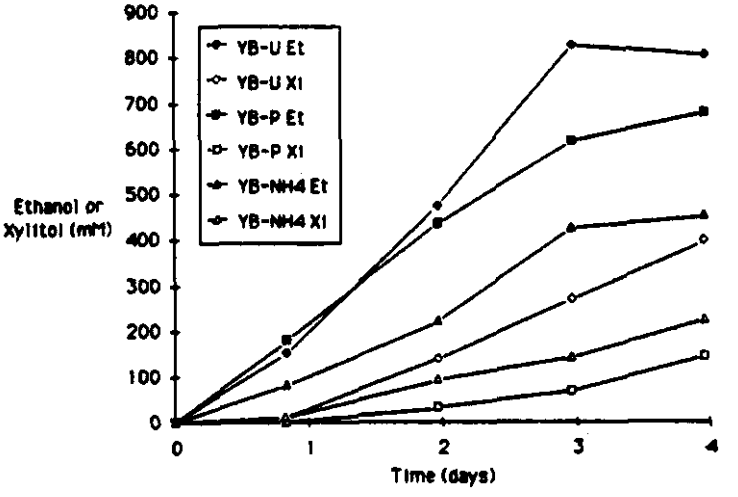

Fig. 2. Comparison of ethanol and xylitol production with three nitrogen sources. Total nitrogen was 1.06 g/L in ease case. Initial xylose = 20% (w/v). YB + 0.05*M* citrate-phosphate buffer, pH 3.5, was used. All other condition were standard. Et = ethanol; Xi = xylitol.

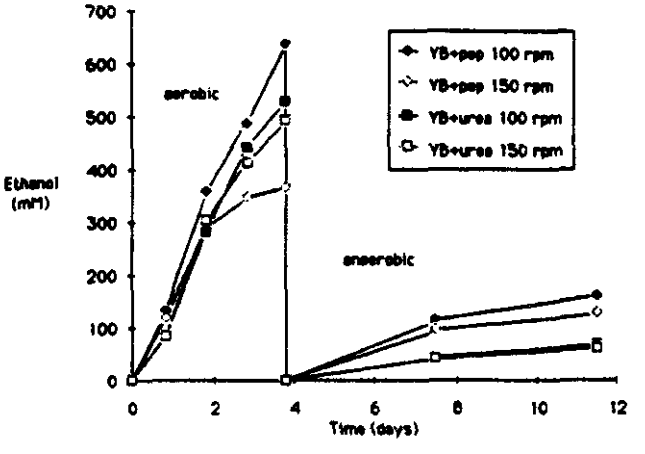

Fig. 3. Effect of aeration on ethanol production with two nitrogen sources. YB was used with either P or U (1.06 g N/L each) under the aeration conditions indicated. Initial xylose = 10% (w/v). No citrate-phosphate buffer was added; initial pH = 4.5. All other conditions were standard.

tion except for C. With this last nitrogen source ethanol production was proportionately much less than that observed with P, U, or NH_4Cl (Fig. 4b). NH_4Cl-grown cells showed the highest specific ethanol production rates.

The total amount of nitrogen present was not as important as the nature of the nitrogen source. For example, RM NH_4-P received 2.12 g N while RM U-P received only 1.06 g N, yet both conditions resulted in essentially the same amount of ethanol production. Also, RM C–Phad half again as much nitrogen as

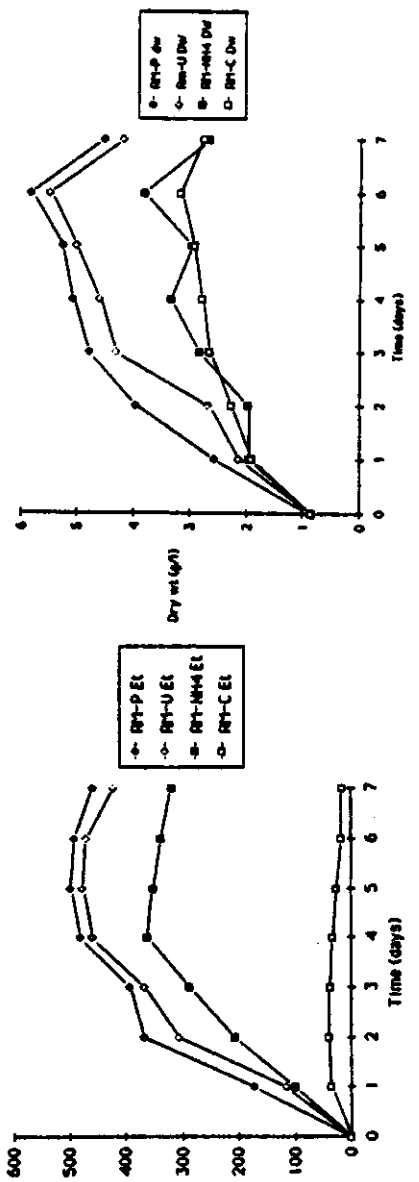

Fig. 4. Effects of four sole nitrogen sources on ethanol production and growth. RM + 0.05*M* citrate–phosphate buffer, pH 4.5, was used. Initial xylose = 20% (w/v). A = ethanol (Et); B = cell dry weight (DW).

RM U-P but still produced less ethanol (Fig. 5a). The increases in ethanol production significantly exceeded proportionate increases in cell mass in the NH_4Cl- and urea-supplemented cultures (Fig. 5b). Mixed nitrogen sources proved to be superior to any sole nitrogen source. As shown in Figure 5a, either NH_4Cl or U supplements to RM P boosted ethanol production by 50-60%

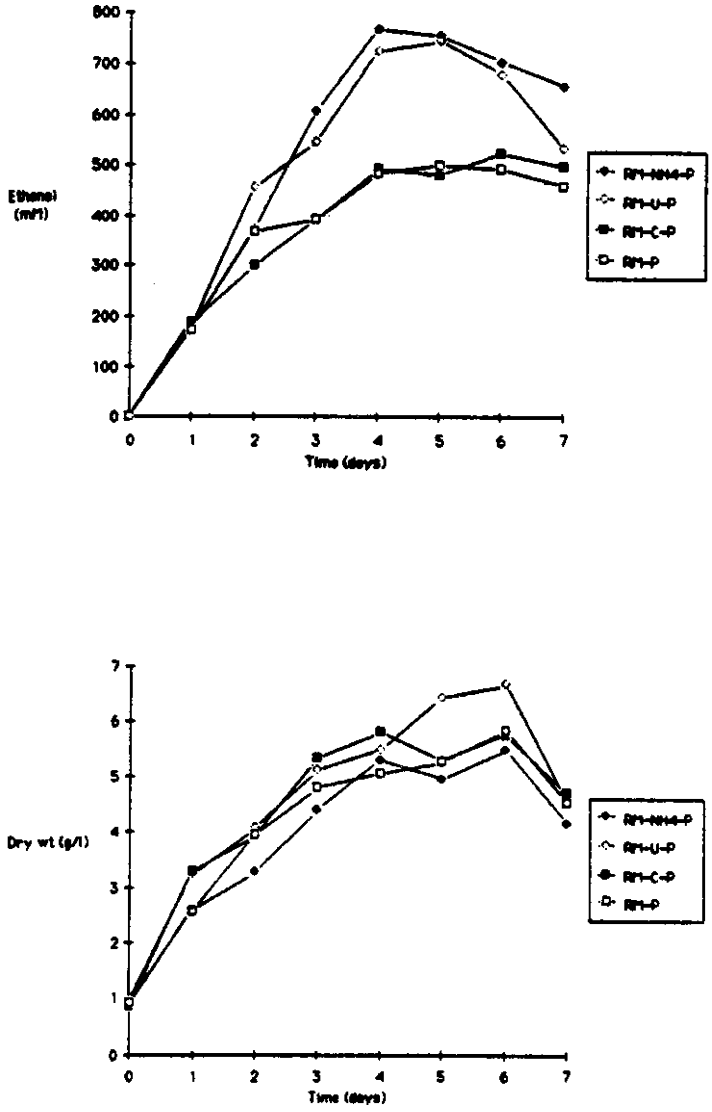

Fig. 5. Effects of mixed nitrogen sources on ethanol production and growth. RM + 0.05*M* citrate-phosphate buffer, pH 4.5-5.5, was used. Total nitrogen (g/L): NH_4-P. 2.12; U–P, 1.06; C–P, 1.56; P, 1.06. Initial xylose = 20% (w/v). A = ethanol; B = dry weight.

whereas a C acid supplement to P had little effect. YB U–P gave results similar to those observed with RM U-P and RM NH_4–P. Other combinations of NH_4Cl with U, U with C, or NH_4Cl with C were less effective for both ethanol production and cell growth (Figs. 6a,b).

Basal Medium

A comparison of RM with YB and U–P with NH_4-C using two different strains of *C. shehatae* showed that the YB medium was better than the RM for ethanol production, that the combination of U and P was better than NH_4CL and

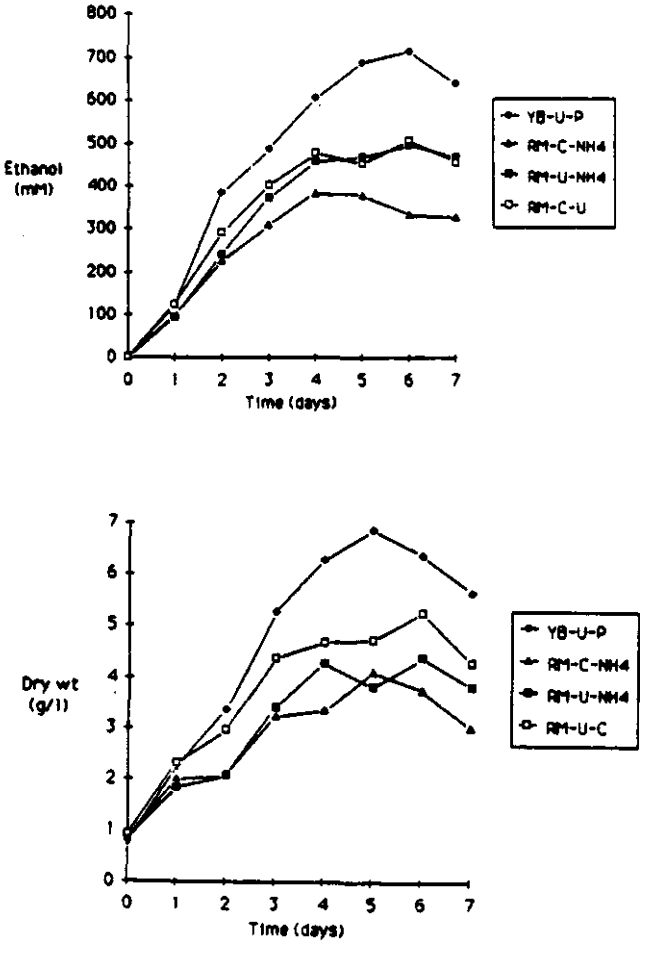

Fig. 6. Effects of mixed nitrogen sources on ethanol production and growth. Total nitrogen (g/L): U-P, 1.06; C–NH_4, 1.56; U–NH_4, 2.12; C–U, 1.56. All other conditions same as Fig. 5. A = ethanol; B = dry weight.

C, and that the strain ATCC 22984 was slightly better than CSIR Y492 (Figs. 7a,b). Observations of relative growth rates on YB xylose agar plates indicated that the Y492 strain could grow more vigorously than ATCC 22984 under aerobic conditions.

Effects of pH

In general, both the fermentation rate and yield tended to increase with decreased pH. With U as the nitrogen source, both the volumetric ethanol production rate (g ethanol/L·h) and the ethanol yield (g ethanol/g cells·h) decreased with increasing pH from pH 2.6 to 5.3. Xylitol production increased

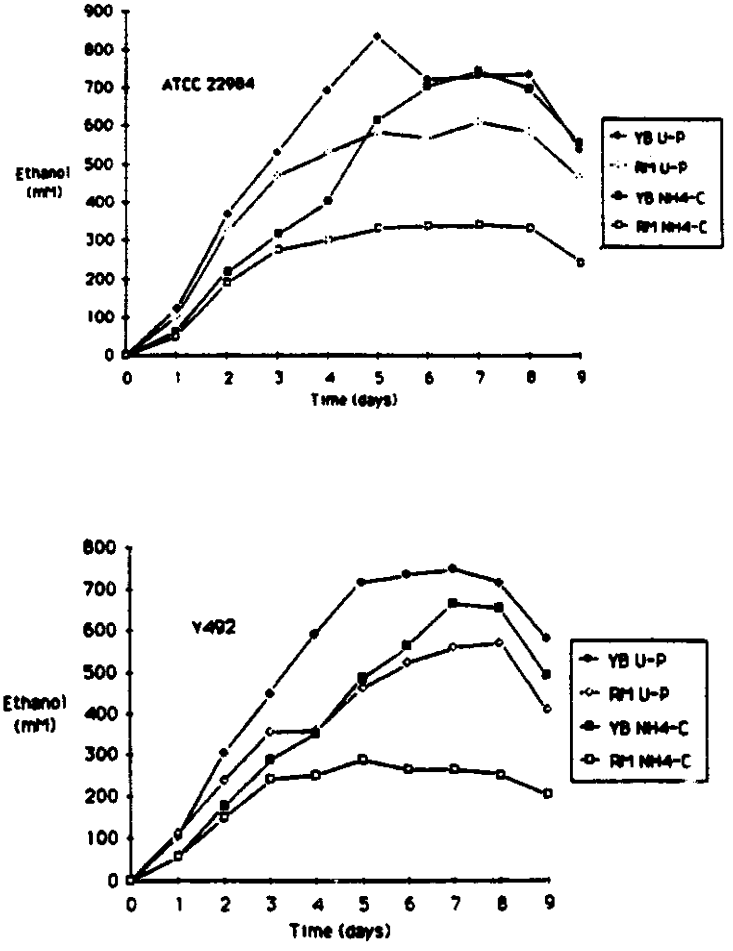

Fig. 7. Comparison of RM with YB plus two different nitrogen mixtures and two yeast strains. Citrate-phosphate buffer, 0.075*M*, pH 4.5, was used. All other conditions were standard.

from pH 2.6 to 3.3 (Figs. 8a,b). Glycerol production followed a pattern similar to that observed with xylitol but was generally much lower. Cell mass decreased with decreased pH, and growth was not observed at pH 2.46. When the effects of pH were examined over a narrower range and at a higher xylose concentration (12%), xylitol production dramatically increased between pH 2.8 and 3.6. This experiment also confirmed a decrease in ethanol yield with increasing pH. At the same time a slight rise in the volumetric ethanol production rate was observed

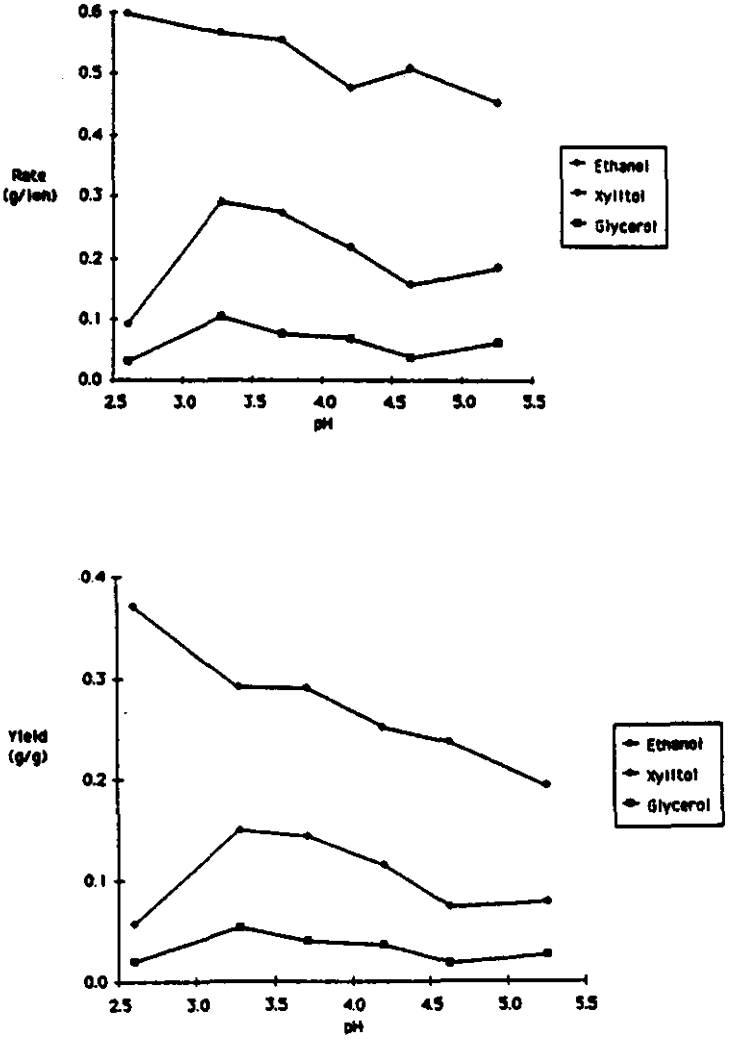

Fig. 8. Effect of pH on volumetric production rate and ethanol yield. YB U (1.06 g N/L) was used with 0.05*M* citrate-phosphate buffer. All other conditions were standard. Initial xylose = 9% (w/v). A = volumetric rates; B = product yields. Rates and yields were calculated from 24–48-h data.

over this pH range (Figs. 9a,b). A third experiment was conducted over the same pH range with 12% xylose but using P as the nitrogen source. In this instance the volumetric ethanol production rates were fairly constant, but the specific rate (g ethanol/g dry wt cells·h) declined about 30%. As observed previously with P (Fig. 1), xylitol production was relatively low, but the xylitol yield still doubled in going from pH 2.6 to 3.6. Although the volumetric rates in this third experiment were about the same as previously observed, the specific rates were somewhat lower, particularly at the lowest pH values tested (data not shown).

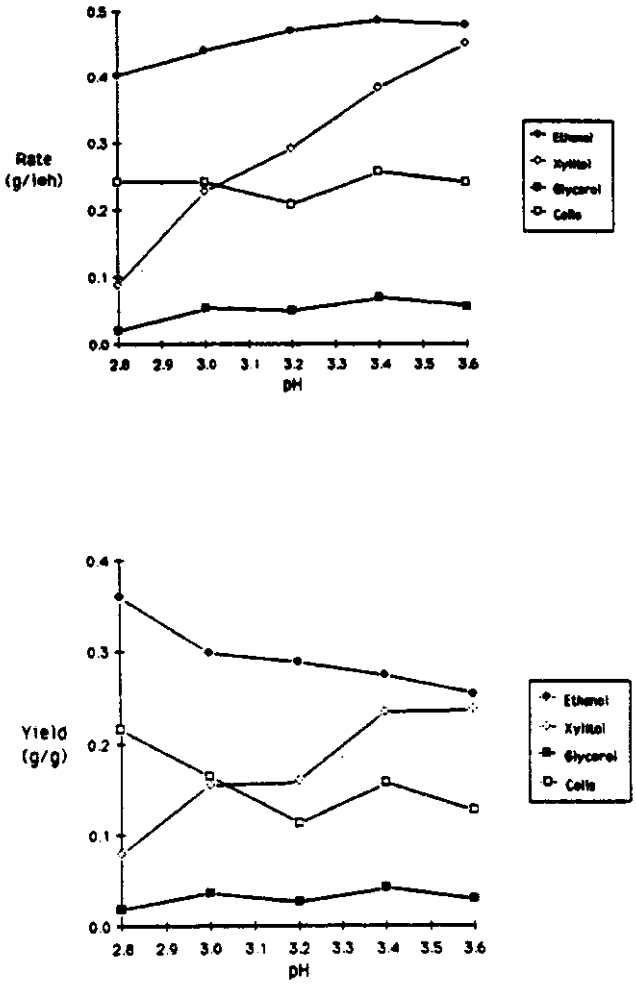

Fig. 9. Effect of pH in a narrow acidic range. Initial xylose = 12% (w/v). YB U was used; all other conditions as in Fig. 8.

Effects of Aeration and Sugar Concentration

The conditions employed in the central point (*F* = 9% xylose, 100 rpm, 50 mL–125-Erlenmeyer)resulted in the highest specific ethanol production rate observed (Fig. 10) and high ethanol yields (Fig. 11). The highest volumetric ethanol production rate—but not the highest specific production rate–occurred at the highest aeration condition, *K* (Fig. 12). Low aeration generally resulted in very low ethanol production rates, and the lowest ethanol yield was observed in condition *B,* where the lowest aeration rate was employed. The effects of aeration were observed most readily by comparing conditions *A, F,* and *K.* Both the specific ethanol production rate and the ethanol yield passed through a maximum with condition *F,* but the volumetric ethanol production rate increased with increased aeration.

Sugar concentration and aeration appeared to be interactive variables. At low aeration increased sugar concentration led to an increased yield of ethanol (cf. *E* and *C*). At higher aeration increased sugar concentration led to a slightly lower ethanol yield (cf. *E* and *C*). At higher aeration increased sugar concentration led to a slightly lower ethanol yield (cf. *J* and *H*). In both pairs the specific rate of ethanol production was essentially unchanged.

Xylitol and glycerol production were also studied (Figs. 10–12).At very low aeration rates the initial specific glycerol production rate and the glycerol yield

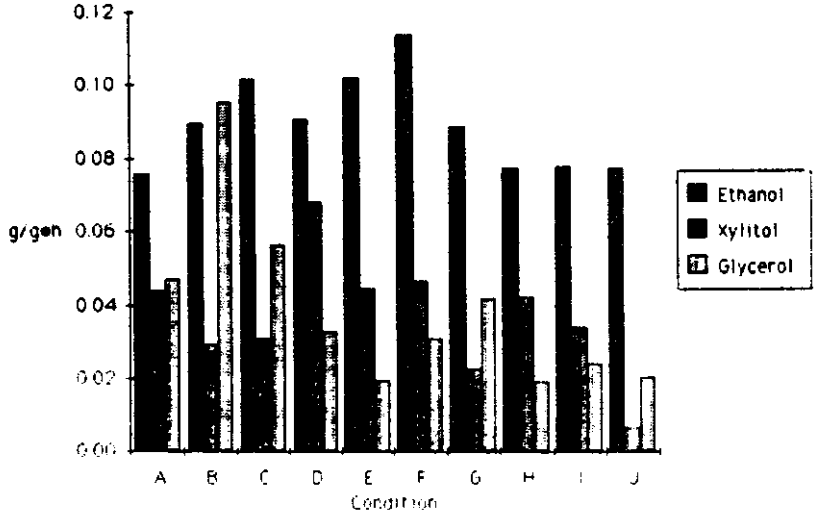

Fig. 10. Effects of aeration and sugar concentration on the specific ethanol, xylitol, and glycerol production rates. YB U–P(1.06 g N/L) + 0.05*M* citrate-phosphate buffer, ph 4.5, was used as the basal medium.

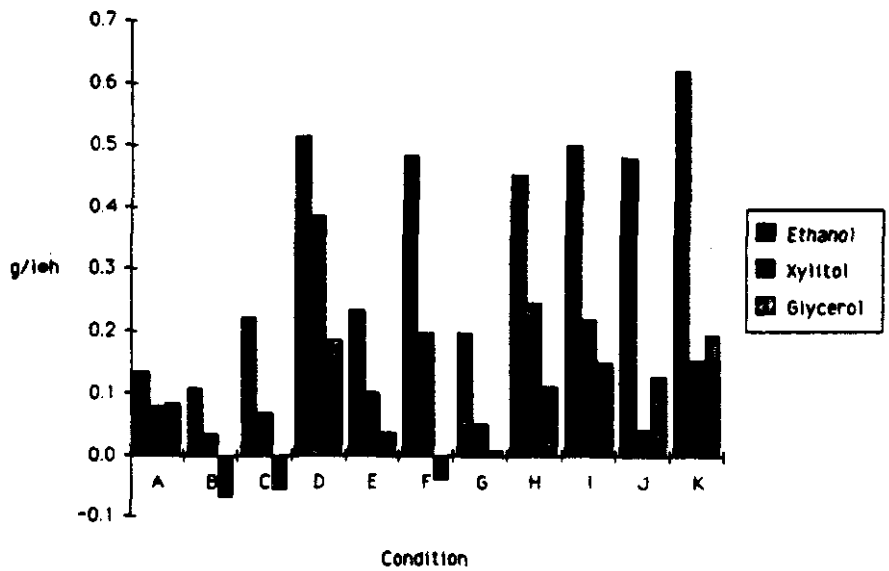

Fig. 11. Effects of aeration and sugar concentration on ethanol, xylitol, and glycerol production rates. All condition same as Fig. 10.

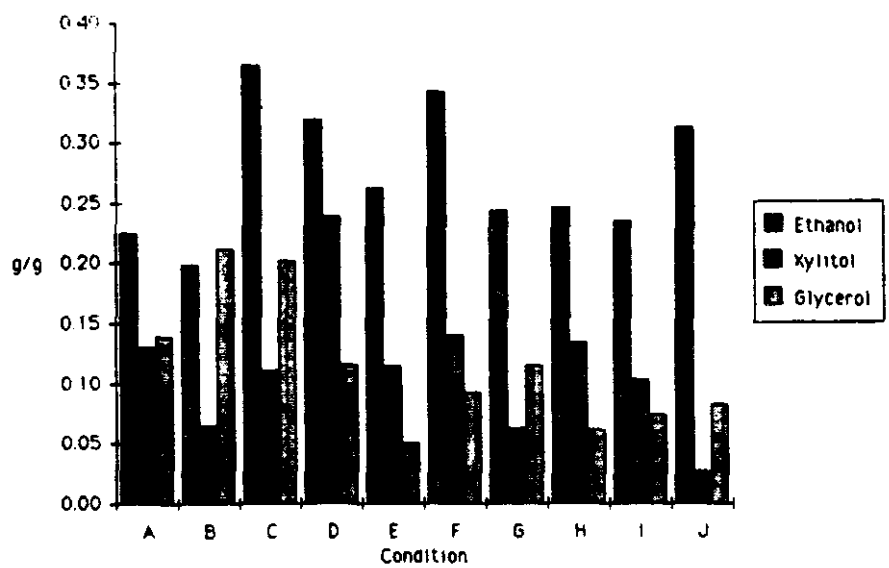

Fig. 12. Effects of aeration and sugar concentration on volumetric ethanol, xylitol, and glycerol yields. All conditions same as Fig. 10.

increased. The apparent discrepancy between volumetric glycerol production rates, specific rates, and yields arises from the fact that 24–48-h data were used to calculate the volumetric production rates for glycerol while the specific rate and yield data employed either 0–24- or 24–48-h data, depending on which gave

the larger value. In three conditions (*B, C,* and *F*) glycerol actually decreased from 24 to 48 h.

The specific xylitol production rate increased with increased sugar concentration. The highest xylitol production rate occurred in condition *D,* which received moderately high aeration. In no instance did ethanol production exceed a yield of 0.4 g–g xylose consumed, but if production of xylitol, glycerol, and cell mass was taken into account, a carbon balance in excess of 90% was routinely achieved.

DISCUSSION

Most of the cultural variables examined here had clear effects on either the production or yield of ethanol, xylitol, and glycerol. The nitrogen source used was particularly important. In some instances. such as in the cases of nitrogen source and aeration or aeration and sugar concentration, the variables were interactive. Probably other variables were interactive as well, and it is unlikely that a true optimum has been obtained in these experiments. Each of the major factors examined are discussed below.

Acidity

The apparent decrease in ethanol yield with increasing pH was most readily attributed to an increase in respiration because there were no increases observed in either cell or other product formations. When du Preez and van der Walt[10] employed a pH of 5.5 in their shake flask studies and of 4.5 in fermentor studies with *C. shehatae* CSIR Y492 and reported somewhat higher maximum volumetric production rates, their aeration rates were also appreciably greater than those used here. It is worthwhile to note that Slininger et al.[11] have also reported significantly higher rates and yields with *P. tannophilus* grown under conditions of low pH.

It was very difficult to separate the effects of pH and nitrogen source in these experiments because different nitrogen sources each affected the course of the medium pH in unique ways. The rise in pH observed with U as a nitrogen source results from U hydrolysis to ammonia and CO_2. The drop observed with NH_4Cl is attributable to the cellular uptake of NH_4^+ balanced by the excretion of H^+, leaving $H^+ + Cl^-$ in the medium. Even with buffered media, observed shifts of 0.2–0.8 pH units after 4–5 days were observed.

Nitrogen Sources

Peptone appears to stimulate cellular respiration and growth. It should be noted that, in general, ethanol production paralleled growth, and higher cell yields with some nitrogen sources were reflected in higher ethanol yields. These results

strongly suggest that, as in the case of *S. cerevisiae,* ethanol production is a by-product of growth.

The stimulation of ethanol production observed by combining U or NH_4Cl with P or some other organic nitrogen source can perhaps be attributed to an effect of NH_4+ on the Embden–Meyerhoff pathway. NH_4^+ is known to stimulate phosphofructokinase in *S. cerevisiae,*[16] and it is possible that a similar effect occurs here.

These results showed that the form of the nitrogen used was far more important than the amount. The total nitrogen levels used here ranged from 0.5 to 2.12 g/L and were lower than the 2.46 g/L used by du Preez and van der Walt.[10] Although the experiments reported here used the same concentration of C, the NH_4Cl levels were about half. Even so, nitrogen was probably always present in excess. The highest cell densities achieved were about 7 g/L. If one assumes that 50% of the total dry weight were protein, then the maximum amount of nitrogen taken up would be about 0.6–0.8 g/L. The lowest nitrogen concentration used here was 0.5 g/L–when C was used alone–and in this instance only 3 g/L dry wt cells were formed, so it is unlikely that nitrogen limited cell growth. It is not known whether the P or C meet certain essential nutritional requirements of these cells.

Media

In general, the differences in ethanol production observed with the two basal media employed were not profound, and the effects of nitrogen and aeration were more significant. The RM employed here actually contained less $MgSO_4$ than the YB, but the trace elements and particularly the vitamin contents were much higher in some cases. Other results not reponed here suggest that further improvements might be obtained by altering the levels of the mineral and vitamin constituents. Dellweg et al.[18] have shown that *P. stipitis* does not require either biotin or thiamine for growth, as does *P. tannophilus,* but that growth is stimulated by yeast extract. Yeast extract is also known to stimulate ethanol production in *P. tannophilus,*[11] and it is likely that the primary effect is on growth. Yeast extract was not used here in the interest of maintaining defined conditions.

Aeration and Sugar Concentration

Ethanol production showed a complex relationship with aeration and sugar concentration. Low volumetric rates of ethanol production could be attributed to low growth rates because both cell growth and volumetric ethanol production rates increased with increasing aeration. Ethanol is produced as a function of growth because the highest volumetric ethanol production and growth rates were obtained with the highest aeration. Moreover, the specific ethanol production did not decrease dramatically under these conditions, even though the yield declined. Growth decreased as volumetric ethanol production decreased at very low aeration rates, and the lowest specific ethanol production rates were observed at

the lowest aeration rates. Moreover, ethanol production under anaerobic conditions was minimal. suggesting that like other *Candida* species, ethanol production by *C. shehatae* is linked to respiration and growth.[17] Although ethanol yields declined at very low aeration rates, the decrease was not in proportion to the decrease in the volumetric rates. Other studies have shown that the volumetric ethanol production rate increases with increased aeration. Du Preez et al.[19] reported that as aeration increased, volumetric production rates increased up to 0.6 g/L·h, about the same as the highest rate obtained here. At very high aeration rates cell growth rates are maximized, but ethanol production is minimal.[10] One can argue that ethanol production is a function of secondary metabolism during the fermentation of xylose by yeasts.[20] However, close examination of Figures 4–7 in the experiments reported here will show that the peak ethanol production tends to follow the peak cell mass, even though accumulation of ethanol and cell mass largely occur at linear rates until the stationary phase is attained.

Specific ethanol production increased with increased sugar concentration at lowaeration. This increase, however, was accompanied by a marked increase in glycerol production, indicating an accumulation of intermediates in the glycolytic pathway. In this multiple-variable experiment YB U–P was used, and it is possible that different results would be obtained with another nitrogen source or basal medium. Overall, the standard conditions employed here gave the best combination of yield and specific production rate, but the specific production rate might not be critical in the final analysis.

CONCLUSION

By observing the time course for ethanol production, it was apparent that for a given condition, ethanol production was essentially linear for the first several days. This linearity occurred in spite of the fact that cell densities increased as much as sevenfold in the same period. Indeed, in most instances ethanol production could better be expressed as a function of the cell growth rate than of the total cell mass present at any given time. The conclusion that ethanol production in *C. shehatae* is a by-product of growth is supported by the observations that both growth and ethanol production ceased under anaerobic conditions and that nitrogen sources. which gave better cell growth, also resulted in better ethanol production.

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