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Influence of oxygen transfer rate and media composition on fermentation of D-xylose by *Pichia stipitis* NRRL Y-7124

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SUMMARY

The optimization of ethanol production by *Pichia stipitis* NRRL Y-7124 was analysed by ATP balance. Ethanol volumetric productivity was maximal (0.5–0.6 g/l h) only over a narrow range of oxygen transfer rates (3–5 mmol O_2/l h). Trace element supplements increased ethanol volumetric productivity 20%. Biotin and thiamine did not significantly affect ethanol yield. Vitamins and trace elements were not synergistic. Organic nitrogen source from yeast extract was used for growth simultaneously to ammonia.

INTRODUCTION

The hemicellulose content of various ligno-cellulosic materials ranges between 25 and 45% of the dry biomass with D-xylose as the major sugar component [8]. The economic exploitation of processes for the microbial conversion of lignocellulosic wastes into liquid fuel would be greatly enhanced if the hemicellulose component could also be utilized. The conversion of D-xylose to ethanol is limited to a small number of yeast strains [3]. Although many studies have been carried out about this process [4,6], much work remains to be done for achieving high yields of ethanol. Low alcohol yields are sometimes due to the formation of products such as xylitol. For this reason, the use of non-xylitol producing strains like *Pichia* sp. is highly attractive.

In this first approach we studied the influence of oxygen transfer rate (OTR), trace elements and vitamins on ethanol production by *Pichia stipitis* NRRL Y-7124.

MATERIALS AND METHODS

Microorganism. The microorganism used was *Pichia stipitis* NRRL Y-7124 which was maintained in yeast extract-peptone-dextrose-agar medium (YEDP medium).

Media. The media used were based on a basal medium, referred to as medium 1, of the following composition (g/l): Yeast extract (Merck), 5; KH_2PO_4, 10; $(NH_4)_2SO_4$, 4.3; $CaCl_2 \cdot 2H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.5; D-xylose, 50. Medium 2 contained D-biotin (0.1 mg/l), and thiamine ClH (5 mg/l), in addition to medium 1. Medium 3 contained medium 1 supplemented with the following trace elements (mg/l): $FeSO_4 \cdot 7H_2O$, 35; $MnSO_4 \cdot H_2O$, 7; $ZnSO_4 \cdot 7H_2O$, 11; H_3BO_3, 2; $CuSO_4 \cdot 5H_2O$, 1; $CoCl_2 \cdot 6H_20$, 2; KI, 0.35; $Na_2MoO_4 \cdot 2H_2O$, 1.3; $Al_2(SO_4)_3$, 0.5. Medium 4 contained medium 1 supplemented with the same concentration of vitamins and trace elements of media 2 and 3 respectively. The initial pH for all media was 5.5.

Inocula. Inocula were carried out with 24-h YEDP slant cultures which were transferred to 250-ml Erlenmeyer flasks each containing 50 ml of medium 1. They were incubated on a rotary shaker operated at 240 rpm for 24 h at 30 °C. Sufficient amounts of this culture, to give an initial concentration of biomass in the fermentation media of 0.06 g/l, were used under high and limited aeration conditions. In the case of 'no aeration' condition a heavy inoculum (2.5 g/l as initial value) was used instead.

Fermentation conditions. To study the influence of oxygen transfer rate (OTR) the following conditions were tested: 5, 13, 36 and 240 mmol O_2/l h. These conditions were achieved using different ratios of Erlenmeyer to media volume. Anaerobic conditions were tested as well using a nitrogen gassed Erlenmeyer flask.

In order to achieve the highest OTR used (240 mmol O_2/l h), experiments were performed in 10-l fermentors containing 3.5 l of medium 1. The operating conditions employed were aeration at 1.3 v.v.m. and agitation at 800 rpm. All experiments were carried out at 30 °C.

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The influence of vitamins and trace elements were studied in Erlenmeyer flasks operated at OTR = 5 mmol O_2/l h, 30 °C, and 240 rpm by using media 1, 2, 3 and 4.

Analytical methods. Ethanol was analysed enzymatically (Sigma Chemical Co. procedure N° 332-U.V.). Xylose was determined with *o*-toluidine reagent [7].

Ammonium was determined by a modification of the Berthelot method [1], and biomass by dry cell weight and optical density. OTR was evaluated by the sulfite method [2].

Statistical methods. For limited aeration conditions the fermentation parameters were calculated as linear regression coefficients of the linear portions of the curves. The results obtained were subjected to co-variance analysis [5]. Those results showing significant differences were also analysed by Dunnet and/or Tuckey tests [5,10]. The anaerobic conditions parameters were calculated as the mean between the initial and final values. For high aeration conditions, μ was estimated as a linear regression coefficient after transformation of data (ln X vs. time) whereas the other parameters were calculated as mean values.

RESULTS AND DISCUSSION

Influence of oxygen transfer rate on growth and product formation

Productivity analysis. The experiments performed at different aeration levels showed that biomass and product formation were strongly dependent on the oxygen transfer rate (Table 1). On anaerobiosis, growth related parameters were almost negligible, in agreement with the data presented by Delgenes et al. [3], while ethanol yield ($Y_{p/x}$, $Y_{p/s}$) were maximal.

Between 5 and 35 mmol O_2/l h, Q_x varied linearly with the OTR value (see Fig. 1); consequently, it can be assumed that growth was limited by the aeration level. The regression of the plot of Q_x as a function of OTR was used to calculate the $Y_{x/o}$ value (59.1 g/mol), which was similar to the value reported by Rizzi et al. [11].

In the conditions described above, biomass varied linearly with the time course (data not shown).

At an OTR value of 240 mmol O_2/l h the culture was not limited by oxygen and was growing exponentially during the period of measurement (data not shown). On the other hand, product yields decreased at increasing OTR's and became almost nil at the maximum OTR attained.

From data presented in Table 1 it was concluded that growth and ethanol yields are inversely related; therefore, it is not possible to optimize biomass and product formation at a unique OTR value (see below).

As ethanol is a 'commodity', the economic feasibility of its production will depend on the maximization of the ethanol volumetric productivity.

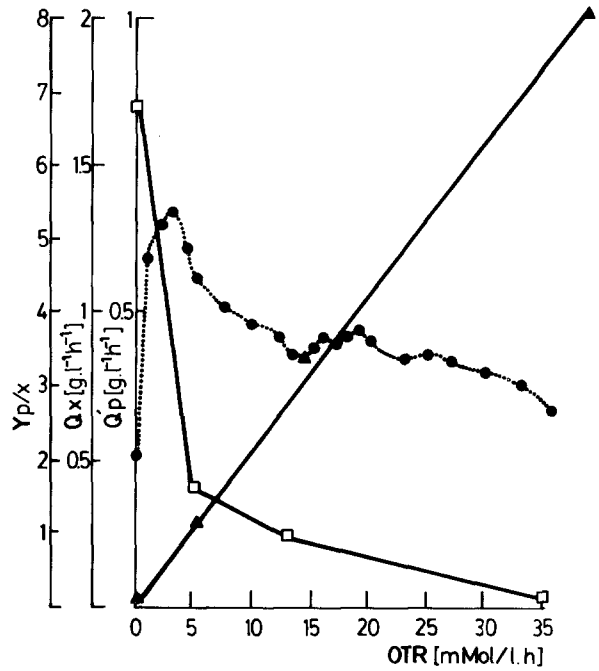

Fig. 1. Biomass volumetric productivity (Q_x), specific product yield ($Y_{p/x}$) and ethanol volumetric productivity growth associated (Q'_p) as function of oxygen transfer rate (OTR). Symbols: Q_x (▲); $Y_{p/x}$ (□); and Q'_p (●).

Despite the results described above, the kinetic parameters of product formation (Q_p; q_p) were not maximal at OTR = 0, showing that growth and product formation

TABLE 1

Influence of Oxygen Transfer Rate (OTR) on fermentation parameters of *P. stipitis* NRRL Y 7124 grown on medium 1

Parameter	OTR (mmol O_2/l h)				
	0	5	13	35	240
μ (h^{-1})	ND	0.11[a]	0.12[a]	0.15[a]	0.45[a]
$Y_{p/s}$ (g/g)	0.34	0.28	0.21	0.11	0.02
$Y_{x/s}$ (g/g)	0.05	0.16	0.34	0.62	0.55
$Y_{p/x}$ (g/g)	7.21	1.70	0.58	0.17	0.02
Q_p (g/l h)	0.24	0.50	0.59	0.35	0.04
Q_x (g/l h)	0.03[b]	0.30[c]	0.88[c]	2.03[c]	[d]
q_p (g/g h)	0.16	0.41	0.34	0.20	0.06

ND: not determined.

[a] Maximum value in the growth conditions tested (see text).

[b] Calculated as a mean value from the initial and final data.

[c] Constant value with time.

[d] Varies exponentially with time.

(Q_x and Q_p, respectively) must be linked to a certain extent. In order to evaluate the relative influence of both parameters on ethanol productivity, the following ATP balance equation was formulated [13], assuming that no ATP was accumulated into the cells and no ATP was lost by futile cycles [15]:

$$a_x \cdot Q_x + q_m \cdot X = a_o \cdot Q_o + a_p \cdot Q_p \quad (1)$$

Under oxygen limitation,

$$Q_x = Y_{x/o} \cdot Q_o \quad (2)$$

then,

$$Q_p = Y_{p/x} \cdot Q_x + q_p \cdot X \quad (3)$$

where:

$$Y_{p/x} = 1/a_p[a_x - (a_o/Y_{x/o})] \quad (4)$$
$$q_p = q_m/a_p \quad (5)$$

According to [3] ethanol volumetric productivity (Q_p) depends on a growth associated component ($Q'_p = Y_{p/x} \cdot Q_x$) and a non-growth associated one ($Q''_p = q_p \cdot X$)

Evaluation of Q'_p. As mentioned above, there are no suitable aeration conditions that can optimize simultaneously $Y_{p/x}$ and Q_x. By multiplying point to point $Y_{p/x} \times Q_x$, at OTR values between 0 and 35 mmol O_2/l h, the Q'_p terminus of equation (3) was calculated (see Fig. 1). The growth-associated component has its maximum (0.5–0.6 g/l h) at a narrow range of OTR, between 3 and 5 mmol O_2/l h.

It must be pointed out that the product yield ($Y_{p/x}$) obtained in anaerobiosis is conceptually different from $Y_{p/x}$ attained under oxygen limitation. The former is only an operational magnitude, variable within the time course of the process, while the latter is independent of time, but variable with OTR. From equation (4), if we assume that a_x = (1/10.3) mol ATP/g biomass [14] and a_p = (1/46) ATP/g ethanol then $Y_{p/x}$ can be calculated as a definite limit value, when OTR decreases progressively to zero. That limit should be a_x/a_p, whose estimated value is 4.46.

The discrepancy between theoretical and experimental yield at OTR = 0 may be attributed either to the period

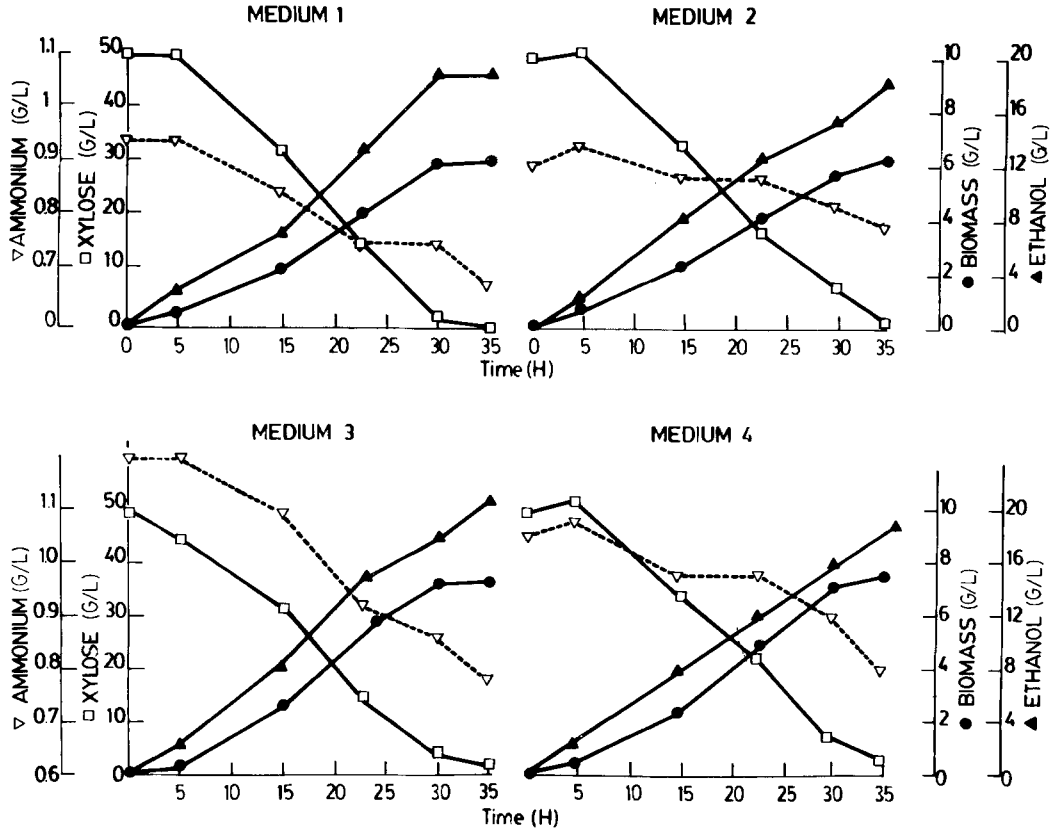

Fig. 2. Effect of media composition on growth and on ethanol yield by *P. stipitis* NRRL Y 7214. Further details in Materials and Methods.

TABLE 2

Fermentation parameters of *P. stipitis* NRRL Y 7124 grown on different media at OTR = 5 mmol O_2/l h and 30 °C during 35 h

Fermentation parameter	Media			
	1	2	3	4
$Y_{p/s}$ (g/g)	0.235 ± 0.019	0.238 ± 0.017	0.301 ± 0.020	0.308 ± 0.023
$Y_{x/s}$ (g/g)	0.169 ± 0.016	0.147 ± 0.003	0.169 ± 0.008	0.157 ± 0.006
$Y_{p/x}$ (g/g)	1.386 ± 0.022	1.614 ± 0.151	1.788 ± 0.066	1.956 ± 0.169
Q_p (g/l h)	0.460 ± 0.041	0.726 ± 0.021	0.583 ± 0.051	0.546 ± 0.042
Q_x (g/l h)	0.331 ± 0.022	0.261 ± 0.013	0.326 ± 0.025	0.288 ± 0.003
q_p (g/g h)	0.354 ± 0.032	0.327 ± 0.061	0.486 ± 0.045	0.496 ± 0.039

Results were expressed as average value ± 1SE.

of measurement, as mentioned above, or to the fact that no strict anaerobiosis was attained (Table 1).

Evaluation of Q_p'' component. Since the q_p value obtained experimentally for OTR = 0 was 0.16 g/g h (Table 1), the resulting maintenance coefficient (q_m) calculated from equation (5) was $3.5 \cdot 10^{-3}$ mol ATP g/h. The value reported by Rizzi et al. [11] for *P. stipitis* CBS 5773 was somewhat lower ($5 \cdot 10^{-4}$ mol ATP/g h). However, both results differ significantly from the q_m value reported for high ethanol producing strains. For instance Watson [16] calculated a q_m value of $5.2 \cdot 10^{-1}$ mol ATP/g h for *Saccharomyces cerevisiae*. Therefore it can be concluded that ethanol productivity is mostly determined by the growth associated terminus Q_p', while the non-growth terminus Q_p'' would become important only at high biomass levels or high maintenance energy.

According to these results, any strategy to increase ethanol productivity by *P. stipitis* should consider the use of a two-step process, performing the first one aerobically to obtain high-density culture, and the second under microaerophilic conditions to maximize ethanol productivity.

Influence of media composition

The fermentation patterns obtained with four different media are shown in Fig. 2, while measurements of the fermentation parameters were summarized in Table 2. These results were subjected to hypothesis tests by statistical analysis, indicating that: (1) the supplementation of media with a pool of trace elements (medium 3) resulted in a statistically significant increase in $Y_{p/s}$ and $Y_{p/x}$ ($P < 0.05$) and in a highly significant increase in Q_p ($P < 0.01$). The magnitude of such increases was 28, 29 and 20%, respectively. No significant changes were detected in growth related parameters, Q_x and $Y_{k/s}$. (2) Supplementation with biotin and thiamine (medium 2) did not influence the ethanol yield. On the other hand, the value of Q_x and $Y_{x/s}$ obtained in media 1 and 3 (without vitamin supplementation) had a better performance than those obtained with medium 2 (with vitamin supplementation). A similar behaviour had been reported by Kamihara et al. [9] who described that excess of thiamine produces a decrease of the respiratory capacity in some *Saccharomyces* strains. Du Preez et al. [6] reported a shortening in time of fermentation with *Pichia* from 99 h to 39 h, while other authors [4] working with the same strain did not detect any change.

The supplementation with trace elements and vitamins together (medium 4) did not produce a better performance than the sole addition of trace elements ($\alpha = 0.10$). Neither synergism or antagonism were noted.

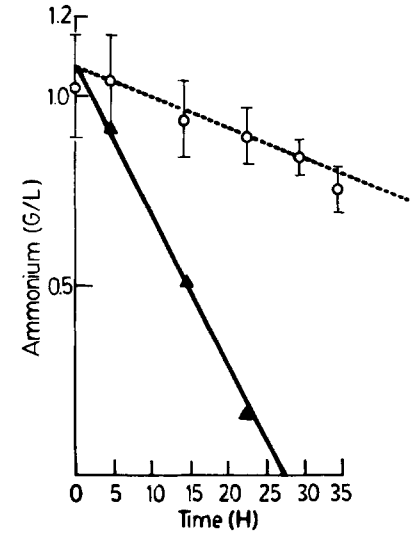

Fig. 3. Experimental and theoretical ammonium uptake during the time course of xylose fermentation by *P. stipitis* NRRL Y 7124. Symbols: experimental value (○); theoretical value (▲).

The ammonia uptake

Theoretical and experimental data on ammonia consumption were plotted versus the time course of a fermentation process carried out at OTR = 5 mmol O_2/l h. Theoretical values were calculated by stoichiometric balance, assuming the biomass elemental composition formulated by Roels [12] and that ammonia was the only nitrogen source.

The Y_{x/NH_4} value calculated from these experiments (32.6 g/g) was much higher than the predicted maximum (7.2 g/g). This result suggests that an organic nitrogen source belonging to the yeast extract was simultaneously and preferentially used for growth.

In order to clarify this point, further research is required.

NOMENCLATURE

a_p	ATP yield per gram ethanol	mol/g
a_o	ATP yield per mol oxygen	mol/mol
a_x	ATP yield per gram biomass	mol/g
OTR	oxygen transfer rate	mol/l h
Q_x	growth volumetric rate	g/l h
Q_o	volumetric respiration rate	mol/l h
Q_p	ethanol volumetric production rate	g/l h
Q'_p	ethanol volumetric growth-linked production rate	g/l h
Q''_p	ethanol volumetric non-growth-linked production rate	g/l h
q_m	specific ATP maintenance coefficient	mol/g h
q_p	specific ethanol production rate	g/g h
X	biomass concentration	g/l
$Y_{p/s}$	ethanol yield per gram xylose	g/g
$Y_{p/x}$	ethanol yield per gram biomass	g/g
Y_{x/NH_4}	biomass yield per gram ammonium	g/g
$Y_{x/o}$	biomass yield per mol oxygen	g/mol
μ_{max}	maximum specific growth rate	h^{-1}

Subscripts: p, product; s, substrate; x, biomass; o, oxygen; NH_4, ammonium; m, maintenance.

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REFERENCES

1 Chaney, A.L. and E.P. Marbach. 1962. Modified reagent for determination of urea and ammonia. Clin. Chem. Acta 8: 130–132.
2 Cooper, C.M., G.A. Fernstrom and S.A. Miller. 1944. Performance of agitated gas liquid contactors. Ind. Eng. Chem. 36: 506–509.
3 Delgenes, J.P., R. Moletta and J.M. Navarro. 1986. The effect of aeration on D-xylose fermentation by *Pachysolen tannophilus*, *Pichia stipitis*, *Kluyveromyces marxianus* and *Candida shehatae*. Biotechnol. Lett. 8: 897–900.
4 Dellweg, H., M. Rizzi, H. Methner and D. Debus. 1984. Xylose fermentation by yeast: comparison of *Pachysolen tannophilus* and *Pichia stipitis*. Biotechnol. Lett. 6: 395–400.
5 Dixon, W.J. and F.J. Massey. 1965. In: Introduction to Statistical Analysis pp. 185–197, Mc Graw Hill U.S.A.
6 Du Preez, J.C., M. Bosch and B.A. Prior. 1986. The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stipitis*. Appl. Microbiol. Biotechnol. 23: 228–233.
7 Hyvarinen, A. and E.A. Nikkila. 1962. Specific determination of blood glucose with o-toluidine. Clin. Chem. Acta 7: 140–143.
8 Jeffries, T.W. 1986. Emerging technology for fermenting D-xylose. Trends in Biotechnol. 3: 208–212.
9 Kamihara, T. and I. Nakamura. 1984. Regulation of respiration and its related metabolism by vitamin B, and vitamin B_6 in Saccharomyces yeast. Adv. Biochem. Eng. Biotechnol. 29: 35–82.
10 Lison, L. 1968. Statisque applique a la Biologie experimentale. Gautier–Villars – Paris. pp. 115–122.
11 Rizzi, M., C. Klein, N.A. Schulze, A. Bui-Thanh and H. Dellweg. 1987. Mathematical model for the semiaerobic fermentation of xylose by the yeast *Pichia stipitis*. In: Proceedings of 4th European Congress of Biotechnology (Neijssel O.M., van der Meer, R.R. and Luyben, K.Ch., eds.), Elsevier, Amsterdam.
12 Roels, J.A. 1983. Macroscopic theory, and Microbial growth and product formation. In: Energetics and Kinetics in Biotechnology. pp. 23, Elsevier Medical Press. Amsterdam.
13 Sinclair, C.G. and B. Kristiansen. 1987. Rate equations. In: Fermentation kinetic and modelling. pp. 18–33. Open University Press, London.
14 Stouthamer, A.H. and C.W. Bettenhaussen. 1975. Determination of the efficiency of oxidative phosphorylation in continuous cultures of *Aerobacter aerogenes*. Arch. Microbiol. 102: 187–192.
15 Tempest, D.W. and O.M. Neijssel. 1984. The status of Y_{ATP} and maintenance energy as biologically interpretable phenomena. Ann. Rev. Microbiol. 38: 459–486.
16 Watson, T.G. 1970. Effects of sodium chloride on steady state growth and metabolism of *Saccharomyces cerevisiae*. J. Gen. Microbiol. 64: 91–99.