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ORIGINAL PAPER

Effect of controlled oxygen limitation on *Candida shehatae* physiology for ethanol production from xylose and glucose

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Abstract Carbon distribution and kinetics of *Candida shehatae* were studied in fed-batch fermentation with xylose or glucose (separately) as the carbon source in mineral medium. The fermentations were carried out in two phases, an aerobic phase dedicated to growth followed by an oxygen limitation phase dedicated to ethanol production. Oxygen limitation was quantified with an average specific oxygen uptake rate (OUR) varying between 0.30 and 2.48 $mmolO_2$ g dry cell weight $(DCW)^{-1}$ h^{-1}, the maximum value before the aerobic shift. The relations among respiration, growth, ethanol production and polyol production were investigated. It appeared that ethanol was produced to provide energy, and polyols (arabitol, ribitol, glycerol and xylitol) were produced to reoxidize NADH from assimilatory reactions and from the co-factor imbalance of the two-first enzymatic steps of xylose uptake. Hence, to manage carbon flux to ethanol production, oxygen limitation was a major controlled parameter; an oxygen limitation corresponding to an average specific OUR of 1.19 $mmolO_2$ g DCW^{-1} h^{-1} allowed maximization of the ethanol yield over xylose (0.327 g g^{-1}), the average productivity (2.2 g l^{-1} h^{-1}) and the ethanol final titer (48.81 g l^{-1}). For glucose fermentation, the ethanol yield over glucose was the highest (0.411 g g^{-1}) when the specific OUR was low, corresponding to an average specific OUR of 0.30 $mmolO_2$ g DCW^{-1} h^{-1}, whereas the average ethanol productivity and ethanol final titer reached the maximum values of 1.81 g l^{-1} h^{-1} and 54.19 g l^{-1} when the specific OUR was the highest.

Keywords Ethanol · Lignocellulosic · *Candida shehatae* · Xylose · Glucose

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Abbreviations

Rxyl,p Ethanol yield over xylose calculated between the beginning and the end of the oxygen limitation phase in Cmol-ETOH Cmol-$xylose^{-1}$ or g-ETOH g-$xylose^{-1}$

Rglc,p Ethanol yield over glucose calculated between the beginning and the end of the oxygen limitation phase in Cmol-ETOH Cmol-$glucose^{-1}$ or g-ETOH g-$glucose^{-1}$

Introduction

The production of ethanol from the biological conversion of renewable feedstocks, such as agricultural or forestry residue, which do not compete with food production, yields an environmentally friendly alternative to petroleum fuel.

As has been thoroughly investigated, these raw materials are mainly composed of cellulose, hemicellulose, lignin and ash [24]. After hydrolysis of sugar polymers, hexoses (glucose, mannose and rhamnose) and pentoses (xylose, arabinose) are released to be fermented. It is crucial to use all the sugar polymers to improve the cost-competitiveness of the ethanol production process. The microbial conversion of pentoses, which represent 25–40% of the total sugars [27], has been identified as the major research challenge to making lignocellulosic ethanol technology mature for industry according to several authors, such as recently Hahn-Hägerdal et al. [17].

Saccharomyces cerevisiae, the most frequently used organism for industrial ethanol production, is not naturally capable of fermenting pentoses and has to be genetically engineered to produce ethanol from xylose or arabinose [22, 20]. Several different strategies have been developed to produce ethanol from xylose with genetically engineered microorganisms, using either thermotolerant yeast and thermophilic bacteria in simultaneous saccharification and fermentation processes [9, 36] or mesophilic bacteria with high growth rates [26].

In parallel, several works have focused on the use of naturally occurring pentose-fermenting yeasts [32, 18, 34] with robust performances. Among the 200 species of yeast examined for the production of ethanol from xylose, *Candida shehatae* and *Pichia stipitis* present the best performances in terms of yield and productivity [37]. Those two yeasts have been studied for ethanol production [11, 3, 28, 2]. One of the best performances was obtained with *C. shehatae* in fed-batch culture with xylose [12]: an ethanol concentration of 44 g l^{-1} was reached with a yield of 0.34 g g^{-1}. Such performance has identified *C. shehatae* as an interesting candidate for industrial fuel-ethanol production from lignocellulosic raw materials. *C. shehatae* is a crabtree-negative yeast [18] that mainly produces biomass and carbon dioxide when there is no nutritional limitation, but can produce ethanol in oxygen-limited conditions. Du Preez et al. [10] suggested that the oxygen availability and particularly the specific oxygen uptake rate (OUR) would be the parameter that determines the partitioning of the carbon flux between ethanol, growth and the production of polyols (xylitol, arabitol, ribitol and glycerol). Alexander et al. [3] performed the physiological characterization of *C. shehatae* in a chemostat in order to control the specific OUR by controlling the stirring rate, the airflow rate and the biomass concentration. The influence of the value of the specific OUR on *C. shehatae* physiology and kinetics in dynamic condition of ethanol production (batch or fed-batch culture) has not been investigated so far.

Previous experimental works carried out with *C. shehatae* in dynamic conditions of ethanol production without control of the specific OUR led to variability in the quantification of the ethanol yield and specific production rate. The ethanol yield over xylose varied from 0.29 g g^{-1} [11] to 0.45 g g^{-1} [35], with a maximum specific productivity of ethanol with xylose as the carbon source in the range of 0.13 g g DCW^{-1} h^{-1} [23] to 0.21 g g DCW^{-1} h^{-1} [3]. Obviously, the identification and understanding of the biological bottlenecks of *C. shehatae* need further investigations under controlled oxygen-limited conditions.

This paper extends the knowledge about *C. shehatae* metabolism during ethanol production. It quantifies the kinetics and carbon distribution in controlled fed-batch cultures with xylose and glucose (separately) as the carbon sources under various conditions, and it monitors and quantifies the effects of specific OURs. Moreover, it helps elucidate the relation among respiration, growth, ethanol and polyols production from xylose and glucose to deduce an innovative culture strategy for highly efficient biofuel production from lignocellulosic resources.

Materials and methods

Strain

Candida shehatae ATCC 22984 was stored in 20% glycerol at −80°C.

Chemicals

Chemicals products (glucose, salts, trace-elements and ammonia) were obtained from VWR International Inc., vitamins from Sigma and glutamate from Merck. All products were of the highest analytical grades.

Medium

The medium was a mineral medium designed in order to avoid nutritional limitations to a biomass concentration of 20 g DCW l^{-1} [13]. The composition of the mineral medium (pH 4.5) was as follows.

In g l^{-1}

KH_2PO_4, 4.5; $(NH_4)_2SO_4$, 3.0; $Na_2HPO_4{\cdot}12H_2O$, 1.5; sodium glutamate, 1; $MgSO_4{\cdot}7H_2O$, 1.

In mg l^{-1}

$ZnSO_4{\cdot}7H_2O$, 40.0; $MnSO_4{\cdot}H_2O$, 3.8; $CoCl_2{\cdot}6H_2O$, 21.0; $CuSO_4{\cdot}5H_2O$, 2.0; $Na_2MoSO_4{\cdot}2H_2O$, 4.1; $CaCl_2{\cdot}2H_2O$, 23.0; $(NH_4)_2Fe(SO_4)_6{\cdot}6H_2O$, 23.0; H_3BO_3, 3.0; panthotenate, 3.7; thiamine, 1.0; nicotinic acid, 1.5; pyridoxine, 0.5;

meso-inositol, 553.9; para-aminobenzoïc acid, 0.2; biotin, 0.01.

Pre-culture conditions

Yeast cells from freshly streaked YPD medium [yeast extract 1% (w/v), bactopeptone 2% (w/v) and 2% glucose (w/v)] and YPX medium [yeast extract 1% (w/v), Bactopeptone 2% (w/v) and 2% xylose (w/v)] were respectively used for inoculation of glucose and xylose cultures.

A colony was then inoculated in a 5-ml tube containing YPD or YPX media, shaken at 100 rpm with a rotary shaker for 12 h at 30°C. The inoculum in rich medium (5 ml) was then transferred in a 250-ml shake flask containing 30 ml of mineral medium plus 100 g l^{-1} of glucose or xylose. This was incubated in the shake flask, placed on a rotary shaker (100 rpm) at 30°C for 12 h, transferred to 250 ml of mineral medium in a 1-l flask and shaken (100 rpm) at 30°C for an additional 12 h.

Fermentations

Six fed-batch experiments were performed in a 5-l fermentor (B Braun International Biotech, Sartorius Group) with a working volume of 3 l. The temperature was regulated at 30°C and the pH maintained at the growth optimal value of 4.5 [31] by addition of a 14% (v/v) ammonia solution. The fermentor was flushed with air and stirred with a Rushton turbine. Gas inlet and outlet compositions were analyzed with a mass spectrometer (Ametek Process Instruments).

Feeding strategy

Experiments were carried out with xylose or glucose as carbon substrates. Pulses of sugar concentrated at 650 g l^{-1} were added during fed-batch culture when the substrate concentration was near 20 g l^{-1} to reach a concentration of 100 g l^{-1} in the reactor in order to be in large excess of carbon sources. Vitamins were added during fermentations as described by Alfenore et al. [5].

Analytical methods

Biomass concentration was measured spectrophotometrically at 620 nm (BIOCHROM® Libra S4) and plotted versus dry cell weight measurements determined as follows: an accurate volume of the culture was harvested and filtered on 0.45-µm-pore-size polyamide membranes, dried at 60°C under a partial vacuum (200 Hg) for 24 h and then weighed. The biomass formula was determined at ENSIACET (Toulouse, France) by elemental analysis of C, H, O, N and ash. The biomass formula used to convert cell dry weight into molar carbon concentration was $C_1H_{2.08}O_{0.66}N_{0.14}$, corresponding to a reduction degree of 4.34. Samples from the fermentor were centrifuged (12,000 g, 3 min) and then filtered on 0.2-µm-pore-size polyamide membranes to be analyzed. Extracellular metabolites (glucose, xylose, ethanol, polyols and organic acids) were quantified by HPLC except for acetate, which was analyzed by gas chromatography. The HPLC system (Alliance 2690, Waters) used an Aminex HPX 87 H 300 × 7.8-mm column coupled with a refractometer (Waters 2414) and a UV detector at 210 nm (Waters 996) under the following operating conditions: oven temperature 50°C, with 5 mM H_2SO_4 as eluant with a flow rate of 0.5 ml mn^{-1} and volume of injection 20 µl. For xylitol, arabitol and ribitol quantification, operating conditions were oven temperature 30°C with 2 mM H_2SO_4 eluant.

Results and discussion

The dynamic behavior of *C. shehatae* during aerobic growth and ethanol production was quantified using six fed-batch cultures on mineral medium with xylose or glucose as a carbon source at the optimum temperature and pH values for growth (respectively 4.5 and 30°C [31]). The cultures were carried out in two phases according to the fermentation strategy of Kastner et al. [21]: an aerobic growth phase (DOT over 20% of saturation value) until a final biomass concentration in the range of [8, 12 g DCW l^{-1}] and a microaerobic ethanol production phase under varying oxygen limitation levels (DOT null).

Oxidative metabolism with xylose or glucose as the carbon source

The main results are reported in Table 1, and the mass evolution of the biomass, sugar, ethanol, polyols cumulated (xylitol, arabitol, ribitol and glycerol) and specific OUR versus time are plotted in Fig. 1.

Growth on xylose

Carbon and reduction degree balances were calculated and were closed within 6% of the error maximum. The maximum growth rate was 0.42 h^{-1} on xylose, a higher value than the 0.28 h^{-1} mentioned by Alexander et al. [3] in similar pH and temperature conditions on rich medium. Concerning carbon distribution, 52% of carbon was affected to microbial growth and 41% of carbon was dedicated to carbon dioxide production. The other products accounted for only 7% of the total carbon consumed (Table 1). The average respiratory quotient defined as the ratio of the carbon dioxide production to the oxygen consumption rate was equal to 1.17, a value very close to 1,

Table 1 *Candida shehatae* growth characteristics in aerobic conditions at pH 4.5 and temperature 30°C in mineral media with xylose or glucose as carbon source

Carbon source	Xylose	Glucose
Yields over sugar (Cmol CmolS^{-1})		
Biomass	0.524	0.564
CO_2	0.408	0.384
Ethanol	0.023	0.003
Glycerol	0.018	0.023
Xylitol	0.007	0.000
Arabitol	0.000	0.024
Ribitol	0.007	0.003
Polyols (glycerol + xylitol + arabitol + ribitol)	0.032	0.050
Yield of biomass over oxygen (g DCW g O_2^{-1})	1.24	1.38
Average Respiratory Quotient RQ (mol CO_2 mol O_2^{-1})	1.17	1.15
Maximum growth rate μmax (h^{-1})	0.42	0.37
Maximum specific OUR (mmolO_2 g DCW^{-1} h^{-1})	9.13	7.46
Maximum specific sugar uptake rate (g g DCW^{-1} h^{-1})	0.67	0.54

which is the theoretical respiratory quotient for oxidative metabolism on sugar with a reduction degree of 4 [29].

The yield of biomass production over oxygen consumed was 1.241 g DCW g O_2^{-1}, below the value of 1.669 g DCW g O_2^{-1} obtained by Alexander et al. [3] in carbon-limiting continuous culture. This difference could be connected to the impact of high sugar concentration in the medium in fed-batch culture (20–100 g l^{-1}) compared to the carbon-limiting continuous culture reported in the literature.

Growth on glucose

The metabolic behavior of *C. shehatae* on glucose as the carbon source in aerobic conditions was similar to that on xylose. Few differences could be observed concerning macroscopic kinetics and yields. The maximum growth rate of 0.37 h^{-1} was lower than the one obtained with xylose as the carbon source (see Table 1), and the biomass over glucose yield was higher (56% of carbon consumed) as a consequence of the lower carbon dioxide and by-product yields over glucose (38% CO_2 and 5% by-product). This carbon distribution was very close to the results obtained by Fonseca et al. [14] with the crabtree-negative yeast *Kluyveromyces marxianus* with glucose as the carbon source: 60% of carbon was dedicated to growth, 34% to carbon dioxide production and 6% to by-product release.

Respiro-fermentative metabolism on xylose or glucose

Following the growth phase, aeration and stirring rates were decreased (Table 2) to shift from fully aerated to microaerated conditions with varying oxygen limitation conditions (Fig. 1 a, b, c, d, e, f). Two criteria were defined to quantify the oxygen limitation level, the average specific OUR $\overline{qO2}$ and its standard deviation.

The carbon distribution shifted from biomass to ethanol and polyols (xylitol, arabitol, ribitol and glycerol) as reported in Fig. 1 a, b, c, d, e and f. Moreover, organic acids (pyruvate, succinate and fumarate) were also excreted during oxygen limitation in low amounts (Table 2). Carbon balance and redox balance were closed within 10%.

For xylose fermentation, the average specific OUR was 0.80 (Fig. 1a), 1.19 (Fig. 1b) and 2.48 (Fig. 1c) mmol-O_2 g DCW^{-1} h^{-1}, which accounted for 7, 13 and 27%, respectively, of the maximum specific OUR obtained in aerobic conditions (Table 1). For glucose fermentation, the average specific OUR was 0.30 (Fig. 1d), 1.77 (Fig. 1e) and 2.46 (Fig. 1f) mmolO_2 g DCW^{-1} h^{-1} corresponding to 4, 24 and 33% of the maximum specific OUR on glucose in aerobic condition, respectively. It was not possible to maintain an average specific OUR much higher than 2.48 mmolO_2 g DCW^{-1} h^{-1} during oxygen limitation without shifting to aerobic conditions.

Ethanol final titer

The maximum ethanol concentration produced from xylose as the carbon source was 48.81 g l^{-1} with a specific OUR value of 1.19 mmolO_2 g DCW^{-1} h^{-1}, whereas the maximum ethanol concentration from glucose as the carbon source was 54.19 g l^{-1} with a specific OUR value of 2.46 mmolO_2 g DCW^{-1} h^{-1} (Table 2). These concentrations were in agreement with the results of Kastner et al. [21] and Du Preez et al. [12] in fed-batch fermentation.

Ethanol yield

The maximum of the ethanol yield on xylose (Rxyl,p) was 0.426 Cmol CmolS^{-1} or 0.327 g g^{-1} (64% of theoretical yield) with an average OUR value of

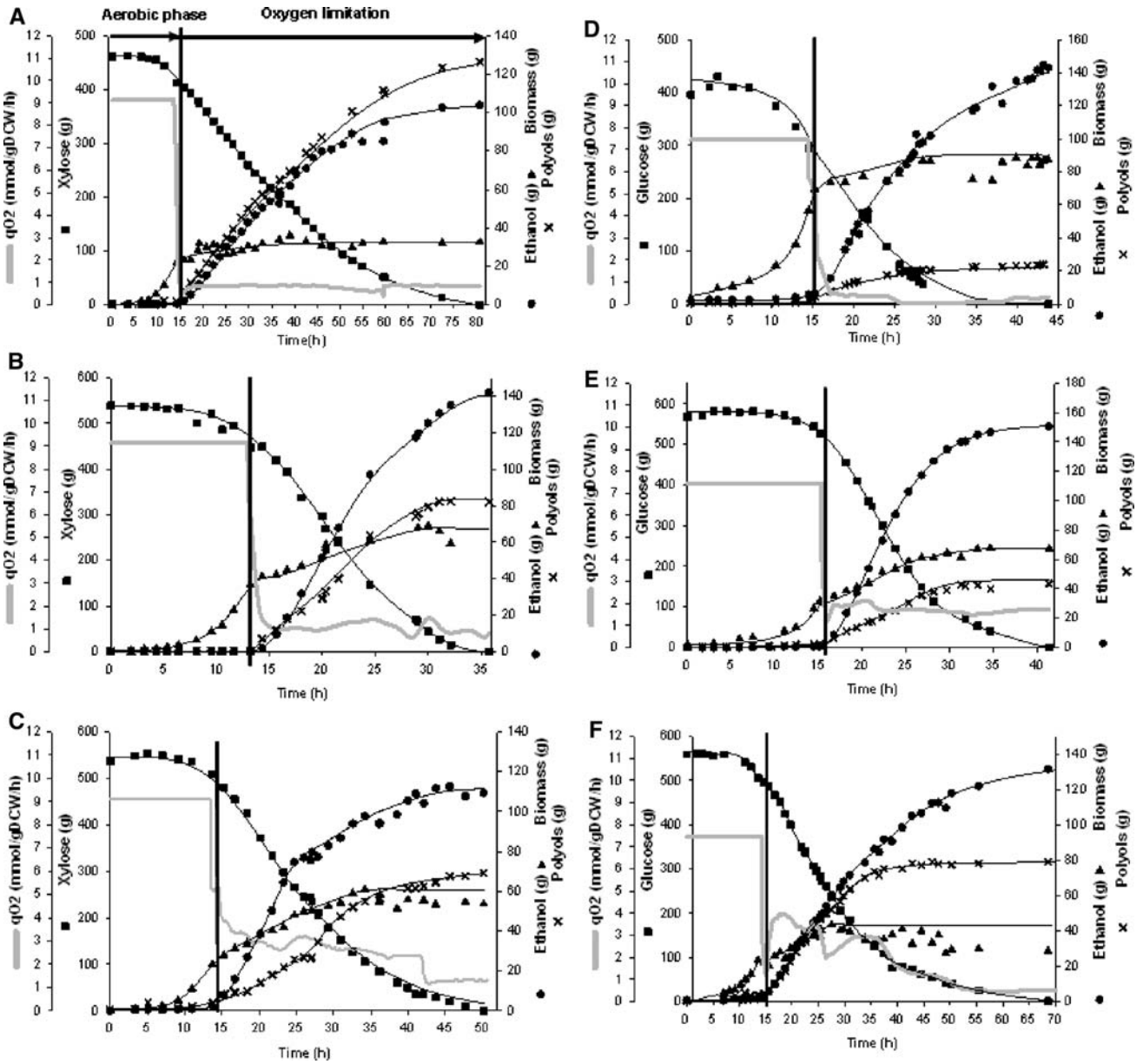

Fig. 1 Mass evolution of xylose, biomass, ethanol, total polyols (xylitol, arabitol, ribitol and glycerol) and specific OUR (qO_2) versus time in fed-batch culture for different oxygen-limited conditions at pH 4.5 and 30°C. Xylose was the carbon source for **a** (xylose-1 experiment, $\overline{qO_2} = 0.80$ mmolO_2 g DCW^{-1} h^{-1}), **b** (xylose-2 experiment, $\overline{qO_2} = 1.19$ mmolO_2 g DCW^{-1} h^{-1}) and **c** (xylose-3 experiment, $\overline{qO_2} = 2.48$ mmolO_2 g DCW^{-1} h^{-1}). Glucose was the carbon source for **d** (glucose-1 experiment, $\overline{qO_2} = 0.30$ mmolO_2 g DCW^{-1} h^{-1}), **e** (glucose-2 experiment, $\overline{qO_2} = 1.77$ mmolO_2 g DCW^{-1} h^{-1}) and **f** (glucose-3 experiment, $\overline{qO_2} = 2.46$ mmolO_2 g DCW^{-1} h^{-1}). During the aerobic phase, we chose to simplify the graph by representing the specific OUR at its maximum value

1.19 mmolO_2 g DCW^{-1} h^{-1} (Xylose-2), an intermediate level of specific OUR investigated in these works (Fig. 2). These results were close to those obtained with *Pichia stipitis* [7, 16, 33, 1]. Moreover, the maximum ethanol yield over xylose was close to the ethanol yield of 0.35 g g^{-1} obtained with xylose engineered *S. cerevisiae* [30]. This maximum yield was reached for an intermediary specific OUR because of the inverse tendencies observed in Fig. 2: when the specific OUR increased, the CO_2 yield increased, but the xylitol yield decreased (biomass yield slightly increased).

When glucose was the carbon source, the ethanol yield on glucose (Rglc,p) reached 0.535 Cmol CmolS^{-1} (80% of theoretical yield) with the lowest specific OUR investigated (Table 2, Fig. 2). When the specific OUR was higher, polyol and CO_2 yield over sugar were higher, and hence as

Table 2 Main results for the oxygen-limited phases of *Candida shehatae* cultures performed at pH 4.5 and temperature 30°C in mineral media with xylose or glucose as the carbon source

Carbon source	Xylose			Glucose		
Experimentation name	Xylose-1	Xylose-2	Xylose-3	Glucose-1	Glucose-2	Glucose-3
Averaged specific OUR (mmolO_2 g DCW^{-1} h^{-1})	0.80 ± 0.35	1.19 ± 0.09	2.48 ± 0.77	0.30 ± 0.18	1.77 ± 0.16	2.46 ± 0.91
Duration (h)	65	22	36	29	27	90
Aeration rate (VVM)	0.6	0.2	0.6	0.7	0.4	0.1
Stirring rate (RPM)	10	500	400	100	450	550
Yields (Cmol $CmolS^{-1}$)						
Ethanol	0.359	0.426	0.322	0.535	0.435	0.394
Xylitol	0.287	0.110	0.041	0	0	0
Arabitol	0	0	0.033	0.013	0.039	0.119
Ribitol	0.013	0.038	0.053	0.010	0.011	0.022
Glycerol	0.016	0.021	0.023	0.035	0.038	0.015
Total polyols (glycerol + xylitol + arabitol + ribitol)	0.316	0.169	0.150	0.058	0.088	0.156
Biomass	0.032	0.074	0.094	0.083	0.093	0.048
Organic acids (pyruvate + succinate + fumarate)	0.009	0.016	0.018	0.026	0.016	0.031
μmax (h^{-1})	0.02	0.05	0.06	0.02	0.08	0.06
qEtOHmax (g g DCW^{-1} h^{-1})	0.12	0.22	0.22	0.14	0.35	0.22
qSmax (g g DCW^{-1} h^{-1})	0.42	0.65	0.67	0.34	0.92	0.88
Average ethanol productivity (g l^{-1} h^{-1})	0.63	2.22	1.04	1.44	1.81	0.60
[ETOH] max (g l^{-1})	41.37	48.81	37.71	42.01	49.23	54.19

biomass yield remained quite constant, the ethanol yield decreased (Fig. 2). Thus, xylose fermentation compared to glucose fermentation was different in terms of carbon distribution. This is in agreement with the experiments of Ligthelm et al. [25] in shake flasks with *C. shehatae* in oxygen-limited conditions where Rglc,p was 0.56 Cmol $CmolS^{-1}$ and an ethanol yield over xylose of 0.48 Cmol $CmolS^{-1}$.

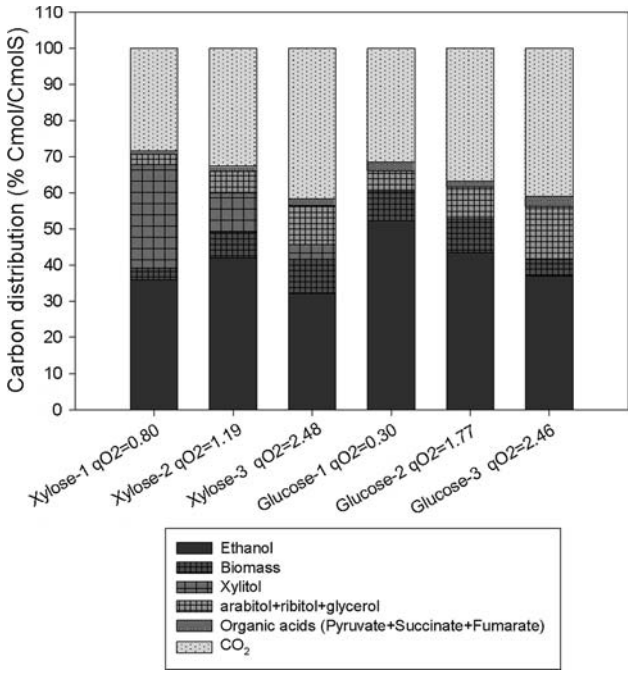

Fig. 2 Carbon distribution in Cmol $CmolS^{-1}$ for xylose and glucose fed-batch fermentations in oxygen-limited conditions of *C. shehatae* at pH 4.5 and 30°C. The average specific OUR is expressed in mmolO_2 g DCW^{-1} h^{-1}

Ethanol yield on glucose and xylose was influenced by polyol production; the maximum polyol production yield on xylose was 0.316 Cmol $CmolS^{-1}$ for the minimum specific OUR (xylose-1 experiment) compared to 0.156 Cmol $CmolS^{-1}$ on glucose obtained for the maximum specific OUR (glucose-3 experiment).

Thus, ethanol yield was specific OUR dependent, as suggested by Du Preez et al. [10], and carbon source dependent (xylose or glucose).

Productivities

The highest average ethanol productivities were 2.22 g l^{-1} h^{-1} on xylose and 1.81 g l^{-1} h^{-1} on glucose (xylose-2, glucose-2, Table 2).

When xylose was the carbon source, the maximum specific productivity of ethanol was 0.22 g g DCW^{-1} h^{-1} with the two highest specific OURs, i.e., 1.19 and 2.48 mmolO_2 g DCW^{-1} h^{-1}, very close to the 0.20 g g DCW^{-1} h^{-1} obtained by Alexander et al. [3]. With glucose the maximum specific ethanol productivity was 0.35 g g DCW^{-1} h^{-1} for a specific OUR of 1.77 mmolO_2 g DCW^{-1} h^{-1} (Table 2).

The specific ethanol productivity difference between xylose and glucose may be attributed to a xylose uptake rate limitation since for the xylose-2, xylose-3 and oxidative phase, the maximum specific xylose uptake rate was in the range of 0.65–0.67 g g DCW^{-1} h^{-1} (Tables 1, 2). It has been reported that the xylose uptake rate could be limiting in *P. stipitis* aerobic culture [25]. The value of the xylose uptake rate obtained was lower than the maximum glucose uptake rate of 0.92 g g^{-1} h^{-1} (Table 2).

In oxygen-limited conditions, the maximum growth rate dropped sharply to 0.06 and 0.08 h^{-1} on xylose and glucose, respectively (Table 2). The specific OUR strongly influenced the maximum growth rate: the higher the specific OUR, the higher the maximum growth rate for a given carbon source except for experiment glucose-3 where biomass yield over glucose was lower than those reached in glucose-2 and xylose-2 experiments (Table 2). Moreover, the maximum growth rate was lower than those obtained with similar specific OURs by Alexander et al. [3] in a chemostat (0.06 h^{-1} vs. 0.11 h^{-1}), probably due to higher ethanol concentrations that inhibited growth [12].

Specific rates of ethanol production (qEtOH) and growth (μ) during ethanol production

The specific rates of ethanol production and growth during fermentation of xylose and of glucose were compared. Typical profiles of the specific rates are plotted in Fig. 3 (xylose-2 experiment, glucose-2 experiment). Within the range of ethanol concentrations from 0 to 15 g l^{-1}, increases in both growth and ethanol production were observed; when the ethanol concentration was higher than 15 g l^{-1}, the specific rates decreased according to the ethanol inhibition effect on growth and ethanol production [12]. Moreover, ethanol production appeared to be coupled to biomass production for both carbon sources, probably to satisfy the energy requirement to sustain growth when energy generation by respiration was limited. This is a known phenomenon in *S. cerevisiae* cultures. Indeed, Alfenore et al. [4] obtained a coupling relation between the specific ethanol production rate and the specific growth rate in oxygen-limited conditions.

In brief, in the range investigated, an intermediary-specific OUR (xylose-2 experiment) maximized Rxyl,p, ethanol productivity and the final ethanol titer. For glucose fermentation, the highest value of Rglc,p was obtained when the lowest specific OUR (glucose-1 experiment) was reached, whereas the highest productivity was obtained for an intermediary specific OUR value (glucose-2 experiment), and the maximum ethanol final titer was obtained for the highest specific OUR (glucose-3 experiment).

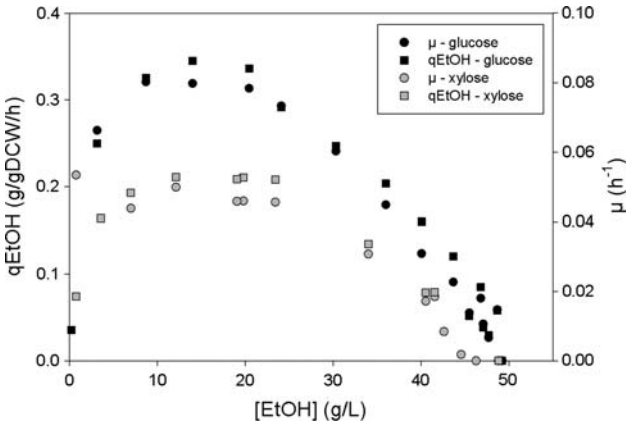

Fig. 3 Evolution of the specific ethanol production rate and of the growth rate for the xylose-2 experiment (xylose as a carbon source) and glucose-2 experiment (glucose as a carbon source) versus the ethanol concentration in the oxygen-limited conditions of fed-batch fermentation of *C. shehatae* at pH 4.5 and 30°C

Polyol production

According to previous studies [8], polyols are involved in carbohydrate storage, in microbial response to osmotic pressure, translocatory compounds and redox sink.

In oxygen-limited cultures, the rate of NADH reoxidation by respiration is limited. The production of one mole of polyol, either xylitol, arabitol or ribitol, could theoretically reoxidize a maximum of one mole of the co-factor NADH [15]. These considerations are similar to those related to *S. cerevisiae* metabolism when the specific OUR in oxygen limitation decreased and the specific glycerol production rate increased [39] in order to reoxidize NADH produced by anabolic reactions.

We investigated the relationship among polyol production, biomass production and respiration by calculating the yield of polyol produced over biomass (in mmolPolyol g DCW^{-1}) in the oxygen-limited phase on xylose and glucose for the six fermentations.

The effect of the specific OUR value and the type of carbon source on each polyol yield over biomass (Fig. 4) was analyzed. Ribitol yield over biomass was mainly carbon source dependent and not specific OUR dependent. It was about four times lower on glucose than on xylose. Glycerol yield over biomass decreased when the specific OUR increased for both carbon sources. Concerning arabitol production, the yield over biomass increased when the specific OUR increased and was much higher on glucose than on xylose. The xylitol yield decreased sharply when the specific OUR increased.

The ratio of the amount of total polyol over biomass produced during oxygen limitation was in the range of 7.5–18.1 mmol polyol g DCW^{-1}. Nevertheless, considering arabitol, ribitol and glycerol production, this ratio was

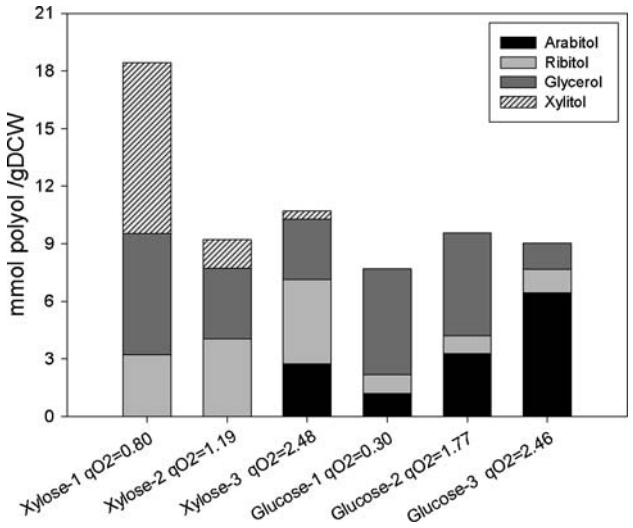

Fig. 4 Evolution of the yield of cumulated polyol over biomass produced during the oxygen limitation phase (amount of polyol produced divided by the amount of biomass produced) for the six fermentations on glucose and xylose with *C. shehatae* on mineral media at pH 4.5 and 30°C. For the glucose-3 experiment as growth stopped at 27.5 h of culture, the yield was calculated only on the growth phase of the oxygen-limited phase. The average specific OURs are expressed in mmolO_2 g DCW^{-1} h^{-1}

between 7.5 and 10.6 mmol polyol g DCW^{-1}. Stoichiometrically, this ratio was equivalent to a maximum of 7.5–10.6 mmol NADH reoxidized per g DCW. These values were very close to the anabolic yield of NADH production over biomass calculated by Verduyn et al. [38] for *S. cerevisiae* (11 mmolNADH g DCW^{-1}) and estimated by Franzén [15] (7.5–12.0 mmolNADH g DCW^{-1}). Hence, arabitol, ribitol and glycerol could contribute to NADH reoxidation within anabolic reactions.

Xylitol production was not correlated to anabolism as the maximum xylitol production yield reached 0.287 Cmol $CmolS^{-1}$ for the lowest specific OUR experiment (xylose-3 experiment) with the lowest biomass production yield over xylose (0.032 Cmol $CmolS^{-1}$). This was in agreement with the assumption that xylitol production was related to the NADH/NADPH imbalance between the enzymes xylose reductase and xylitol dehydrogenase [6, 12, 19, 20].

Conclusions

The dynamic physiological behavior of *Candida shehatae* was evaluated in fed-batch fermentations with xylose or glucose as the carbon source in various aeration conditions corresponding to quantified specific OUR values, without any nutritional limitations. These experimental works, under strictly controlled conditions, gave new insights into the effect of oxygen limitation on ethanol production with *C. shehatae*. It is a key parameter affecting the kinetics and carbon distribution of the microbial metabolism during intensive ethanol production.

Without oxygen limitations, oxidative metabolism occurred with mainly biomass and carbon dioxide production with yields over sugar of 0.52 Cmol $CmolS^{-1}$ and 0.41 Cmol $CmolS^{-1}$ on xylose, and 0.56 Cmol $CmolS^{-1}$ and 0.38 Cmol. $CmolS^{-1}$ on glucose, respectively. The maximum specific growth rate on xylose was 0.42 h^{-1} and on glucose 0.37 h^{-1}.

From the analysis of xylose and glucose fermentations, it has been proposed that the lower specific ethanol productivity on xylose compared to glucose could be attributed to a xylose uptake rate limitation: for the three different aeration conditions tested in these works, the maximum specific xylose uptake rate was in the range of 0.65–0.67 g. g DCW^{-1}. h^{-1} compared to a maximum glucose uptake rate of 0.92 g. g DCW^{-1}. h^{-1}.

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