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The yeast *Candida tenuis*: assessment of the organism for xylose fermentation and stereospecific reduction of ketones

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Contents

Anaerobic and microaerobic fermentation of xylose by native *Candida tenuis*

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Abstract

One of the main drawbacks of the known xylose fermenting yeasts for utilization in the ´lignocellulose to ethanol` process is the requirement of well-defined oxygen levels for the efficient conversion of xylose. Maintaining this critical low oxygenation is unsuitable for industrial purposes. Altering the aeration rate as well as setting anaerobic conditions results in an unfavorable impact on yields. While ethanol productivity declines to levels not economically viable, by-product formation (especially xylitol yield) emerges.

We investigated the effect of different oxygen transfer rates as well as anaerobic conditions on the ethanol yield and by-product formation with native yeast *Candida tenuis* CBS 4435. Low oxygenation levels (<10 µM h^{-1}) resulted in $Y_{ethanol}$ with highest values up to 0.45 g g^{-1}, while by-product formation remained at very low levels. $Y_{xylitol}$ and $Y_{glycerol}$ never exceeded values above 0.08 and 0.04 g g^{-1}, respectively.
New perspectives emerge when looking at anaerobic conditions where yields even remained at levels akin. Highest $Y_{ethanol}$ reached a value of 0.42 g g^{-1} and yet $Y_{xylitol}$ and $Y_{glycerol}$ never exceeded 0.07 g g^{-1} and 0.04 g g^{-1}, respectively. The average value of q_{xylose} was 0.16 g (g_{CDW} h) $^{-1}$. We also detected formation of small amounts of ribitol in the late phase of fermentation both in anaerobic as well as microaerobic xylose conversions.
Furthermore, we found a relationship between the morphogenic states of *Candida tenuis* and the fermentation efficiency. We achieved best results with cells in the teleomorph state while in anamorph state efficiency declined at high degrees.

Introduction

Elaborate research has driven knowledge of alternative energy sources forward over the last four decades. A prospect towards fuelling cars with carbon dioxide neutral energy is the green milestone `bioethanol´ from abundant, non nutrient-competitive lignocellulosic material [2]. Being one of the main carbohydrate constituents, xylose is metabolized by native xylose fermenting yeasts to ethanol under stringent conditions [7], [14], [25].

It has been supposed that there is a critical level of oxygenation at which productivity and ethanol yield are reaching their maximum value. Below this level, the ethanol yield drops significantly as reported so far for *Pachysolen tannophilus, Pichia stipitis and Candida shehatae* [4], [5], [6], [14], [15]. A lot of effort has been devoted to the characterization and optimization of microorganisms capable of metabolizing xylose to ethanol. Interest in prime choice *P. tannophilus* diminished subsequently when fermentation knowledge of *Candida* and *Pichia* species emerged with their evidently better ethanol yield combined with less by-product formation.

Under well-defined low aeration conditions, which are problematic for industry purposes [25], the highest ethanol yield settles between 0.36 and 0.48 g g^{-1} for *C. shehatae* [11] and for closely related *P. stipitis* [4], respectively. While there is hardly any formation of by-products (small amounts of glycerol) with *P. stipitis*, the xylitol yield arises to 0.15 g g^{-1} with *C. shehatae* [11] and even higher values for other yeasts [7], [11]. This approximates nearly half of the ethanol yield of *C. shehatae*. Under industry-favorable anaerobic conditions, the ethanol yield of both organisms drops below values of ≤0.25 g g^{-1} while by-product formation remains at or arises to inauspicious levels akin [11]. Under anaerobiosis a lot of metabolic related disadvantages emerge. In *P. stipitis* for example, the lack of oxygen seems to decrease ethanol production due to a redox imbalance.

This problem is common among many native yeast strains capable of fermenting xylose [12]. This redox imbalance under anaerobic conditions is caused by the two main enzymatic steps of xylose metabolization in native yeasts due to oxygen dependent regeneration effects [2], [12]. The first reduction step to xylitol is catalyzed by xylose reductase (EC 1.1.1.21), which is further oxidized via xylitol dehydrogenase (EC 1.1.1.9) to xylulose. While xylose reductase is NAD(P)H preferring, xylitol dehydrogenase is NAD^+ dependent.

Ceasing mitochondrial function and consequential energy loss, which would be needed for xylose import [12] appear to be the consequence of the redox imbalance. This also results in severely curtailed growth and decreased specific growth rates. During stationary growth at the late phase of fermentation also levels of specific enzymatic activities decrease greatly and formation of xylitol increases [11], [12], [13]. Furthermore, in Kluyver positive yeasts like *Candida utilis* anoxic conditions completely annihilate the fermentative capability for xylose [12]. This might be ascribed to oxygen dependent transport mechanisms as well as to a redox imbalance.

In order to overcome this redox imbalance, a lot of strategies have been developed through scientific efforts in cost-intensive and time consuming genetic work, leading to both reduced by-product formation as well as increased ethanol yields [24].

So far detailed investigation of *Candida tenuis* was only reported under aerobic conditions with the aim of improving enzymatic induction [1], [22]. While *Candida tenuis* xylose reductase (*Ct*XR) has been extensively examined and modified in the context of building efficient host systems for xylose utilization and biocatalysis, less work was attributed to the native whole cell system in the context of xylose utilization [3], [16], [17], [23]. Only one common screening of yeasts for the capability of xylose conversion ascribed *Candida tenuis* to be one of the better xylose fermenting yeasts [14]. However,

the effects of the oxygen transfer rate (OTR) on xylose utilization and by-product formation were not investigated therein [14].
Though there are many different *Candida tenuis* strains with varying ethanol production efficiency [14], further work focused on other ones like *Pichia stipitis* because of the evident high ethanol yield and low by-product formation.

This thesis covers a detailed physiological characterization of *Candida tenuis* concerning the effects of low oxygen transfer rates (<10 µM h^{-1}) and anaerobic conditions on the ethanol yield and by-product formation from xylose. This includes the effects of morphogenic switches on fermentation performance in preculture as well as in bioreactor experiments. Furthermore the multiple relationships and impact of morphology on enzymatic expression levels of *Ct*XR and *Ct*XDH under different oxygen transfer rates as well as under anaerobiosis have been investigated.

Despite dual-coenzyme specificity of *Ct*XR, low by-product yields have been observed under anaerobic conditions [3], [19]. This implies efficient coenzyme regeneration not only under microaerobic but a fortiori under anoxic conditions.

Materials and methods

Chemicals

NADH (sodium salt; ≥98 % pure) and NAD^+ (free acid; ≥97.5 % pure) were purchased from Roth (Karlsruhe, Germany). Glass beads (0.5 mm diameter) were obtained from BioSpec Products (Bartlesville, USA). All other chemicals used were of the highest quality and purity available and obtained from Sigma-Aldrich / Fluka (Gillingham, Dorset, U.K.) or Roth (Karlsruhe, Germany). N_2 and CO_2 for aeration, as well as the reference gas for calibration of off-gas analytics were obtained from Linde GAS GmbH (Graz, Austria). Two mL tubes were purchased at Eppendorf Austria GmbH (Wien, Austria). Fifteen mL and 50 mL tubes were obtained from Sarstedt (Wr. Neudorf, Austria).

Organism and culture conditions

Candida tenuis CBS 4435 strain was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). Unless otherwise indicated, all precultures were grown on 20 g L^{-1} D-xylose as C-source in a defined mineral medium containing 14.4 g L^{-1} KH_2PO_4, 5 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4 \times 7H_2O$ and 0.025 % (v/v) Antifoam 204 (Sigma-Aldrich). As described elsewhere in full detail [10], trace elements and vitamins were added, except for riboflavin and folic acid. Precultures were incubated at 25℃ on a rotary shaker at 125 rpm. They were inoculated by transferring a loopful of cells from an agar plate into a baffled 300 mL Erlenmeyer flask containing 50 mL of medium and were then transferred into baffled 1000 mL shake flasks containing 300 mL of medium. The later cultures were grown from an estimated start optical density at 600 nm of 0.05 until a value of 3.5 was reached and harvested by centrifugation (4400 g, 15 min, 4℃).

Bioreactor experiments

All fermentations were carried out in a Labfors 3 bioreactor (Infors HT, Bottmingen-Basel Switzerland) equipped with two six bladed disc impellers. The bioreactor had a working volume of 2 L, wherein the ratio of impeller to reactor diameter was 0.4.

Twenty g L^{-1} D-xylose was used as C-source in an optimized buffered medium containing the same ingredients as the preculture medium except for 3 g L^{-1} KH_2PO_4, 10 mg L^{-1} ergosterol and 0.42 g L^{-1} Tween-80. The pH was adjusted to 4.5 and further controlled via the bioreactor unit by the addition of 1 M H_3PO_4 or 1 M NaOH at a setpoint of 4.5±0.1.

The batch process was operated at 25℃, 200 rpm and at different aeration conditions. Anaerobic conditions where maintained by sparging the bioreactor with 0.16 vvm N_2. Surface gassing was performed by diffusion of surrounding air trough an air filter with 50 mm of diameter (Acro 50, PALL Corporation, USA), which was connected to the top plate of the vessel unit.
When bioreactor experiments were done at least in duplicate, results show mean values and the corresponding standard deviation (S.D.).

Shakeflask experiments

Anaerobic and microaerobic (≤1 µM h^{-1}) shake flask cultivations were carried out in 100 mL non-baffled Penicillin-flasks (Lactan GmbH, Austria). Microaerobic conditions, in that case, were set up by plunging a needle equipped with a filter trough the rubber part of the tightly closed cap. Anaerobiosis was maintained during fermentation by a tightly closed shake flask. These experiments were done on a rotary shaker at 180 rpm at 25℃.

For employing an OTR of 7±1 µM h^{-1} 300 mL baffled shake flasks that were tightly closed with rubber stoppers have been used. Two glass tubes were inserted in the stopper, one with a valve for purging with N_2 and another with a needle equipped with a filter, plugged in at its far end that served as gas exchange. These baffled shake flasks were incubated at 25℃ on a rotary shaker at 110 rpm.

Anaerobic conditions before inoculation were always reached by gassing the medium with N_2.
The medium used as well as the carbon source were the same as in preculture medium, except for 10 mg L^{-1} ergosterol and 0.42 g L^{-1} Tween-80 added.
Shake flask experiments were done in duplicate or triplicate and results show mean values as well as the corresponding S.D.

Analytical methods

Samples taken from the bioreactor or shake flasks were immediately worked-up by centrifugation (10 min, 15700 g, 4℃). After storage at -20℃, the supernatant was analyzed by HPLC and the remaining cell pellet was used for enzymatic studies. The volume of the bioreactor sample was 10 mL, whereas the volume of the shake flask sample was 2 mL.

HPLC analysis was performed on a LaChrome HPLC system (Merck-Hitachi) with an L-7400 UV-detector, L-7490 RI-detector and a thermo-stated column oven. An Aminex HPX-87H column (BioRad, Hercules, CA, USA) with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}, thermostated at 65℃, was used for determination of metabolites (et hanol, xylitol, acetate, pyruvate, lactate, succinate, ribitol) and xylose concentrations [17]. For further determination of glucose, xylose and xylitol concentrations an Aminex HPX-87C column with water as the mobile phase at a flow rate of 0.4 mL min^{-1}, thermostated at 85℃, was employed [16].

Cell dry weight (CDW) was determined gravimetrically. The sample work-up was done by centrifugation (10 min, 15700 g, 4℃), discarding the supernatant, resuspending and washing the pellet in distilled H_2O. After another centrifugation step and discarding the supernatant, the pellet was resuspended in distilled H_2O, transferred into glas vials and dried at 105℃ for at least 24 h. Cooling of vials including samples was performed in an exsiccator.

Cell growth was estimated from the optical density at 600 nm in both shakeflask and bioreactor experiments. Cell growth was further investigated by colony forming units (CFU). Bioreactor probes were used for dilution streaks (quadruplicates, 1E-3 to 1E-7) on YPD-agar plates. Plates were incubated at 25℃ for at least 48 hours, evaluated by counting of colonies and calculation of mean values.

Off gas analytics were done using an Innova 1313 acoustic gas analyzer (LumaSense Technologies, Ballerup, Denmark). It was calibrated with reference gas containing 0.1 % ethanol and 5.0 % CO_2, the reminder being N_2. For *carbon balance calculations* where off gas analytic was inaccessible, it was assumed that one mol of CO_2 was formed per mol of ethanol or acetate.

Preparation of cell extract

Equal amounts of cell wet weight and of glass beads were mixed together. 200 µL of 50 mM potassium phosphate buffer (PPB) pH 7.0 was added and cells were mechanically disrupted by vortexing for 30 seconds and subsequent storage on ice for 30 seconds. This process was repeated tenfold. By further centrifugation (2 min, 4400 g, 4℃) cell debris was removed and enzyme activities were measured in this crude cell extract.

Enzyme assays

Xylose reductase (XR) and xylitol dehydrogenase (XDH) activities were assayed spectrophotometrically at 25℃ by following the formation or depletion of cofactor NADH at 340 nm (ε_{NADH} = 6.22 cm^{-1} mM^{-1}). The XR assay contained 0.35 mM NADH and 700 mM xylose in a 50 mM PPB at pH 7.0. The XDH assay was comprised of 3.5 mM NAD^+ and 700 mM xylitol in 50 mM Tris HCl buffer at pH 9.0.

Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad Laboratories; Hercules, USA) referenced against known concentrations of BSA.

Results

Morphology and fermentation performance

As a yeast, dimorphism is one of the morphogenic characteristics of *C. tenuis*. In the growth time course of a preparatory culture we investigated the relation of the parameters optical density (OD) at 600 nm, pH-value and morphology via phase contrast microscopy.

There is a switch in morphogenic states from the yeast form (teleomorph) at low values of OD, passing a transition state at an OD of about 6 where a mixture of yeast and pseudohyphae forms are found, to mainly pseudohyphae forms at high OD values (anamorph) (Fig. 1).

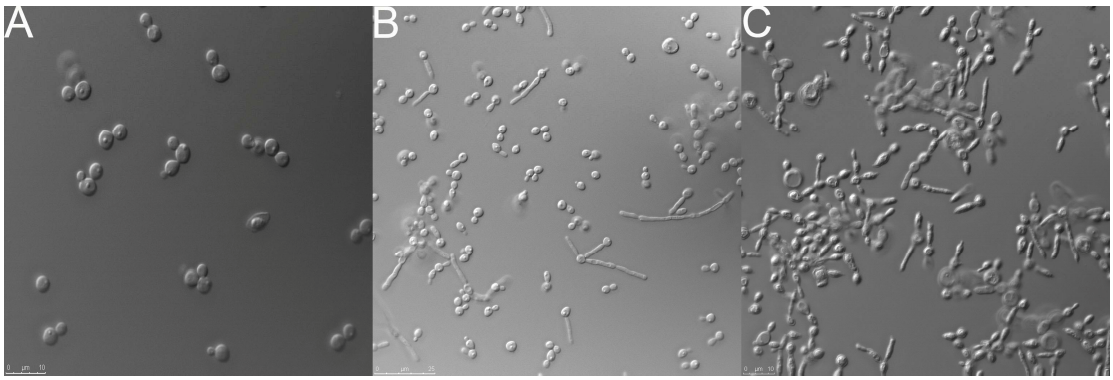

Fig. 1 Switch in morphogenic states on xylose from yeast (A) OD_{600} = 3 via transition state (B) OD_{600} = 6 to pseudohyphae forms (C) OD_{600} = 10.

In correlation to exponential growth there is a comparable pH shift in preparatory culture when grown in liquid media on xylose (Fig. 2) as well as on glucose. No detectable shift in amounts of acids (pyruvate, lactate, acetate) was found by HPLC analysis over the whole growth time of preparatory cultures.

A morphogenic switch from yeast to pseudohyphae forms could also be observed in the late phase of bioreactor experiments after about 75 hours of fermentation, wherein the pH was kept at its initial value of 4.5±0.1.

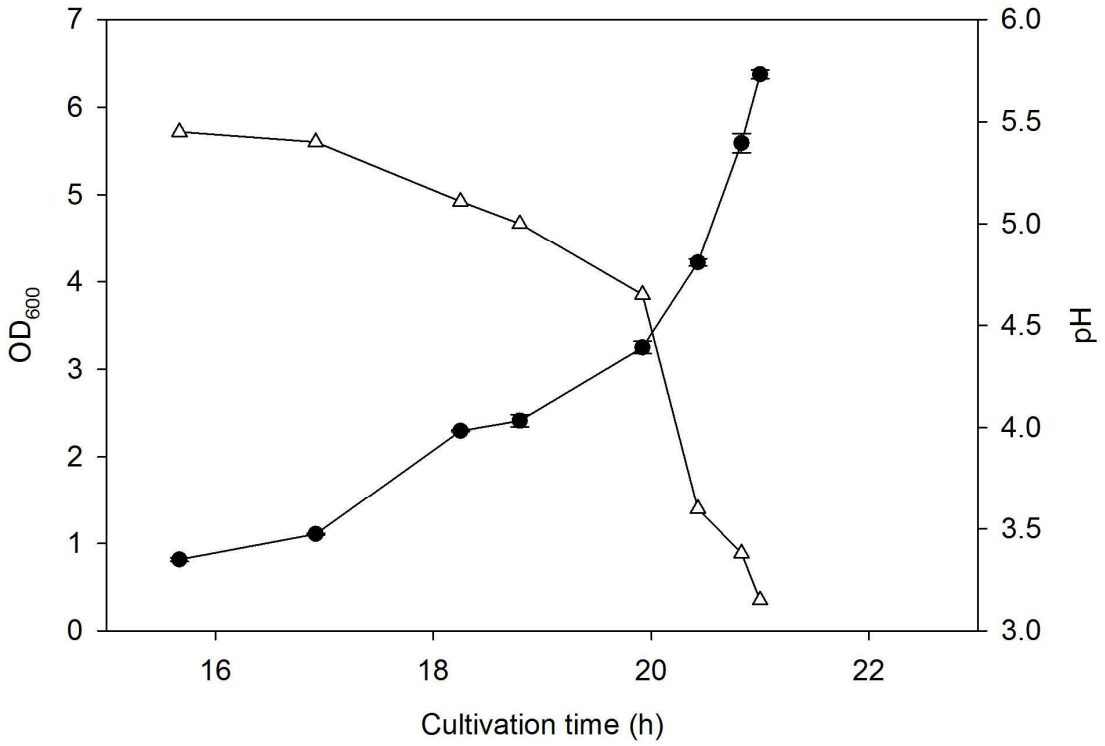

Fig. 2 Correlation of pH (open rectangle) and optical density at 600 nm (black dots) in a preparatory culture on xylose.

We encountered problems with transferring cells from preculture at high OD values (≥6) into bioreactor for anaerobic xylose conversion. High OD values in the precutures resulted in low metabolic activity during fermentation. To get accurate and morphogenic state independent data (only yeast form), precultures were therefore harvested below an OD value of 4.

Effect of aeration rate

Various oxygen transfer rates (OTR) in the range from 1 up to 10 µM h^{-1} (details are given in the methods section), as well as complete anaerobic conditions, obtained by N_2 aeration, have been tested.
Xylose uptake rates and yield coefficients were calculated based on the linear range up to a maximum fermentation time of 70 h.

We did not find a sharp inflexion point with *C. tenuis* between anaerobic and microaerobic conditions. There is hardly any correlation between varying OTR in the range from 0 up to 10 µM h^{-1}, yield coefficients (g g_{xylose}^{-1}) and xylose uptake rate (g g_{CDW}^{-1} h^{-1}). as given in (Table 1). Throughout the different tested OTRs the average $Y_{ethanol}$ is 0.42, which comes along with very low by-product formation. The average $Y_{xylitol}$ and $Y_{glycerol}$ is 0.04 and 0.03, respectively, and there is no effect ascribable to varying oxygenation levels. By-product formation of pyruvate, lactate, acetate or succinate remained at yields beyond values of 0.01 g g^{-1}.

During the late phase of fermentation (≥85 h), small amounts of ribitol are formed with an average maximum value of 35±2 mg L^{-1} in anaerobic shake flask and 12±1 mg L^{-1} in microaerobic fermentation experiments. This has so far only been reported for closely related *Candida shehatae* under microaerobic conditions [9]. The average value of q_{xylose} is 0.16±0.03.

Microaerobic conversion rates with an OTR of 7±1 µM h^{-1} using glucose as carbon source showed comparable uptake rates and ethanol yields ($q_{glucose}$ = 0.14±0.01, $Y_{ethanol}$ = 0.41±0.01). The only accountable by-product formation of glycerol comes up to a $Y_{glycerol}$ of 0.04±0.01.

In anaerobic bioreactor experiments a small increase in OD values might rather be attributed to stalling cells than to growth (μ_{max} = 0.004 h^{-1}). These results are further reinforced by nearly consistent CDW (Fig. 3) and CFU values.

Very little growth (μ_{max} = 0.01 h^{-1}) occurs under microaerobic conditions with an OTR of about 4 µM h^{-1} and it seems that higher OTRs (comparision of anaerobic and microaerobic) stimulate growth in small fields, concluded from a small increase in CDW (ΔCDW 0.35±0.05 g L^{-1}).

Previous shake flask experiments coincide with consecutive bioreactor experiments (Fig. 3): low by-product formation comes along with high ethanol yield.

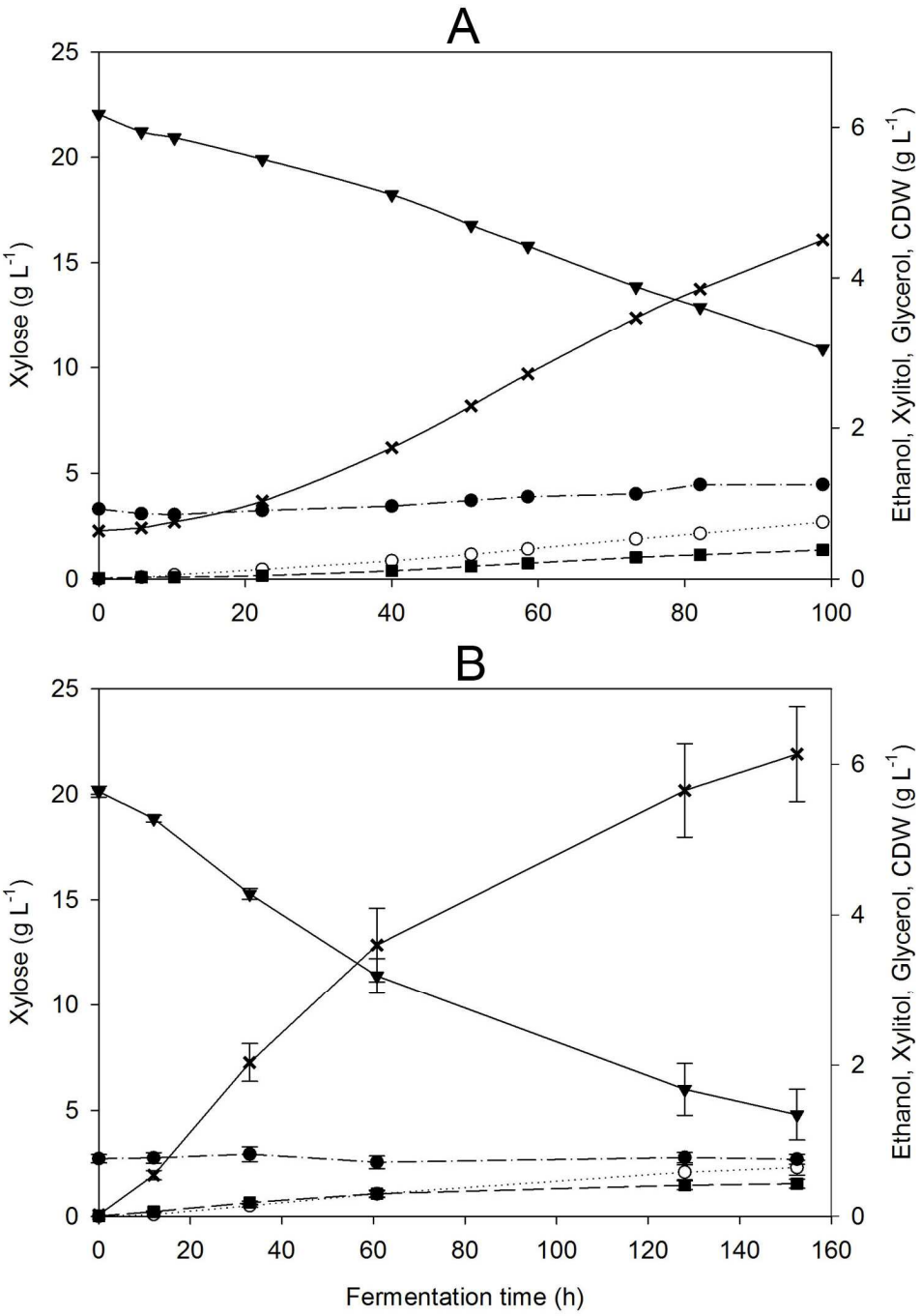

Fig. 3 Time course of xylose conversion in an anaerobic bioreactor (A) and an anaerobic shake flask (B) experiment. Symbols: triangles, D-xylose; crosses, ethanol; closed circles, CDW; open circles, xylitol; squares, glycerol.

Enzymatic expression levels

In the studied range of OTRs, xylose reductase and xylitol dehydrogenase (*Ct*XDH) activities remained at levels akin (Table 1) and were comparable to previously reported values for xylose conversion with *C. tenuis* under aerobic conditions [1]. A time course of enzymatic activities shows an increase of *Ct*XR and *Ct*XDH activities in the first hours (Fig. 4). In general a peak of enzymatic activities at 60 hours and a decline at about 100 hours of fermentation was observed.

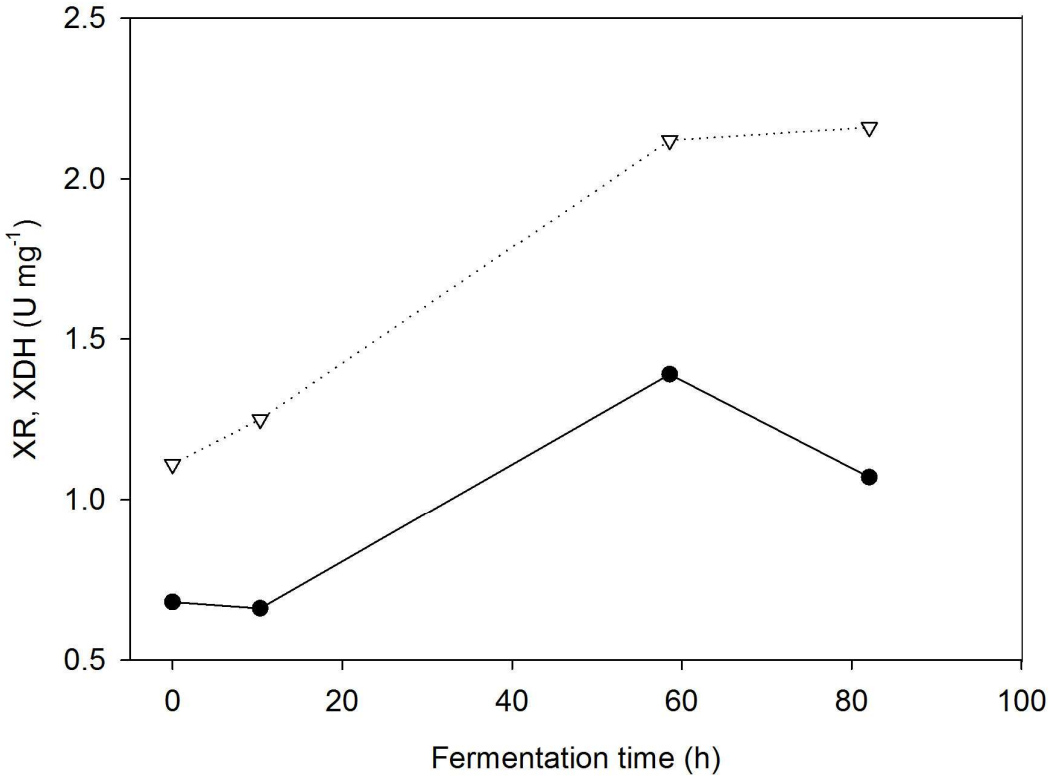

Fig. 4 Time course of enzymatic activities in an anaerobic batch fermentation with N_2 aeration. Triangles, XDH; closed circles, XR.

Table 1 Comparison of aeration influence on xylose fermentation and product formation by *C. tenuis* CBS 4435 strain

	Shake flask			Bioreactor	
OTR	7±1 µM h^{-1} [e]	≤1 µM h^{-1}	anaerob	4±0.1 µM h^{-1}	anaerob
q_{xylose}[a]	0.12 ± 0.02	0.19± 0.02	0.20± 0.01	0.16± 0.01	0.13
$Y_{ethanol}$[b]	0.45± 0.01	0.41± 0.01	0.41± 0.01	0.42± 0.01	0.40
$Y_{xylitol}$[b]	0.07± 0.01	0.03± 0.01	0.03± 0.01	0.03± 0.01	0.06
$Y_{glycerol}$[b]	0.03± 0.01	0.03± 0.01	0.04± 0.01	0.03± 0.01	0.04
C-recovery[c]	99%	90%	90%	96%	90%
XR[d]				1.1± 0.2	1.4± 0.1
XDH[d]				2.7± 0.3	2.2± 0.1

[a] Initial xylose uptake rates (q_{xylose}) are given in (g (g_{CDW} $h)^{-1}$) calculated from time-course data obtained from linear data range of fermentation, see Fig. 3

[b] Yield coefficients (Y) are given in (g (g xylose)$^{-1}$); they were calculated using data obtained after maximum fermentation time of 70h

[c] For calculation of the carbon balance it was assumed that one mol CO_2 was formed per mol of ethanol or acetate

[d] Xylose Reductase (XR) and Xylitol Dehydrogenase (XDH) activities are given in (U mg^{-1}) and were measured after 60h of fermentation; measurement parameters as follows: XR 700 mM xylose, 0.35 mM NADH, in 50 mM potassium phosphate buffer pH 7.0, 25°C; XDH 700 mM xylitol, 3.5 mM NAD^+, in TrisHCl buffer pH 9.0, 25°C

[e] Note that preculture was made with 20 g L^{-1} glucose instead of xylose

Discussion

In the 1980s *Candida tenuis* has been proven to be one of the ´better xylose fermenters´. Only carbon dioxide formation from xylose and volumetric ethanol production were the test criteria [14]. Further work reported for *Candida ´tenuis´* CBS 2883 investigated yields under aerobic, semi-aerobic and anoxic conditions [13]. According to CBS-KNAW Database (Utrecht, The Netherlands) this tested strain with the CBS number 2883 turns out to be *Candida sake (Candida parapsilosis)*. Nevertheless, intermediate ethanol yields of 0.27 g g^{-1} were found for anoxic conditions and xylitol formation reached nearly levels akin [13].

Picha stipitis and *Candida shehatae* were also tested under semi-aerobic and anoxic conditions. Ethanol yields under anoxic conditions never exceeded levels of 0.38 g g^{-1} with increased formation of xylitol (up to 0.13 g g^{-1} for *Candida shehateae*) compared to semi-aerobic fermentations [14].

In our studies we found that the high ethanol yield of 0.45 g g^{-1} in microaerobic fermentation is indeed comparable to those of the best native xylose fermenting yeasts, like *Pichia stipitis* [4], [24]. Under these conditions the high yield of ethanol with *Candida tenuis* comes up to 88 % of the theoretical limit [2]. The advantage of the investigated *Candida tenuis* strain over many other types of yeast is the high ethanol yield and low by-product formation that remain unaltered under microaerobic and anoxic conditions [7], [24].

There is a widely held notion that ethanol yield as well as by-product formation is directly linked to the cofactor specificity of the enzymes involved in the xylose conversion pathway [2], [12]. According to that it was suggested that for most yeast strains anaerobic fermentation is negligible due to a redox imbalance [2], [12]. This redox imbalance is caused by different and partially

stringent cofactor requirements of the two prime enzymatic steps of xylose metabolism via xylose reductase (XR) and xylitol dehydrogenase (XDH) [2], [12], [18]. Both enzymes, XR catalyzing reduction of xylose to xylitol and XDH reoxidizing xylitol to xylulose (Fig. 5), are well-characterized [3], [12], [18]. While XR may be dual-specific with preference for NADPH, XDH is strictly dependent on NAD^+ [2], [12]. The cofactor specificity of XR may alter between different yeast strains [3], [12]. Following the theory, ethanol yield and by-product formation is strongly subjected to the ability of cofactor regeneration [2], [12]. While regeneration is accessed through mitochondrial activity when oxygen is available, at oxygen limited or even anaerobic conditions cofactor recycling becomes a function of the prime enzymatic steps of XR and XDH. Under anaerobic conditions it was suggested that a strictly NADPH dependent XR is resulting in a blocked metabolic pathway with annihilated ethanol productivity, as it was reported for *Candida utilis* [12]. A dual-specific XR promotes partial regeneration of NADH. The evident drawback of this dual-specificity is the by-product formation (xylitol) whose amount is mainly depending on the ratio of cofactor preference [2], [12].

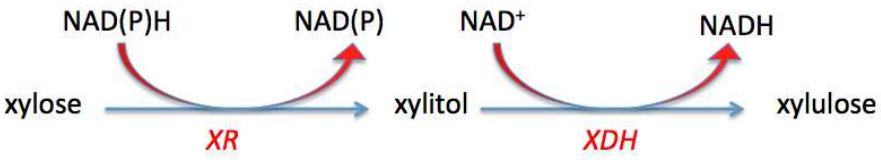

Fig. 5 First steps of xylose metabolization via xylose reductase (XR) and xylitol dehydrogenase (XDH)

In this context high ethanol yield combined with low by-product formation for xylose conversion with *C. tenuis* under anaerobic conditions is somewhat surprising. The dual-specificity of *Ct*XR [3] is expected to lead to reduced ethanol and increased xylitol yields under anaerobic conditions. This might be the result of an efficient internal, non oxygen-dependent ability of cofactor regeneration.

Hence, cofactor regeneration in *C. tenuis* might be obtained by metabolic pathways leading to a transhydrogenase like net reaction or by a NADH-dependent *Ct*XR reaction *in vivo* [2], [12]. Reported data for implemented *Ct*XR in *Saccharomyces cerevisiae* show an equal utilization of NADH and NADPH *in vivo* [19]. As a result, higher xylitol formation occurs with native *Ct*XR in recombinant *Saccharomyces* than with the altered, NADH preferring variant [3], [17], [19]. A clear requirement for a NADH-dependent XR reaction in *C. tenuis* would be that levels of intracellular NADH and NADPH concentrations undergo a large shift from the situation found in *S. cerevisiae* to another one that favors NADH [19]. This large shift towards NADH is very unlikely to reflect the true *in vivo* situation in *C. tenuis*.

By following a time course of enzymatic expression levels of *Ct*XR and *Ct*XDH we observed a lag phase in the first hours, ensued by a maximum and a decline during the late phase of fermentation, as it was also described previously [1], [13]. Likewise a decrease in levels of both XR and XDH were found during the late phase of xylose fermentation with *Pichia stipitis* and *Candida shehatae* [13]. Reports claimed that this might be attributed to a change in morphology [13], [15].

As it is with closely related yeasts [20], we observed different morphogenic states in *Candida tenuis*. The switch in morphogenic states from yeast towards pseudohyphae forms might be in relation to a decline in enzymatic expression levels. Furthermore, fermentation was only efficient when cells were transferred mainly in yeast forms from preculture into bioreactor. The multiple relationships of oxygen transfer, pH shift and morphogenic switches are currently unclear but beyond the scope of this study.

Albeit of the high ethanol yield, very little growth (μ_{max} = 0.004 h^{-1}) occurs under anaerobic conditions compared to aerobic conditions (μ_{max} = 0.28 h^{-1}) [22]. This is may be due to abated energy yield per mol xylose under the lack of oxygen. Xylose uptake rate with an average value of 0.16 g $(g_{CDW}\ h)^{-1}$ throughout microaerobic and anaerobic conditions is more than two-fold higher than for *Candida shehatae* or *Pichia stiptis* under anoxic conditions [13]. Glucose and xylose uptake rates under anaerobic conditions reach similar levels in *Candida tenuis*. Compared to glucose uptake under aerobic conditions it is only twofold smaller [8].

Our results show that *Candida tenuis* outstanding xylose fermentation performance offers very high ethanol yields with low by-product formation throughout the tested range of OTRs and even under anaerobic conditions. The latter establishes easier to handle process conditions without rendering yields.

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Harnessing *Candida tenuis* for whole-cell reductions of *o*-chloroacetophenone

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Abstract

The use of whole cell-catalyzed enantioselective production of chiral alcohols is an upcoming (bio-)technology. Especially pharmaceutical manufacturing harnesses microbial cell factories and their enzymatic units for the efficient production of optically pure synthons. Herein the *Candida* spp. dehydrogenases are used as key players with very high specificities and stereoselectivities towards keto-compounds.

We have optimized a whole-cell bioreduction system based on native *Candida tenuis*. Enzymatic conversion of *o*-chloroacetophenone is achieved by intracellular reduction via well-investigated *Candida tenuis* xylose reductase (*Ct*XR). The reduction product, (*S)*-1-(*o*-chlorophenyl)-ethanol is used as chiral key intermediate in the preparation of polo-like kinase 1 (PLK1) inhibitors, a class of new chemotherapeutics. The yeast cells provide a high level *Ct*XR expression with inherent cofactor regeneration and concomitant protection of intracellular enzymes.

Highest expression levels of CtXR were found in xylose-grown, anamorph cells (OD_{600} ≤ 5) and determined to 2.0±0.2 U mg^{-1}. The pronounced cell-toxicity of (*S)*-1-(*o*-chlorophenyl)-ethanol limited productivities of aqueous batch conversions to 0.11±0.01 g g_{CDW}^{-1}. *In situ* substrate supply and product removal by 40 % (v/v)s *n*-hexane was applied to increase productivities to 0.25±0.01 g g_{CDW}^{-1}. In a biphasic batch conversion of 100 mM *o*-chloroacetophenone and under optimized reaction conditions with respect to physiology of cells, aqueous phase composition and type of co-substrate, a product concentration of 59 mM was obtained.

The co-substrate yield for D-glucose and productivities were determined as 3.3±0.1 g g^{-1} and 0.28±0.01 g g_{CDW}^{-1} under these conditions. The resulting enantiomeric purity of the (*S)*-1-(*o*-chlorophenyl)-ethanol was always >99.9 % throughout varying process conditions. The construction of recombinant whole cell catalysts is tedious and application of native hosts in the development of new pharmaceuticals enables fast production of synthons at the lab-scale.

Introduction

Whole-cell bio-catalysis has gained increasing interest in in the production of chiral intermediates for chemo-enzymatic multistep synthesis of enantiomerically pure pharmaceuticals [4-9]. Cheap catalyst production, high selectivities and excellent enantiomeric purity of products are clear advantages over common organic synthesis [6]. Hence whole-cell bio-catalysts progressively replace counter-part organic catalysts in pharmaceutical industry [6]. While conditions in organic chemistry synthesis are very far from mild, process parameters of bio-catalysis are operated near physiological and environmentally friendly conditions [6], [20]. The enzyme(s) performing conversion of the chemical substance of interest are ´immobilized´ and protected inside the cells. The evolutionary optimized cell system provides ideal reaction conditions and in situ cofactor regeneration [20].

General bottlenecks in whole-cell biocatalysis are biocatalyst stability, ratio of biocatalyst to substrate and co-substrate, toxicity and solubility of substrate and product as well as solvent compatibility [4], [9]. Engineering approaches to overcome the bottlenecks of bio-catalytic systems at the cell, reaction and process level have been reported recently [2], [4-9].

We investigated the natural whole-cell reduction system based on *Candida tenuis* CBS 4435 for the synthesis of enantiomerically highly pure ketones. While different types of keto-substrates were tested for their conversion by cells of *Candida tenuis*, process conditions were optimized for *o*-chloroacetophonone and the production of (*S)*-1-(*o*-chlorophenyl)-ethanol.

The stereoselective reduction of the precursor ketone to the chiral key intermediate (*S)*-1-(*o*-chlorophenyl)-ethanol is of great demand for the synthesis of polo-like kinase 1 (PLK1) inhibitors [17], [18]. The efficacy of PLK1 inhibitors as chemotherapeutics has recently been shown in xenograft tumor models [16], [17]. The efficient and stereoselective reduction of *o*-chloroacetophonone is rare among chemo- and enzymatic catalysts [20], [21]. *Candida tenuis* xylose reductase (*Ct*XR) is one of the few reductases capable of converting *o*-chloroacetophonone with absolute stereoselectivity (> 99.9% ee) and useful specificity (Fig. 6) [1], [6].

A clear advantage of the *Candida tenuis* whole cell system is the high level expression of xylose reductase, the inherent ability for cofactor regeneration and the thereof lacking need for laborious genetic engineering.

We studied the impact of co-substrate type and concentration, composition of aqueous phases, co-solvents and different morphological states of the cells on the productivity and on the enantiomeric purity of the product. Reaction engineering was applied to circumvent product and substrate toxicity of aromatic ketones in whole-cell reductions using *Candida tenuis*.

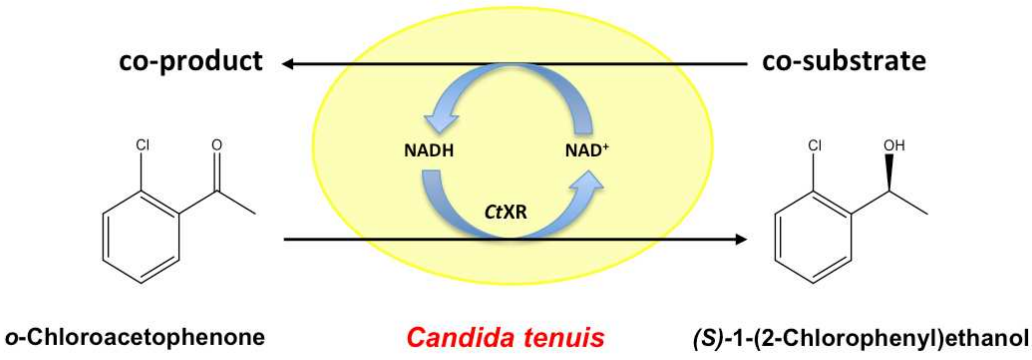

Fig. 6 Biocatalytic reduction of *o*-chloroacetophenone by whole cells of *Candida tenuis* CBS 4435 with internal co-factor recycling

Materials and methods

Chemicals

NADH (sodium salt; ≥98 % pure) and NAD^+ (free acid; ≥97.5 % pure) were purchased at Roth (Karlsruhe, Germany). 0.5 mm glass beads were obtained from BioSpec Products (Bartlesville, USA). Racemic 1-(2-chlorophenyl)ethanol was from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). All other chemicals used were of the highest quality and purity available and obtained from Sigma-Aldrich / Fluka (Gillingham, Dorset, U.K.) or Roth (Karlsruhe, Germany). Two mL tubes were purchased at Eppendorf Austria GmbH (Wien, Austria). Fifteen mL and 50 mL tubes were obtained from Sarstedt (Wr. Neudorf, Austria).

Organism and culture conditions

Candida tenuis CBS 4435 strain was obtained from the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands). Unless otherwise indicated, all cells were grown on 20 g L^{-1} D-xylose as C-source in mineral medium containing 0.106 M KH_2PO_4, 0.038 M $(NH_4)_2SO_4$, 0.002 M $MgSO_4$ x $7H_2O$, and 0.025 % (v/v) Antifoam 204 (Sigma-Aldrich). As described elsewhere in full detail [10], trace elements and vitamins were added, except for riboflavin and folic acid.

Precultures were incubated at 25℃ on a rotary shaker at 125 rpm. They were inoculated by transferring a loopful of cells from an agar plate into baffled 300 mL Erlenmeyer flasks containing 50 mL of medium. Precultures were used to inoculate main cultures (300 mL) in 1000 mL baffled shake flasks to an optical density of 0.05 (OD_{600nm}). To investigate the effect of cell morphogenic states on whole cell reductions, cells were harvested at different optical densities, maximally at an OD_{600nm} of 5.

Cells were harvested by centrifugation (4400 g, 15 min) prior to dissolution into buffer to defined cell dry weights (CDW, (g_{CDW} L^{-1})). Buffer concentrations and pH values are given in detail at the corresponding experimental setups.

Preparation of cell extract

Equal amounts of cell wet weight and of glass beads were mixed together. Two hundred µL of 50 mM potassium phosphate buffer (PPB) pH 7.0 was added and cells were mechanically disrupted by vortexing for 30 seconds and subsequent storage on ice for 30 seconds. This process was repeated ten times. By further centrifugation (2 min, 4400 g, 4°C) cell debris was removed and enzyme activities were measured in the crude cell extract.

Enzyme assays

Xylose reductase (XR) and xylitol dehydrogenase (XDH) activities were determined on a Beckman DU 800 spectrophotometer at 25℃ by following the depletion or formation of cofactor NADH at 340 nm (ε_{NADH} = 6.22 cm^{-1} mM^{-1}). XR assays contained 0.35 mM NADH and either 700 mM xylose or 9.6 mM *o*-chloroacetophenone as substrate in a 50 mM PPB (pH 7.0). The sparingly water soluble *o*-chloroacetophenone was pre-dissolved in ethanol prior to dilution into buffer to give a final ethanol concentration of 5 % v/w.

Reaction mixtures for the determination of XDH activities consisted of 3.5 mM NAD^+ and 700 mM xylitol in 50 mM Tris HCl buffer at pH 9.0. The initial rate measurements were started by the addition of cell extract. Enzyme units were defined as µmol NADH reduced per minute.

Whole-cell bioreduction of ketones

All anaerobic batch and fed-batch experiments were carried out in 2 mL Eppendorf reaction tubes that were incubated at 25°C and rotated on an end-over-end rotator (model SB3, Stuart, VWR, Austria) at 30 rpm. The total volume of the reaction mixture was 1 mL in batch experiments. Oxygen limited or anaerobic conditions predominated in batch experiments performed

in closed tubes with cell dry weights above 10 g_{CDW} L^{-1}. Excess carbon dioxide prevailed when tubes were opened after batch conversion.
In aerated fed-batch setups, 300 mL baffled Erlenmeyer flasks sealed with cotton plugs with at a total reaction volume of 50 mL were incubated in a rotary shaker at 75 rpm and 25℃.

Aqueous phases were buffered with either potassium phosphate or citrate-phosphate buffer. Buffer molarities were varied between 50 and 100 mM and initial pH-values were set to 6.0 or 5.5. Furthermore mineral medium as described under *Organism and culture conditions* was used. Ten mg L^{-1} ergosterol and 0.42 g L^{-1} Tween-80 were added to the mineral medium when employed as solvent in whole cell reductions. Concentrations of co-substrate (xylose or glucose) were 0, 200 or 300 mM.

Molar yields of alcohol production on co-substrate consumption (co-substrate yields) were calculated over the whole conversion time starting with addition of *o*-chloroacetophenone. All experiments were done in quadruplicate and results show mean values and the corresponding standard deviation (S.D.).

Analytical methods

Reactions were stopped by the addition of 9 volume equivalents of ethanol and subsequent vortexing (1 min). Cell debris was removed by centrifugation (4400 g, 10 min, 4℃) and the supernatant was used for HPLC analysis. Probes were stored in a cooling unit at 4℃ until a nalysis.

Chiral HPLC was performed on a LaChrome HPLC system (Merck-Hitachi) equipped with an L-7400 UV-detector, an L-7490 RI-detector and a thermo-stated column oven. For enantiomeric separation and quantitative analysis of the *R*- and *S*-alcohols a reversed phase CHIRALPAK AD-RH column from Daicel (purchased at VWR International, Vienna, Austria) was used.
Baseline separation of the *R*- and *S*-alcohols was obtained with acetonitrile and water (20:80, by volume) as the mobile phase at a flow rate of 0.5 mL min^{-1} at a temperature of 40℃. The detection was done a t 210 nm.

Metabolites and sugars were analyzed on the same LaChrome HPLC system described above. An Aminex HPX-87H column (BioRad, Hercules, CA, USA) with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}, thermostated at 65℃, was used for identification a nd quantification of metabolites (ethanol, xylitol, acetate, pyruvate, lactate, succinate, ribitol) and xylose. Five mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL min^{-1} and an operating temperature of 65℃. For quantifi cation of glucose, xylose and xylitol concentrations an Aminex HPX-87C column with water as the mobile phase at a flow rate of 0.4 mL min^{-1} and a temperature of 85℃, was employed.

Results

We tested whole cells of *Candida tenuis* in bio-reductions of 100 mM ethyl 4-cyanobenzoylformate (ECBF) or *o*-chloroacetophenone. Enantiomeric purities of the reduction products were determined to 65±2 and ≥99.9 % for ethyl (*R)*-4-cyanomandelate and (*S)*-1-(*o*-chlorophenyl)-ethanol, respectively. The low optical purity of ethyl (*R)*-4-cyanomandelate renders a synthetic application sparsely attractive and therefore further work focused on *o*-chloroacetophenone as substrate.

Influence of morphogenic state on productivity

We investigated the morphogenic states of a batch culture of *Candida tenuis* by phase contrast microscopy and correlated the results to OD_{600nm} (Fig. 7). Predominantly yeast forms were found until an OD_{600nm} of 6 was reached (teleomorph); at an OD_{600nm} of about 6 a switch in morphology takes place and yeast as well as pseudohyphae forms coexist. After the transition state mainly pseudohyphae are formed and prevail at an OD_{600nm} of about 10 (anamorph).

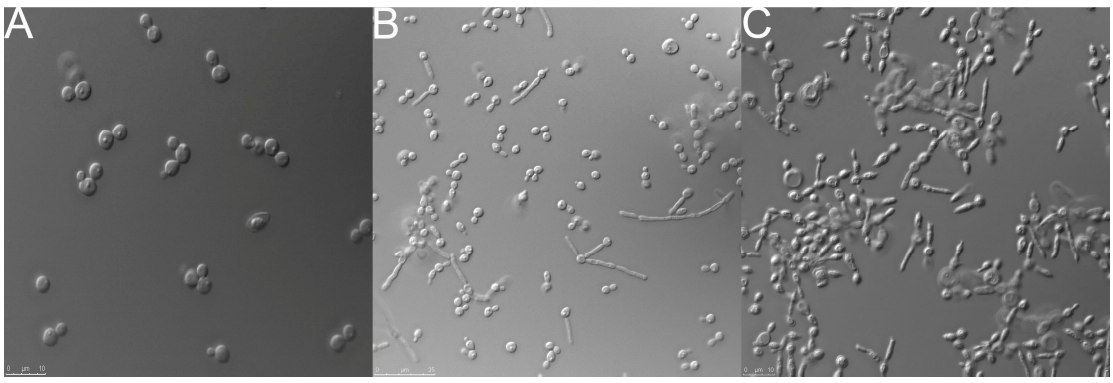

Fig. 7 Switch in morphogenic states on xylose from yeast (A) OD_{600} = 3 via transition state (B) OD_{600} = 6 to pseudohyphae forms (C) OD_{600} = 10.

The morphological state shows high impact on the expression level of xylose reductase and hence bioreduction efficiencies. Reduction yields of monophasic *o*-chloroacetophenone batches drop at an OD_{600nm} of about 11 at least 10-fold when compared to an OD_{600nm} of about 5.

Table 2 Influence of different morphogenic states of *Candida tenuis* on expression levels of *Ct*XR and on the productivity.

OD_{600nm}	Morphology	g_{CDW} L^{-1}	Yield	U mg^{-1} [a]	g g_{CDW}^{-1} [b]
4.5	teleomorph			2.0±0.2	
5	teleomorph	18	12 %	-	0.10±0.01
11.2	anamorph			0.19±0.02	
11.0	anamorph	20	1 %	-	0.01±0.002

[a] Specific activities measured with D-xylose in crude cell extract, details see Methods section

[b] Reaction conditions: 100 mM *o*-chloroacetophenone, 50 mM PPB

A constant ratio of specific activities to productivities for teleomorph and anamorph cells suggests no change in resistance towards toxicity of substrate and product due to changes in morphology and physiology. In all further experiments *Candida tenuis* cells were harvested below an OD_{600nm} of 6 to obtain teleomorph cells with high reduction capacity.

In situ substrate supply and product removal

We investigated toxicity effects of substrate and product in a fed-batch by adding 5 mM of substrate each 30 minutes or 10 mM each 25 minutes (data not shown). Results suggest a toxicity limit of product at a conversion level of 0.11 g g_{CDW}^{-1}. Above the critical limit, added substrate accumulates without further product formation requiring the separation of toxic product and substrate from the aqueous, cell-containing phase.

We tested *n*-butyl acetate and *n*-hexane as *in situ* extracting reagents. Low cell-toxicities and high extraction capabilities for substrate and product are requirements for organic solvents used in whole cell bioreductions. *n*-Hexane

turned out as best choice in *o*-chloroacetophenone whole-cell reductions. Variation of the *n*-hexane content from 20 to 50 % lead to an improvement of productivity up to 3.5-fold as compared to aqueous, monophasic batches (Fig. 8).

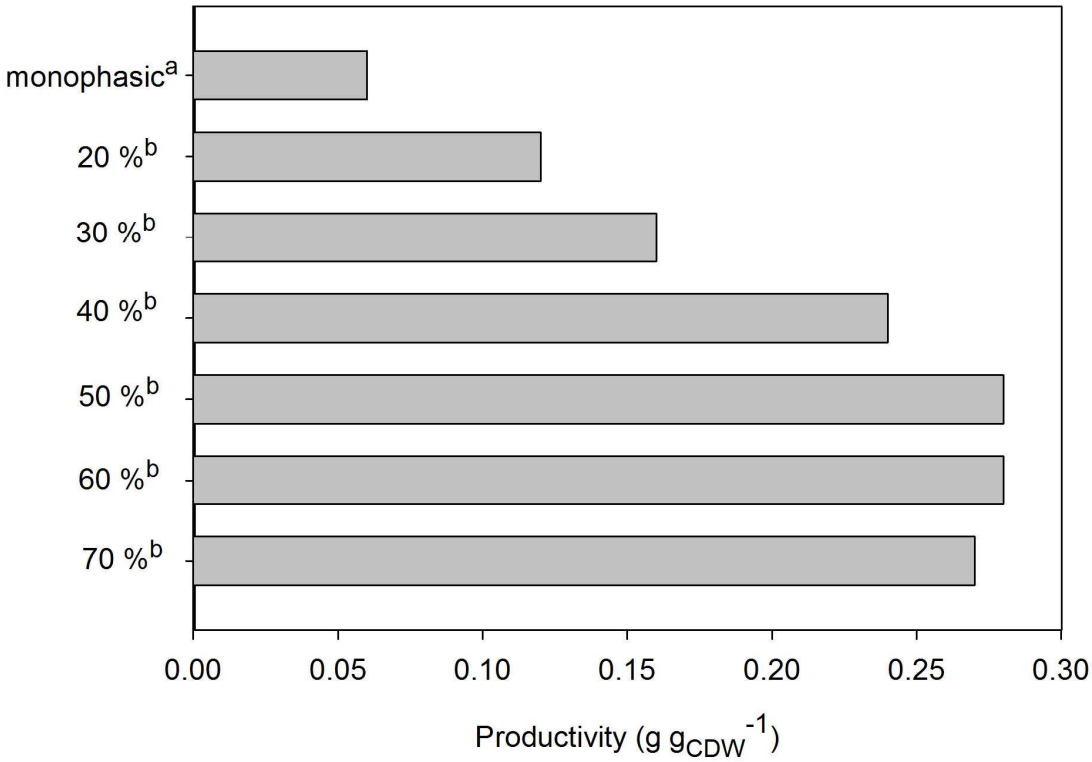

Fig. 8 Productivities of *o*-chloroacetophenone reductions by whole cells of *Candida tenuis* in potassium phosphate buffer supplemented with 0-70 % (v/v) *n*-hexane as co-solvent. Reaction conditions: 200 mM glucose as co-substrate, 25℃, 1 5h; [a] 50 mM PPB, [b] 50 mM PPB and varying percent by volume of *n*-hexane

We obtained highest productivities of 0.25±0.1 and 0.22±0.1 g g_{CDW}^{-1} in mineral medium and 100 mM phosphate buffer supplemented with 40 % (v/v) *n*-hexane (Table 3). Lowest conversion of *o*-chloroacetophenone in *n*-butyl acetate lead to productivities below 0.01 g g_{CDW}^{-1} (Table 3).

Forty % (v/v) *n*-hexane was used in further experiments varying the concentration of *o*-chloroacetophenone (Table 4).

Table 3 Comparison of different aqueous and organic phases in 100 mM *o*-chloroacetophenone batches [a]

Aqueous phase	Molarity (mM)	Organic phase	Percent by volume (%)	Productivity (g g_{CDW}^{-1})	Product (mM)
Potassium phosphate	50	-	-	0.08±0.01	13
Potassium phosphate	50	*n*-hexane	40	0.20±0.02	26
Potassium phosphate	100	*n*-hexane	40	0.17±0.02	22
Citrate-phosphate[a]	100	*n*-hexane	40	0.22±0.01	34
Mineral medium[b]	100	*n*-hexane	40	0.25±0.01	32
Potassium phosphate	50	*n*-butyl acetate	40	≤0.01	≤1

[a]Reaction conditions: 200 mM glucose as co-substrate, 25℃, 1 5h conversion time
[b]Mineral medium for *C. tenius*, see methods section for details
[c]Buffer containing 0.1 M Na_2HPO_4 and 0.2 M citric acid

Equal product concentrations and productivities (g g_{CDW}^{-1}) in biphasic batches of 100 and 200 mM *o*-chloroacetophenone stress furthermore a critical toxicity limit of product as main reason for catalyst deactivation. The toxicity limit of (*S*)-1-(*o*-chlorophenyl)-ethanol is increased 2.2-fold to 0.24 g g_{CDW}^{-1} in 40 % *n*-hexane as compared to monophasic batches as a reason of partition of product into the organic phase. Doubling of catalyst loading from 20 to 40 g_{CDW} L^{-1} lead to doubled product concentration and hence equal productivities within the experimental error (Table 4, Fig. 9).

Varying substrate concentrations show no impact on the enantiomeric ratio of the product, which was always above 99.9 % throughout the tested range (Fig. 9).

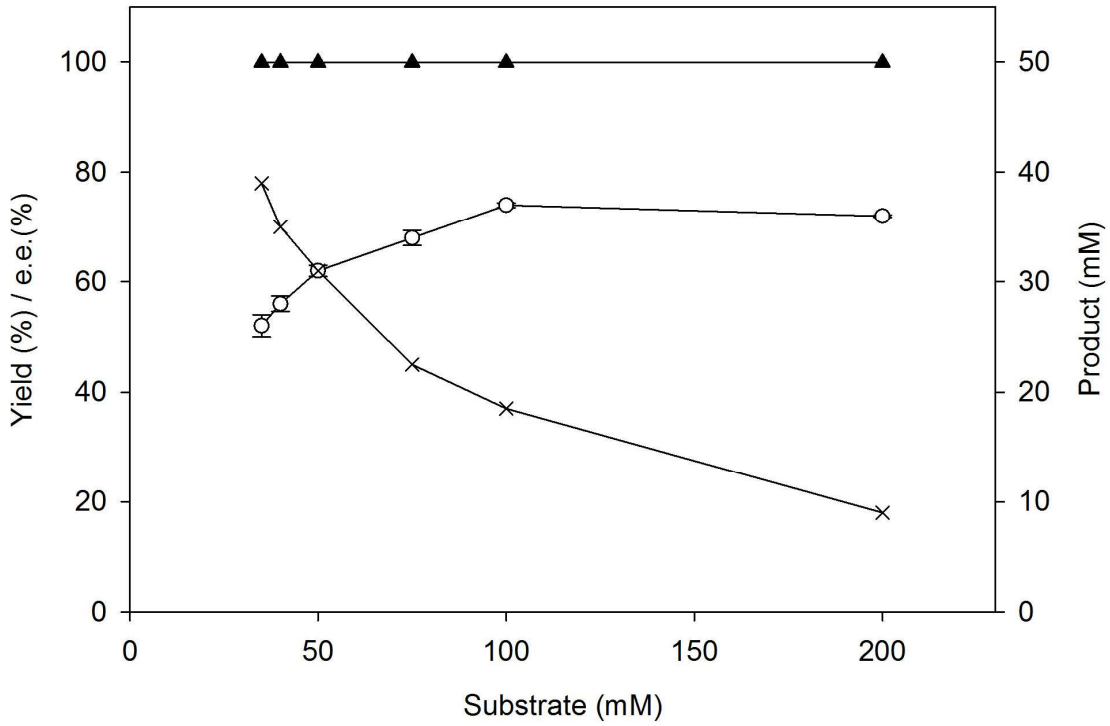

Fig. 9 Influence of substrate concentration on product formation, yield and enantiomeric excess (e.e.) in biphasic 40 % *n*-hexane *o*-chloroacetophenone batches with mineral medium, Reaction conditions: biphasic 40 % *n*-hexane and mineral medium, 200 mM glucose as co-substrate, 25℃, 10 h conversion time, 24 g $_{CDW}$ L^{-1}; open circles, product; triangles, e.e.; cross, yield;

Influence of carbon source on enzymatic expression levels and on conversion yields

The effect of carbon source on xylose reductase expression was studied by growing cells on either 20 g L^{-1} D-xylose or D-glucose. Xylose reductase activities were 9-fold higher in crude extracts obtained from xylose-grown as compared to glucose-grown cells. Ketone reductase activities determined with the xenobiotic substrate *o*-chloroacetophenone were even 21-fold higher when xylose was used as carbon source. This indicates a very low constitutional expression level of xylose reductase in the absence of xylose (Fig. 10).

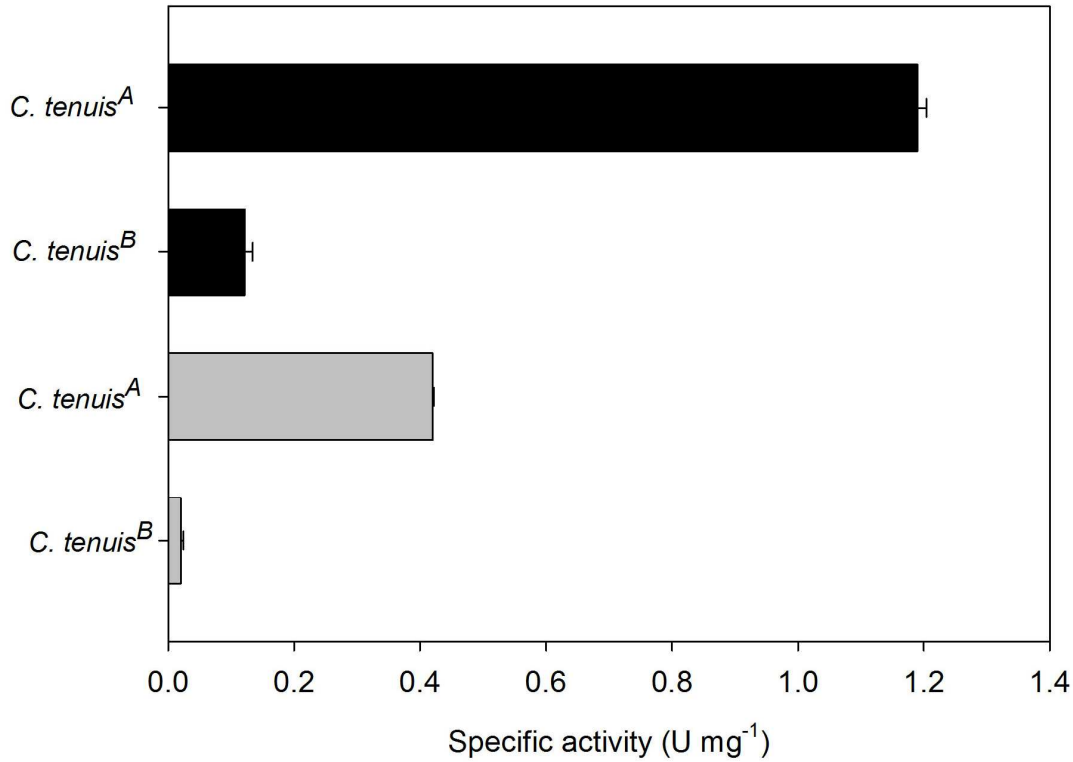

Fig. 10 Influence of 20 g L^{-1} xylose (^{A}) or glucose (^{B}) as carbon source in culture medium on enzymatic expression levels and reduction capability of xylose and *o*-chloroacetophenone (*o*-Chl); Reaction conditions: cells were grown on either 20 g L^{-1} xylose or glucose, enzymatic activities were determined in cell debris according to materials and methods section; black bar, D-xylose; grey bar, *o*-Chl;

Different concentrations of D-xylose or D-glucose were tested as co-substrates of *o*-chloroacetophenone whole-cell reductions with respect to reduction yield. Best results were achieved with a glucose concentration of 200 mM. Analysis of co-substrate consumption showed that 18 mmol g_{CDW}^{-1} of glucose is consumed by the cells during the period of whole-cell conversion. Although xylose is necessary for high expression levels of xylose reductase (Fig. 10), it lowers the amount of product formation when applied as co-substrate (see Appendix).

The co-substrate yield was calculated over the time span of chiral alcohol formation. It was mainly influenced by the amount of cells (g_{CDW}) and the type of co-substrate applied. The highest $Y_{co\text{-}substrate}$ 3.34±0.1 was reached with 200 mM glucose in biphasic 40 % *n*-hexane batches. Conversion yield was increased at least 1.6-fold with glucose as compared to xylose. With xylose $Y_{co\text{-}substrate}$ was also 2.6±0.2 but the amount of product formation dropped nearly 2-fold. A switch in the molarity of added co-substrate from 200 mM to 300 mM did not affect reduction. Hence 200 mM glucose turned out as optimal co-substrate for the conversion of 100 mM *o*-chloroacetophenone.

We finally investigated the influence of buffer-type and salt concentration of the aqueous phase on the product yield (Table 3). Phosphate ions are indispensable for growth of *Candida tenuis*, thus always a phosphate buffer was used despite its low buffer capacity at pH 5.5. To increase buffer capacity we used higher phosphate concentrations, a citrate-phosphate buffer (CPB) or the phosphate-buffered mineral medium optimized for culturing *Candida tenuis*. There is a promptly occurring, acidic pH-shift in the early phase of co-substrate consumption and ketone reduction (Fig. 11, Fig. 12). Strong buffering using 100 mM citrate phosphate buffer reduced the acidic shift to only 0.2 pH units, resulting in an 1.2-fold increase in productivity compared to 50 mM potassium phosphate buffer (Table 3).

Table 4 Productivities, reduction and co-substrate yields for *o*-chloroacetophenone whole-cell reductions with different catalyst to substrate ratios[a]

Cell dry weight (g_{CDW} L^{-1})	Substrate (mM)	Product (mM)	Yield (%)	Productivity (g $gCDW^{-1}$)	$Y_{Co\text{-}substrate}$ (g g^{-1})
24	35	26±1.0	75	0.16±0.01	2.00±0.2
24	100	37±0.2	37	0.25±0.01	3.34±0.1
24	200	36±0.1	18	0.23±0.01	3.27±0.1
13	100	23±0.5	23	0.28±0.01	2.17±0.2
20	100	32±1.0	32	0.25±0.01	2.56±0.1
40	100	59±1.5	59	0.23±0.01	3.20±0.1

[a] Reaction conditions: concentrations in biphasic 40 % *n*-hexane and mineral medium, 200 mM glucose as co-substrate, 25℃, 10 h conversion time

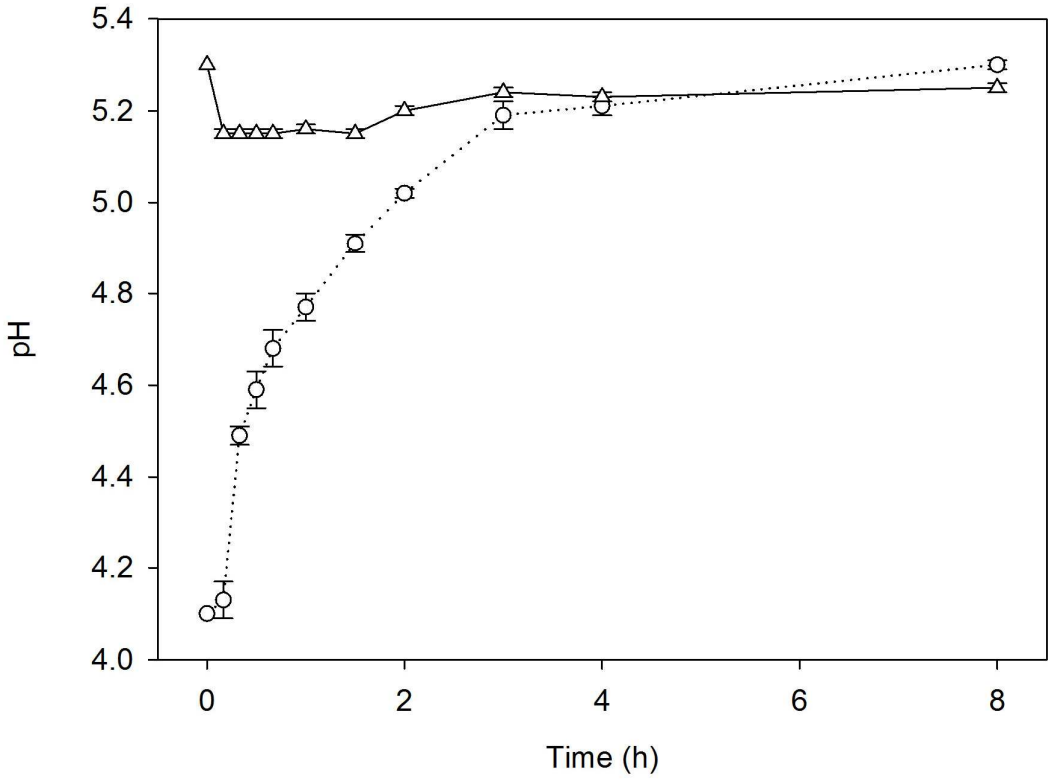

Fig. 11 pH shifts during batch conversion of 100 mM *o*-chloroacetophenone in biphasic 40 % *n*-hexane batches with different aqueous phases, Reaction conditions: 20 g_{CDW} L^{-1}, initial pH 5.5, measurement start with addition of *o*-chloroacetophenone; triangles, 100 mM CPB; circles, 100 mM PPB;

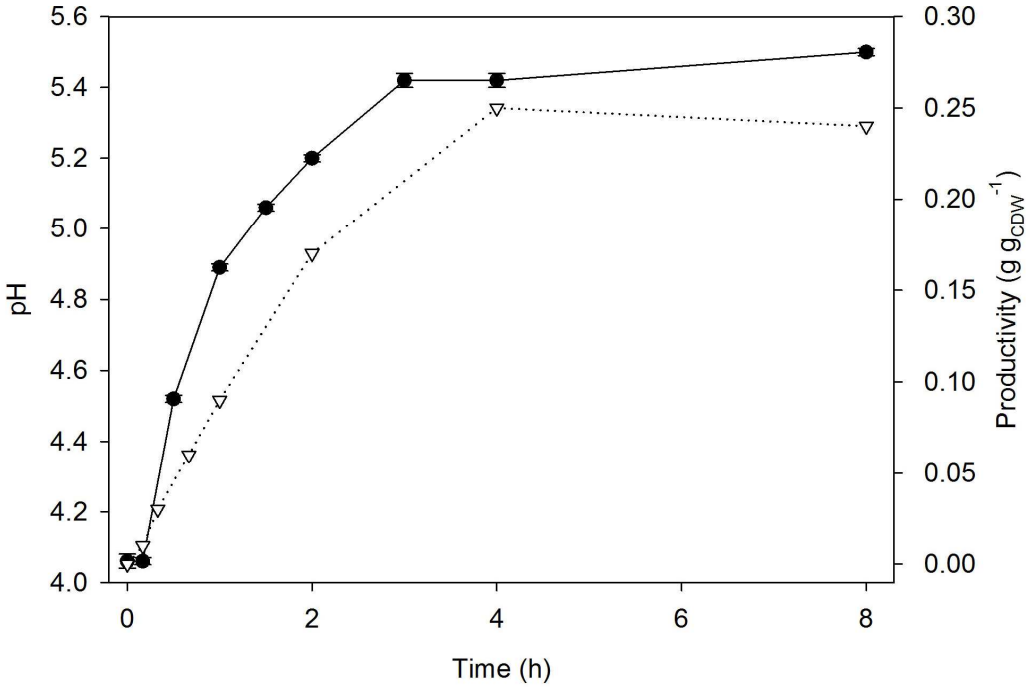

Fig. 12 Relationship of productivity and pH shift during biphasic 100 mM *o*-chloroacetophenone batch conversion in 100 mM PPB, 40 % *n*-hexane, 200 mM glucose as co-substrate, 18 g_{CDW} L^{-1}; circles, pH; triangles, productivity;

Optimized conditions

Highest productivities were obtained in a biphasic batch system composed of 40 % *n*-hexane and mineral medium supplemented with 200 mM glucose as co-substrate and a substrate concentration of 100 mM. Productivitiy and $Y_{co\text{-}substrate}$ were determined to 0.28 and 3.3, respectively (Table 4). Increasing catalyst loading to a concentration of 40 g_{CDW} L^{-1} resulted in a final product concentration of 59 mM (Table 3). Highest initial rates of *o*-chloroacetophenone conversion in whole-cell bioreduction with *Candida tenuis* were determined to 30±4 U g_{CDW}^{-1}.

Discussion

Candida tenuis xylose reductase as biocatalyst

Intensive characterization of *Ct*XR over the last years showed outstanding enantioselectivities for the NAD(P)H-dependent reduction of aromatic α-hydroxy esters and phenylethanols [1], [2], [4]. *Ct*XR converts *o*-chloroacetophenone with useful specificity (k_{cat}/K_m= 340 M^{-1} s^{-1}) and perfect *S*-stereoselectivity (enantiomeric excess of over 99.9 %) [4]. Specificity towards aromatic α-hydroxy esters was even enhanced by a rational engineering approach to enlarge the substrate-binding pocket [1].

Wild-type and modified *Ct*XRs were successfully overexpressed in the most common hosts, *Escherichia coli* and *Saccharomyces cerevisiae* and used as whole-cell catalysts [2], [4]. Stoichiometric addition of the raw cofactor is not economically viable due to high costs of NAD(P)H. Hence to overcome cofactor shortage in *E. coli*, formate dehydrogenase was co-expressed as *in vivo* NADH-recycling system [2]. The reductase background of recombinant *S. cerevisiae* was suppressed by reaction conditions that allowed the regeneration of reduced cofactor mainly in the form of NADH, thereby silencing the NADPH-dependent reductase background [22].

In this study we showed the advantages of the native host, *Candida tenuis,* in whole-cell reductions. *Candida tenuis* offers both high native expression of *Ct*XR and inherent cofactor regeneration for the asymmetric conversion of ketones. Hence there was no need for laborious genetic engineering. We tried to overcome the two main problems with (native) whole-cell systems. The operational stability of the catalyst is very low due to toxic effects of aromatic substrate and product [4], [5]. Furthermore, internal cofactor regeneration must be efficient for high product yields [2], [6].

Toxicity of aromatic substrates

The high toxicity of aromatic substrates towards biocatalysts requires the separation of the biocatalyst from hydrophobic substrates and products. Biphasic co-solvent systems as well as resin-based technologies were used to circumvent the toxicity problem of α- and β-keto esters by *in situ* substrate removal and product removal [4], [9], [11]. The toxicity of organic compounds correlates negatively to the corresponding octanol−water partition coefficient (log*P*) values [23]. Although *o*-chloroacetophenone is a very hydrophobic substrate (log*P* = 2.1), catalyst half-life of similar yeast host systems like *Saccharomyces cerevisiae* is only 30 minutes in the presence of 100 mM [4]. Still no microorganism has been found capable of withstanding α-hydroxy ester or *o*-chloroacetophenone compounds for a longer time or at higher concentrations (>10 mM) without a massive decrease of productivity [8], [9], [11]. Catalyst half-life time and hence amount of product formation depends in a great extend on the choice of the in-situ extraction technology applied.

Also *Candida tenuis* cells were immediately affected by toxicity of substrate and furthermore by formation of product. Experiments showed that catalyst half-life time is very low due to toxic effects caused by the accumulation of (*S)*-1-(*o*-chlorophenyl)-ethanol rather than by *o*-chloroacetophenone. This may be due to the higher solubility of the product because of its reductively introduced alcoholic group. Moreover the formation of product occurs intracellular and seems to have a great impact on reduction capability, as it was in whole-cell reduction with closely related *Candida pseudotropicalis* [8].

Likewise, in-vitro data of enzymatic activity show very low half-life time of *Ct*XR when directly exposed to *o*-chloroacetophenone [1], [4]. For prolongation of catalyst half-life time we implemented an organic phase as *in situ* extraction solvent in the whole cell reduction process. Because of its boiling point and capability to extract the main part of substrate and partially also the product from the aqueous phase, *n*-hexane was the prime choice as co-solvent. Productivity was increased significantly (>3.5-fold) with *n*-hexane as co-solvent compared to monophasic whole cell reduction systems. As for

Candida magnoliae a mixture of water and *n*-butyl acetate seems to work well in a whole-cell bioreduction process, we also tried to apply this to our system [7]. However, *Candida tenuis* showed no conversion of *o*-chloroacetophenone under similar conditions.

A previous approach to use *o*-chloroacetophenone above its solubility limit in the form of small droplets (0.643 µm) lead to productivities of only 0.15 g g_{CDW}^{-1} in whole-cell reductions with *Candida pseudotropicalis* cells. While an organic co-solvent was necessary for product extraction in the process with *Candida pseudotropicalis*, we could show that direct implementation of *n*-hexane during whole-cell reduction leads to increased and high productivity of 0.28 g g_{CDW}^{-1} [15].

Effect of co-substrates on whole-reductions

The specific reduction capacity of the cells depends on the expression level of intracellular reductases and the availability of reduced NAD(P)H. The choice of co-substrate is therefore an important process parameter that promotes regeneration of cofactor. Glucose was the prime co-substrate in most reported yeast systems as its metabolization provides NAD(P)H through the pentose phosphate pathway and the citric acid cycle [7], [8], [9], [15].

We could show that internal regeneration of NADH is enhanced by the addition of co-substrate. While xylose seems to compete against *o*-chloroacetophenone as substrate of *Ct*XR, the exchange by glucose resulted in an 1.5-fold increase of productivity. This result suggests a faster glucose uptake and better energy yield per mol glucose compared to mol xylose [12], [13], [14].

Acidic pH-shift during whole cell reductions

The formation of CO_2 due to respiration of cells might contribute to the large pH-shift downwards observed in aerated precultures and anaerobic conversion of *o*-chloroacetophenone. While a switch of pH was found at OD_{600} values of approximately 5 in precultures, the same pH value of 4 was reached after 10 minutes of incubation during whole-cell bioreductions (in biphasic batches). High cell densities suggest oxygen limited conditions in the late phase of preculture cultivation (>3.5 g_{CDW} L^{-1}) and during conversion of *o*-chloroacetophenone (>20 g_{CDW} L^{-1}).

HPLC metabolite analysis of a xylose preculture broth showed no formation of acids (pyruvate, acetate, lactate) but small amounts of ethanol (0.03±0.01 g L^{-1}; $Y_{ethanol}$ = 0.01) at an OD_{600} value of about 10 (~ 3.5 g_{CDW} L^{-1}). The cultures were incubated in a rotary shaker (125 rpm) and sealed with cotton plugs. Formation of ethanol indicates anoxic conditions during precultures of crab-tree negative *Candida tenuis* [15]. The main reason for catalyst deactivation in whole cell reductions was assigned to the toxicity of substrate and product, pH shifts were of minor importance.

The relationship of intracellular (*S)*-1-(*o*-chlorophenyl)-ethanol formation, the pH-shift towards the initial pH of the buffer and the thereof resulting influence on the productivity is currently unclear but beyond the scope of this study.

The outstanding enantiomeric purity of the product, (*S)*-1-(*o*-chlorophenyl)-ethanol, was not compromised by variations of reaction conditions such as pH, temperature, type of co-solvent or co-substrate.

Conclusion

High-level expression of *Ct*XR and internal cofactor regeneration renders *Candida tenuis* an efficient whole-cell conversion system for α-hydroxy esters and *o*-chloroacetophenone reduction. In this work we demonstrated good productivities combined with the absolute enantiomeric purity of the (*S)*-1-(*o*-chlorophenyl)-ethanol product.

The use of the native system is advantageous in cases where established reductase systems, either isolated or as whole-cell catalysts, are missing. Products that are rarely formed by abundant reductases often show high enantiomeric purities when assayed with reductase-rich organisms.
o-Chloroacetophenone is therefore converted with absolute ee-values in the native host *Candida tenuis*. Construction of recombinant whole-cell catalysts is tedious and application of native hosts in the development of new pharmaceuticals enables parallel process engineering.

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Appendix

Anaerobic and microaerobic fermentation of xylose by native *Candida tenuis*

Materials and Methods

Materials and Methods are according to the main section of this work. Modifications of protocols are listed consecutively.

Organism and culture conditions

For a growth test on arabinose, cells of *Candida tenuis* were incubated with 20 g L^{-1} arabinose instead of xylose in the same media as described in the main section.

To investigate the morphological influences of *Candida tenuis* cells on the xylose fermentation performance, cells were harvested in the anamorph state (OD_{600} value ≥8.0). All other experiments were done with cells in the teleomorph state (preculture OD_{600} value ≤5.0). The OD_{600} values are given at the corresponding result section.

Bioreactor experiments

Anaerobic conditions were either maintained by sparging the bioreactor with 0.16 vvm N_2 or CO_2. Microaerobic conditions (OTR = 68 µM h^{-1}) were maintained by sparging the reactor with 0.12 vvm N_2 and 0.31 vvm compressed air. Surface gassing (OTR = 4±0.1 µM h^{-1}) was performed by diffusion of surrounding air trough an air filter with 50 mm of diameter (Acro 50, PALL Corporation, USA), which was connected to the top plate of the vessel unit.

Results

A comparison of bioreactor experiments under different aeration conditions shows the influence of oxygen transfer rate or the gas used to maintain anaerobic conditions (N_2 or CO_2) on the xylose fermentation performance (Fig. 13, Table 5).

Using cells in the early anamorph state results in a decrease of fermentation performance (Table 5). Results of harvesting and using preparatory cultures at an OD_{600} of 8.7 in xylose fermentations indicate a change in physiology, which is in correlation with the impact of morphological changes on the observed xylose conversion performance (Fig. 1, Table 5). A decrease in the ethanol yield and xylose uptake rate suggests a reduced xylose fermentation performance due to a change in morphology from the teleomorph towards the anamorph state (Table 5). Low enzymatic activities measured in bioreactor experiments with OD_{600} values ≥5 (Table 5) and in preparatory cultures (Table 2) imply furthermore a relation between the morphological state of the cells and the xylose fermentation performance. Using cells in the teleomorph state shows increased ethanol yields, xylose uptake rates and high levels of specific enzymatic activities compared to cells in the anamorph state (Table 2, Table 5). A twofold increase in cell dry weight might be due to higher OTR rates resulting in a carbon balance value of 130 % (Table 5).

Cells of *Candida tenuis* are affected by the carbon source used in the preculture media. Xylose grown cells form smaller bodies with changed outer morphology compared to glucose grown cells (Fig. 14). The exchange of xylose by glucose as main carbon source lowers specific enzymatic expression levels for xylose utilization in precultures (Table 8). While glucose and xylose are used efficiently as carbon source, no growth was detected on arabinose.

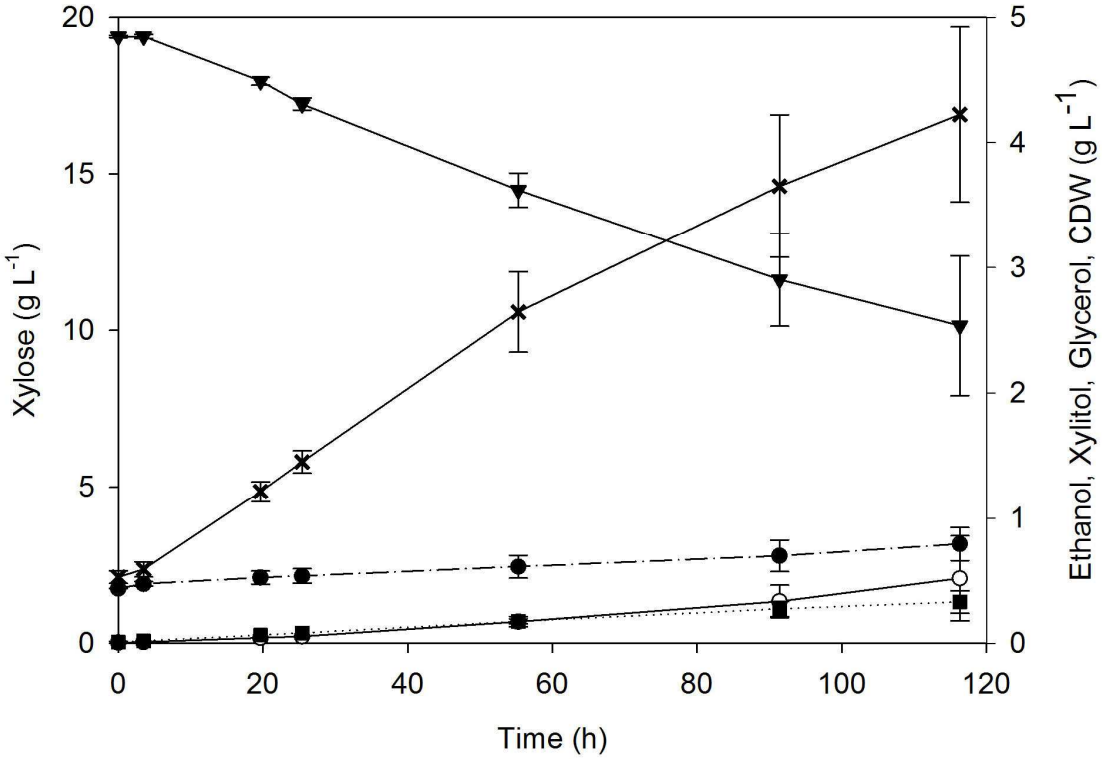

Fig. 13 Time course of xylose conversions in microaerobic bioreactor experiments (OTR = 4±0.1 µM h^{-1}). Symbols: triangles, D-xylose; cross, ethanol; closed circles, CDW; open circles, xylitol; squares, glycerol.

Table 5 Bioreactor experiments with different aeration conditions; note that one preparatory culture was transferred in its early anamorph state.

	CO_2	N_2	Air	N_2/Air
OTR	0 µM h^{-1}	0 µM h^{-1}	4±0.1 µM h^{-1}	68 µM h^{-1}
q_{xylose}[a)]	0.05	0.10	0.16± 0.01	0.05
$Y_{ethanol}$[b)]	0.39	0.38	0.42± 0.01	0.23
$Y_{xylitol}$[b)]	0.05	0.06	0.03± 0.01	0.14
$Y_{glycerol}$[b)]	0.02	0.05	0.03± 0.01	0.15
C-recovery	56%	75%	96%	130%
t-OD_{600}[c)]	3.5	3.2	3.2	8.7
XR[d)]	0.98	0.69	1.1± 0.2	0.08
XDH[d)]	1.44	1.19	2.7± 0.3	0.2

[a]Initial xylose uptake rates (q_{xylose}) are given in (g (g_{CDW} $h)^{-1}$) and were calculated from time-course data obtained from linear data range of fermentation

[b]Yield coefficients (Y) are given in (g (g xylose)$^{-1}$); they were calculated using data obtained after maximum fermentation time of 70h

[c]Shows (transfer-) OD_{600} value of preparatory culture before transferring into bioreactor

[d]Xylose Reductase (XR) and Xylitol Dehydrogenase (XDH) activities are given in (U mg^{-1}) and were measured after 60h of fermentation; measurement parameters as follows: XR 700 mM xylose, 0.35 mM NADH, in 50 mM potassium phosphate buffer pH 7.0, 25°C; XDH 700 mM xylitol, 3.5 mM NAD^+, in TrisHCl buffer pH 9.0, 25°C

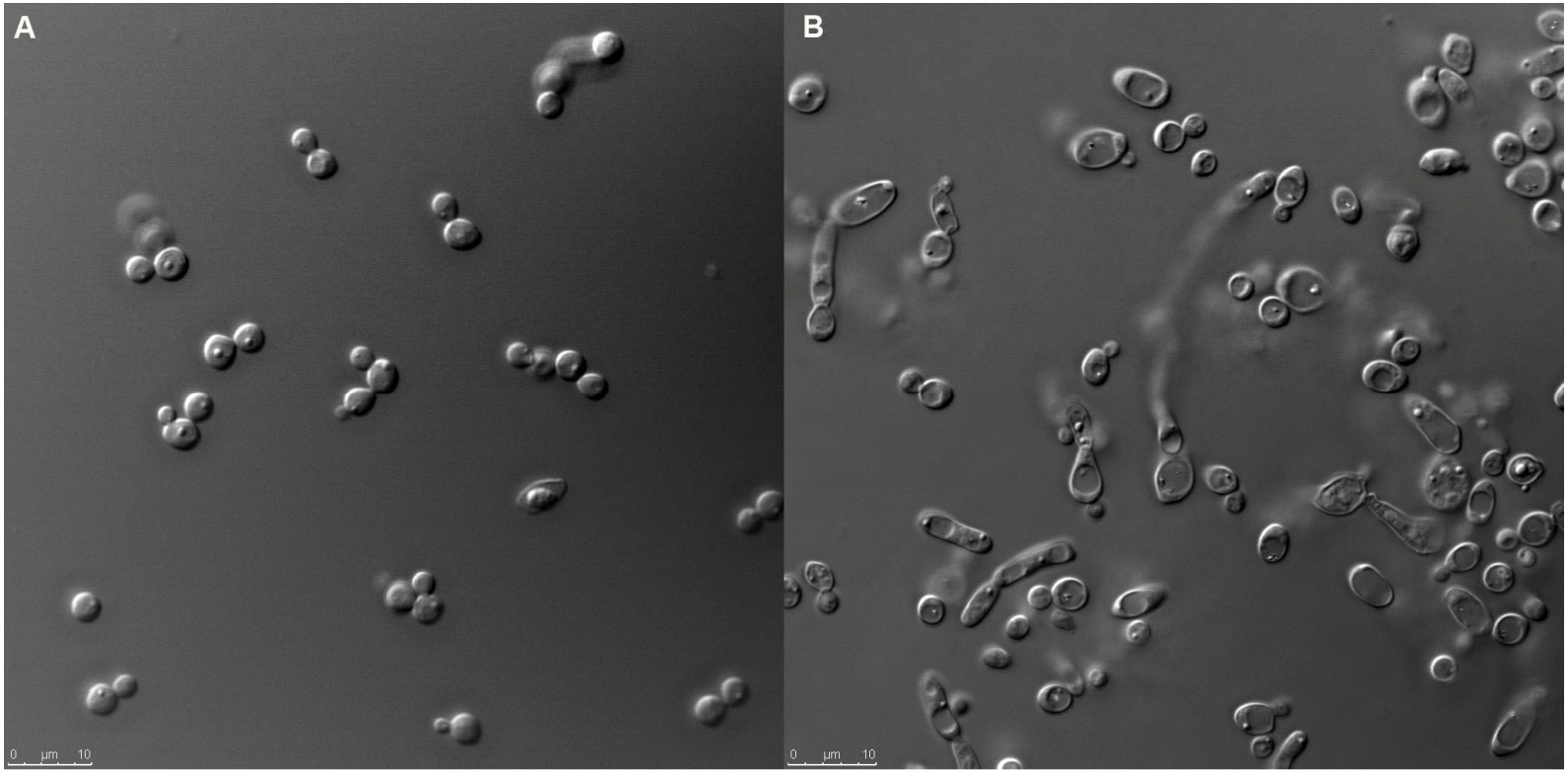

Fig. 14 Phase contrast microscopy picture of a xylose grown preparatory culture OD_{600} = 4 (*A*) and a glucose grown culture OD_{600} = 5 (*B*) with different morphological shape and size.

Whole-cell bioreduction of aromatic ketones

Materials and Methods

Materials and Methods are according to the main section of this work. Modifications of protocols are listed consecutively.

Supplementation with cofactor. 500 µM NAD^+ was added to the aqueous phase (50 mM PPB) for enhancement of enzymatic performance in a 100 mM *o*-chloroacetophenone biphasic 40 % *n*-hexane batch.

Cell permeabilisation. Cell permeabilisation reagent Triton X-100 (Sigma-Aldrich, Gillingham, Dorset, U.K.) was tested for its influence on the productivity at concentrations from 0.05 to 0.1 % in 100 mM *o*-chloroacetophenone biphasic batches with 40 % *n*-hexane.

Partition of o-chloroacetophenone and (S)-1-(o-chlorophenyl)-ethanol between n-hexane and buffer. Solubility of substrate and product were tested in whole cell reductions of 100 mM substrate in biphasic *n*-hexane batches with *Candida tenuis*. After 2 hours of batch conversion amounts of substrate and product were determined in the aqueous and in the *n*-hexane phase. Furthermore, distribution of substrate without the addition of cells was determined using 100 mM *o*-chloroacetophenone in biphasic systems with 50 mM PPB. *n*-Hexane was varied by volume between 20 and 40 % in both experiments.

Effect of culture media carbon source on productivity. Cells were either grown on 20 g L^{-1} glucose or xylose as carbon source in preculture media. The influence of the carbon source on the productivity was investigated in monophasic and biphasic 40 % (v/v) *n*-hexane 100 mM *o*-chloroacetophenone batches with 50 mM PPB as aqueous phase.

Results

Partition of o-chloroacetophenone and S-1-(o-chlorophenyl)-ethanol between n-hexane and buffer. 94 % of the substrate was found in the 40 % *n*-hexane phase without the addition of cells. When cells were added and substrate was converted to the corresponding alcohol, a volume of 40 % *n*-hexane lead to an increased productivity of 1.7-fold compared to 20 % *n*-hexane (Table 6). Partition of substrate and product also changed with increasing volumes of *n*-hexane (Table 6). The total recovery of substrate and product achieved by extraction with ethanol was always above 90 % (calculated using the relation of substrate concentration before conversion and obtained HPLC data of product and substrate concentrations after conversion).

Table 6 Partition of substrate and product between the catalyst, the aqueous phase and the organic phase; reaction conditions: 100 mM *o*-chloroacetophenone batches with varying volumes of *n*-hexane, 2 h conversion time.

***n*-Hexane**			**Potassium phosphate 50 mM**		**Catalyst**		
n-Hexane (%)	Substrate	Product	Substrate (mM)	Product	Substrate	Product	Productivity (g g_{CDW}^{-1})
20[a]	64.12	14.80	3.79	7.02	11.29	1.59	0.13
30[b]	37.88	12.12	3.02	9.17	23.20	5.57	0.17
40[c]	38.61	9.06	2.09	7.57	45.90	9.69	0.19

[a] Initial substrate concentration was 119.66 mM

[b] Initial substrate concentration was 100.26 mM

[c] Initial substrate concentration was 120.96 mM

Effect of NAD^+ and Triton X-100

There is no detectable change in productivity after the addition of NAD^+.

Reduction performance was negatively influenced by the addition of Triton X-100. Productivity dropped at least 2.1-fold with 0.05 % and 4.2-fold with 0.1 % Triton X-100 when added to biphasic batches.

Comparision of Candida tenuis and Pichia stiptis. *Candida tenuis* and *Pichia stipitis* were tested in parallel for their conversion performance with ethyl 4-cyanobenzoylformate and *o*-chloroacetophenone (Table 7). While enantiomeric purity of the product and the productivity were determined to be at levels akin, initial rates were at least 3-fold higher with *Candida tenuis* (30±4 U g_{CDW}^{-1}) as compared to *Pichia stipitis* for *o*-chloroacetophenone (100 mM *o*-chloroacetophenone monophasic batches, 50 mM PPB, no co-substrate added).

Investigation of reductase activities showed 2-fold higher activity for *o*-chloroacetophenone with crude cell extract obtained from *Candida tenuis* compared to *Pichia stipitis* (Table 8).

Effect of culture media carbon source on productivity. When cells were grown on xylose compared to glucose productivity was increased 2-fold in monophasic and a 2.2-fold in biphasic conversions of *o*-chloroacetophenone (Fig. 17).

Table 7 Substrate screening in 100 mM aqueous batches with *Candida tenuis* and *Pichia stiptis;*

Host	Substrate	Product *(S)* (mM)	Product *(R)* (mM)	Productivity (g g_{CDW}^{-1})	ee-value (%)
P. stipitis	ECBF[a]	1.9	9.1	0.51±0.02	81±3
C. tenuis	ECBF[a]	2.9	13.2	0.41±0.01	65±2
P. stipitis	*o*-chl[b]	3.6	-	0.10±0.02	≥99.9
C. tenuis	*o*-chl[b]	3.5	-	0.08±0.01	≥99.9

[a)] Ethyl 4-cyanobenzoylformate (ECBF)

[b)] *o*-Chloroacetophenone (*o*-chl)

Table 8 Reductase expression levels of *C. tenuis* and *P. stiptits.* Cells were grown on different carbon sources, assayed with either D-xylose or *o*-chloroacetophenone;

Host	Substrate[a)]	Enzyme activity (U mg^{-1})	C-source Culture medium[b)]
P. stipitis	D-xylose	0.67±0.03	D-xylose
P. stipitis	*o*-chloroacetophenone	0.18±0.02	D-xylose
C. tenuis	D-xylose	1.20±0.07	D-xylose
C. tenuis	*o*-chloroacetophenone	0.42±0.04	D-xylose
C. tenuis	D-xylose	0.14±0.02	D-glucose
C. tenuis	*o*-chloroacetophenone	0.02±0.00	D-glucose

[a)] Determination of enzymatic activities according to *Materials and Methods* section

[b)] Either 20 g L^{-1} D-xylose or D-glucose was used for culturing cells

Results obtained from whole-cell bioreductions with cells of *Candida tenuis* are shown in the following section (Table 9, Table 10, Table 11, Table 12, Table 13, Table 14, Table 15, Table 17, Table 18; Fig. 15, Fig. 16, Fig. 17, Fig. 18).

Table 9 Productivites of whole cell reductions using different co-substrates; reaction conditions 100mM *o*-chloroacetophenone batches, 50mM PPB, initial pH 5.5, 25℃, 15h conversion;

Co-substrate	Co-substrate (mM)	*n*-Hexane (%)	$Y_{co\text{-}substrate}$ (g g^{-1})	Productivity (g g_{CDW}^{-1})	Product (mM)
-[a)]	-	-	-	0.08±0.01	13
D-xylose	200	0	2.1±0.3	0.06±0.01	9.5
D-xylose	300	0	-	0.06±0.02	12
D-xylose	200	40	2.6±0.2	0.16±0.02	19
D-glucose	200	0	1.9±0.4	0.09±0.01	12
D-glucose	200	40	3.3±0.1	0.25±0.01	32

[a)] Aqueous phase only, no co-substrate added

Table 10 Different *o*-chloroacetophenone concentrations in biphasic batches; reaction conditions: 40 % *n*-hexane with mineral medium and 200 mM glucose as co-substrate, 24 g_{CDW} L^{-1}, 25℃, 10 h conversion time;

Substrate (mM)	Product (mM)	Conversion (%)	Productivity (g g_{CDW}^{-1})	$Y_{Co\text{-}substrate}$ (g g^{-1})
35	26±1.0	75	0.16±0.01	2.00±0.2
40	28±0.7	70	0.19±0.01	2.45±0.2
50	31±0.5	62	0.21±0.02	2.59±0.1
75	34±0.7	45	0.22±0.01	2.80±0.1
100	37±0.2	37	0.25±0.01	3.34±0.1
200	36±0.1	18	0.23±0.01	3.27±0.1

Table 11 Increasing catalyst loading in biphasic batches; reaction conditions: 100 mM *o*-chloroacetophenone, 40 % *n*-hexane and mineral medium with 200 mM glucose, 10 h conversion time;

CDW ($g\ L^{-1}$)	Product (mM)	Productivity ($g\ g_{CDW}^{-1}$)	$Y_{co\text{-}substrate}$ ($g\ g^{-1}$)
9.4	16±0.3	0.27±0.01	2.02±0.1
12.5	23±0.5	0.28±0.01	2.17±0.2
17	30±1.0	0.28±0.01	2.38±0.1
20	32±1.0	0.25±0.01	2.56±0.1
24	37±0.2	0.24±0.02	3.34±0.1
40	59±1.5	0.23±0.01	3.20±0.1

Table 12 Aerated fed-batch; reaction conditions: 10 mM *o*-chloroacetophenone was added each 25 min, 36 $g_{CDW}\ L^{-1}$, 50 mM PPB, 75 rpm, reaction volume 50 mL, 200 mM glucose as co-substrate, 25℃

Time (h)	Substrate (mM)	Product (mM)	Yield (%)
0.42	0.75	4.80	71.10
0.83	0.64	13.89	83.60
1.25	3.01	18.82	72.30
1.67	10.61	20.06	55.60
2.09	18.95	19.13	39.90

Table 13 Aerated fed-batch; reaction conditions: 5 mM *o*-chloroacetophenone was added each 30 min, 26 g_{CDW} L^{-1}, 50 mM PPB, 75 rpm, reaction volume 50 mL, 200 mM glucose as co-substrate, 25℃

Time (h)	Substrate (mM)	Product (mM)	Yield (%)
0.50	0.49	3.83	84.90
1.00	0.06	8.63	91.40
1.50	0.10	12.48	88.80
2.00	1.63	15.50	81.70
2.50	4.41	14.86	57.80
3.00	8.82	15.53	53.90
3.50	12.83	15.65	46.30
4.00	16.07	14.95	38.60
4.50	19.96	15.49	35.80
5.00	23.81	15.18	31.60

Table 14 Productivities of *o*-chloroacetophenone reductions by whole cells of *Candida tenuis* with 0-70 % (v/v) *n*-hexane as co-solvent; reaction conditions: 50 mM potassium phosphate buffer, 200 mM glucose as co-substrate, 25℃, 15h;

Conditions	Time (h)	Productivity (g g_{CDW}^{-1})	Product (mM)	Catalyst[c] (g_{CDW} L^{-1})
70v% *n*-hexane[b]	15	0.27±0.01	16.5	11
60v% *n*-hexane[b]	15	0.28±0.01	22.5	14
50v% *n*-hexane[b]	15	0.28±0.01	27	18
40v% *n*-hexane[b]	15	0.24±0.02	28.5	22
30v% *n*-hexane[b]	15	0.16±0.02	27	25
20v% *n*-hexane[b]	15	0.12±0.01	23	29
monophasic[a]	15	0.08±0.03	12	30

[a]50mM PPB initial pH 5.5
[b]50mM PPB initial pH 5.5, different percent by volume *n*-hexane
[c]Catalyst concentration in batch (g_{CDW} L^{-1})

Table 15 pH shifts and glucose uptake during batch conversions with different aqueous phases; reaction conditions: 100 mM *o*-chloroacetophenone in biphasic 40 % *n*-hexane, 100 mM PPB or 100 mM CPB with 200 mM glucose, initial pH 5.5, measurement start with addition of *o*-chloroacetophenone;

Time (h)	PPB [a] (pH)	CPB [b] (pH)	Time (h)	Glucose in [a] (mM)	Glucose in [b] (mM)
0.000	4.10±0.00	5.30±0.01	0.000	201	206
0.167	4.13±0.04	5.14±0.01	0.167	120	116
0.330	4.49±0.02	5.14±0.01	0.330	117	115
0.500	4.59±0.04	5.14±0.01	0.667	116	116
0.667	4.68±0.04	5.14±0.01	1.000	116	114
1.000	4.77±0.03	5.15±0.02	2.000	113	110
1.500	4.91±0.02	5.14±0.01	4.000	110	107
2.000	5.02±0.01	5.19±0.01	8.000	111	106
3.000	5.19±0.03	5.23±0.01			
4.000	5.21±0.02	5.22±0.01			
8.000	5.30±0.01	5.24±0.01			

[a] Productivity 0.19 g g_{CDW}^{-1}, 18 g_{CDW} L^{-1}
[b] Productivity 0.21 g g_{CDW}^{-1}, 24 g_{CDW} L^{-1}

Table 16 Time course of xylose uptake in whole-cell reductions with xylose as co-substrate; reaction conditions: 100 mM *o*-chloroacetophenone batches, 200 mM xylose as co-substrate, 50 mM PPB; [a] monophasic batches; [b] biphasic 40 % *n*-hexane batches;

Time (h)	Xylose in [a] (mM)	Xylose in [b] (mM)
0.000	202	202
0.167	141	136
0.330	139	131
0.667	134	128
2.000	131	126
4.000	132	126

[a] Productivity 0.10 g g_{CDW}^{-1}, 32 g_{CDW} L^{-1}
[b] Productivity 0.16 g g_{CDW}^{-1}, 19 g_{CDW} L^{-1}

Table 17 Relationship of productivity and pH shift during biphasic batch conversions; reaction conditions: 100 mM *o*-chloroacetophenone, 100 mM PPB, 40 % *n*-hexane, 200 mM glucose as co-substrate;

pH-measurement		Productivity	
(h)	(pH)	(h)	(g g_{CDW}^{-1})
0.000	4.06±0.02	0.000	0.00
0.167	4.06±0.01	0.167	0.01
0.500	4.52±0.01	0.330	0.03
1.000	4.89±0.01	0.667	0.06
1.500	5.06±0.01	1.000	0.09
2.000	5.20±0.01	2.000	0.17
3.000	5.42±0.02	4.000	0.25
4.000	5.42±0.02	8.000	0.24
8.000	5.50±0.01		

Table 18 Comparison of monophasic and biphasic batches with cells of *Candida tenuis* grown on glucose or xylose as carbon source; culture conditions: cells were grown on either 20 g L^{-1} glucose or xylose; reaction conditions: 100 mM *o*-chloroacetophenone, 200 mM glucose as co-substrate, 50 mM PPB, 15 h conversion time;

C-Source[a]	*n*-Hexane (v/v)	Catalyst (g_{CDW} L^{-1})	Productivity (g g_{CDW}^{-1})
Glucose	-	30	0.03
Glucose	40 %	18	0.06
Xylose	-	26	0.09
Xylose	40 %	16	0.20

[a] Either 20 g L^{-1} D-xylose or D-glucose was used for culturing cells

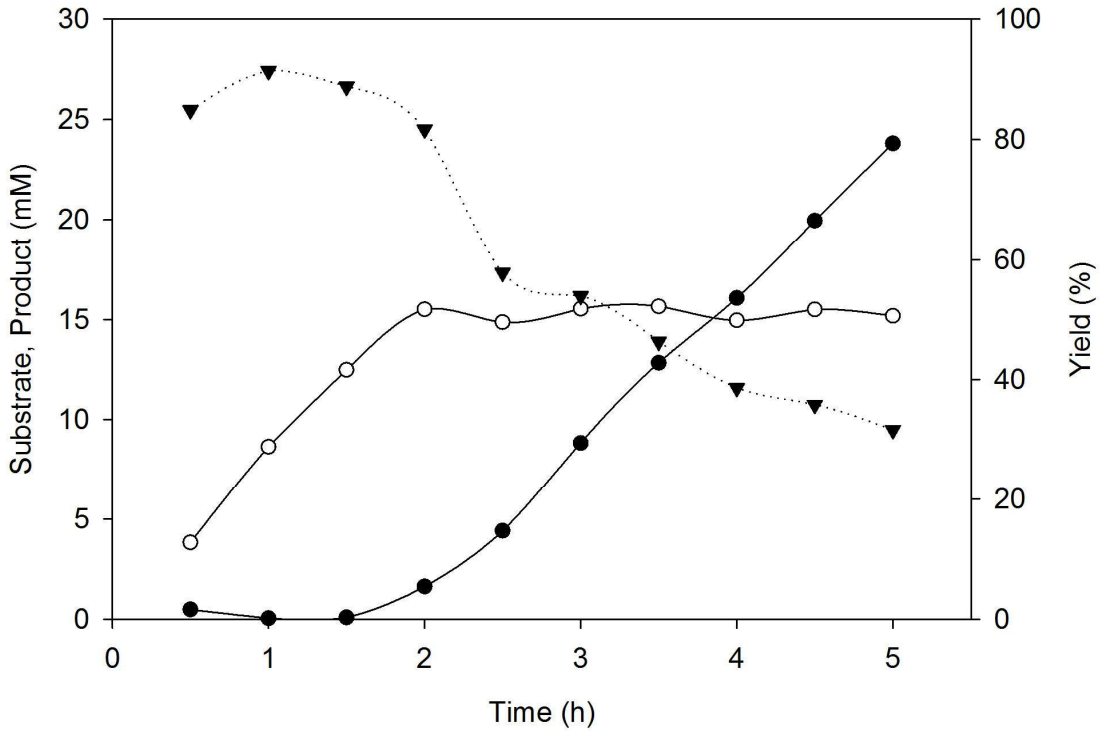

Fig. 15 Areated aqueous *o*-chloroacetophenone Fed-Batch; reaction conditions: 5 mM 30 min^{-1} *o*-chloroacetophenone, 26 g_{CDW} L^{-1}, 25℃, 75 rpm; triangles, conversion yield; open c ircles, (*S*)-1-(*o*-chlorophenyl)-ethanol; closed circles, *o*-chloroacetophenone;

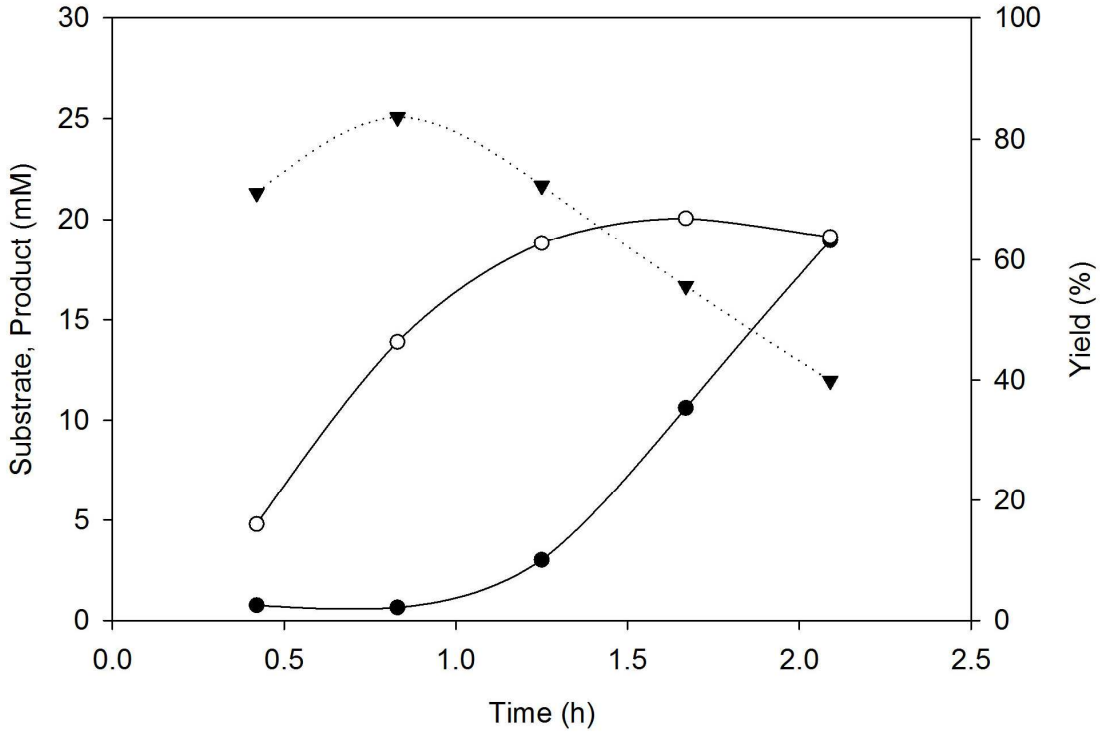

Fig. 16 Areated aqueous *o*-chloroacetophenone fed-batch, reaction conditions: 10 mM 25 min^{-1} *o*-chloroacetophenone; 36 g_{CDW} L^{-1}, 25℃, 75 rpm; triangles, conversion yield; open circles, (*S*)-1-(*o*-chlorophenyl)-ethanol; closed circles, *o*-chloroacetophenone;

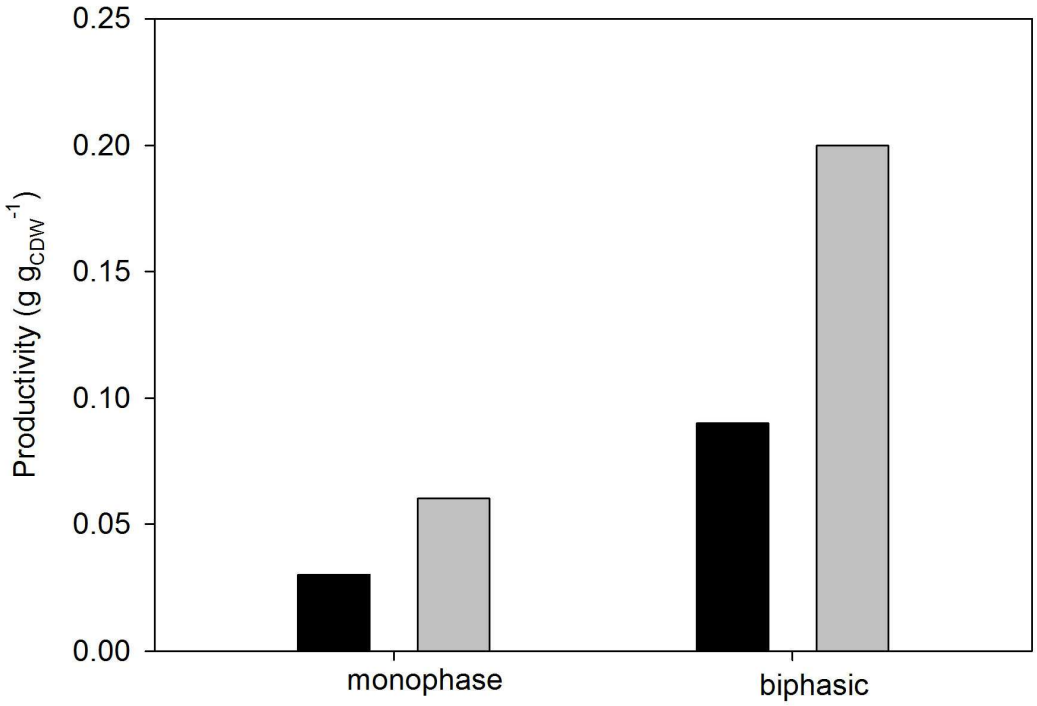

Fig. 17 Comparison of monophasic and biphasic batches with cells of *Candida tenuis* grown on glucose or xylose as carbon source; culture conditions: cells were grown on either 20 g L^{-1} glucose or xylose; reaction conditions: 26 and 16 g_{CDW} L^{-1} (monophasic, biphasic) glucose grown cells; 30 and 18 g_{CDW} L^{-1} (monophasic, biphasic) xylose grown cells; 100 mM *o*-chloroacetophenone, 200 mM glucose as co-substrate, 50 mM PPB, 15 h conversion time; black bars, glucose; grey bars, xylose;

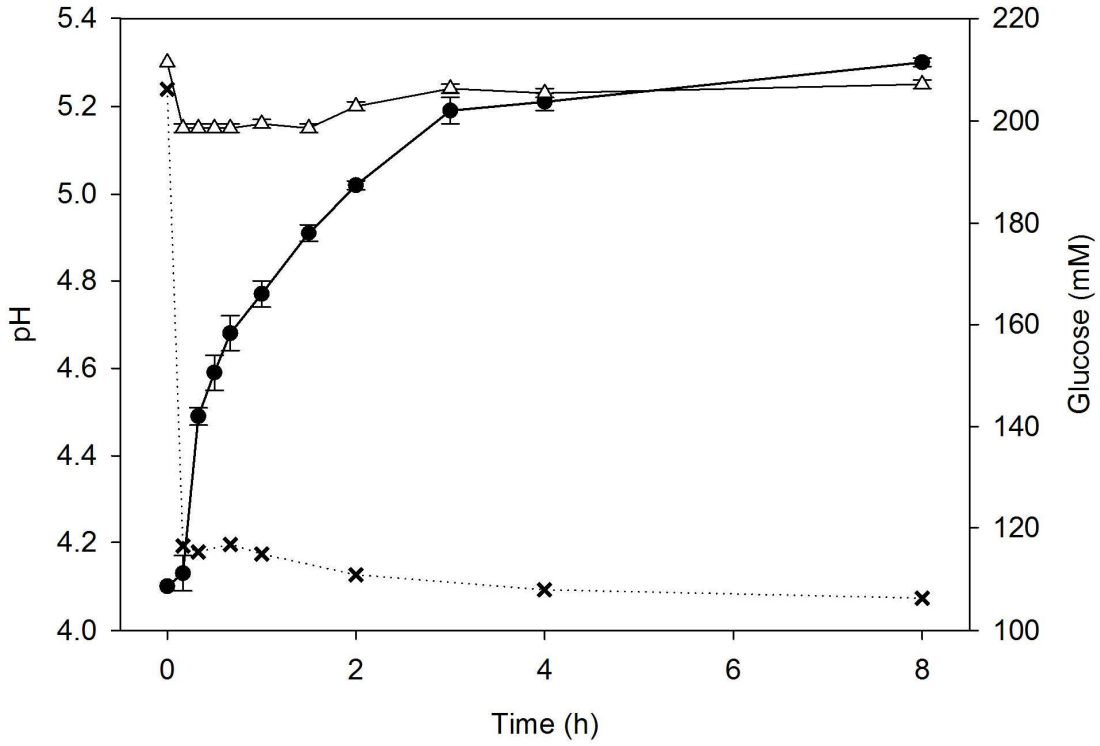

Fig. 18 Determination of co-substrate consumption and pH shifts in whole-cell reductions; reaction conditions: 100 mM *o*-chloroacetophenone, 100 mM CPP; pH curves of whole-cell conversions in 100 mM CPP and PPB; 40 % *n*-hexane, 30 g_{CDW} L^{-1}, 200 mM glucose as co-substrate, 8 h conversion time; cross, glucose; circles, PPB; triangles, CPP;