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Inhibition aspects of the bioconversion of L-phenylalanine to 2-phenylethanol by *Saccharomyces cerevisiae*

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Abstract

The inhibitory impacts of the bioconversion of L-phenylalanine (L-Phe) to 2-phenylethanol (PEA), a very important natural aroma compound, on the metabolism of *Saccharomyces cerevisiae* Giv 2009 were investigated in batch and chemostat cultures. The bioconversion was found to be completely growth associated and lead to a maximal final PEA concentration of 3.8 g/l of PEA. This was attained in a fed-batch procedure on glucose in order to prevent the formation of ethanol, which generally reduced the final achievable PEA concentration by its synergistic inhibitory effect. Chemostat cultures revealed that the bioconversion uncoupled the catabolism from anabolism of *S. cerevisiae* especially under oxidative growth conditions and thereby reduced the critical dilution rate D_{crit}. In addition, higher specific oxygen uptake rates q_{O_2} were found in the presence of the bioconversion at oxidative growth than the maximal respiratory capacity $q_{O_2}^{max}$ found in continuous cultures without bioconversion.

Keywords: 2-Phenylethanol; L-Phenylalanine; *Saccharomyces cerevisiae*; Inhibition kinetics; Metabolic uncoupling

1. Introduction

Biotechnological production of flavors is becoming increasingly attractive because, being classified as natural by the European and US food agencies, this label represents a strong marketing advantage. Numerous examples such as vanillin and benzaldehyde are known to have been already successfully commercialized [1]. 2-Phenylethanol (PEA) is also one of the most important flavors. It posses an aromatic appeal described as a rose-like aroma and is added to modify certain flavor compositions, especially fruit formulas, where it contributes organoleptically. Natural PEA is mainly extracted from rose petals. Because of their rarity these rose oils command a high price [2]. Inspite of the high cost of such an aroma, the demand for natural PEA from vegetal sources continues to outstrip the supply. Accordingly, the flavor industry is turning to the production of aromas obtained by biotechnological processes. A promising approach is the bioconversion of L-phenylalanine (L-Phe) via the Ehrlich pathway to PEA using yeast [3]. Ehrlich described the first step as a deamination that yields ammonia. However, it is now accepted that the first step is a transamination with the formation of glutamate from α-ketoglutarate [4,5]. The decarboxylation of phenylpyruvate results in the formation of phenylacetaldehyde, which is subsequently reduced by an alcohol dehydrogenase to PEA (Fig. 1). The formation of fusel alcohols from amino acids is restricted to the growth phase of the yeast [6]. It is probably the transamination step that is growth associated, since it needs α-ketoglutarate from the TCA cycle.

There is a large variety of yeast that are known to produce PEA. *Saccharomyces cerevisiae*, *Hansenula anomala* [7] and several *Kluyveromyces* strains [8] are known to form important amounts of PEA from L-Phe. The wild strain *S. cerevisiae* Giv 2009 was screened by Givaudan Flavors Ltd. (Dübendorf, Switzerland) for its good production performance of PEA in the presence of the precursor L-Phe.

PEA has been used for many years as a bactericide in pharmaceutical preparations [9]. Concentrations between 2 and 3 g/l have been found to inhibit completely the growth of several species of bacteria and fungi [9–13]. Seward et al. [14] have reported a decrease of the growth rate by 75% in the presence of 2.5 g/l of PEA for *S. cerevisiae*. Several possible mechanisms of inhibitory actions of PEA are mentioned in literature. Cell membranes seem to be an important target site for PEA in general, since aromatic alcohols

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Nomenclature

c	concentration (g/l)
c_i^{inh}	inhibitory threshold concentration of product i in Holzberg model (g/l)
D	dilution rate (h^{-1})
D_{crit}	critical dilution rate (h^{-1})
F	volumetric feed rate (ml/min)
ISPR	in situ product recovery
k	slope in the Holzberg model (l/g)
L-Phe	L-phenylalanine
PEA	2-phenylethanol
P/O ratio	moles of ATP per oxygen atom used in the oxidative phosphorylation
q	specific consumption or production rate (mmol/g/h, g/g/h)
$q_{O_2}^{max}$	maximal respiratory capacity of the yeast (mmol/g/h)
r	volumetric productivity (mmol/l/h, g/l/h)
RQ	respiratory quotient (q_{O_2}/q_{CO_2})
t	time (h)
V	volume (l)
Y	yield (g/g, mol/mol)
Y'	yield (C-mol/C-mol)
α	slope in the Ludeking–Piret equation (g/g)
β	intercept in the Ludeking–Piret equation (g/g/h)
μ	growth rate (h^{-1})
μ_{max}	maximal growth rate (h^{-1})

Subscripts

EtOH	ethanol
S	glucose
X	biomass
0	initial condition

Superscripts

i	referred to compound i
in	referred to reactor inlet flow
ox	referred to oxidative conditions

in general increase the membrane fluidity [12]. As a result, leakage of ions [14] and a reduced uptake of amino acids and glucose [10] are reported in the literature. Wilkie and Maroudas [11] have shown that PEA inhibits the growth of *S. cerevisiae* by inducing a respiratory deficiency. They attributed the deficiency partly to the induction of petite mutations (cells synthesize non-functional mitochondria), and partly to a direct inhibition of respiration. Increased mitochondrial permeability was proposed as the reason inhibition of respiration. PEA has also been investigated as a bacterial inhibitor of macromolecular synthesis [13]. They found that PEA inhibits protein and RNA synthesis in *Escherichia coli*, probably via specific mechanisms of action [15].

The aim of this work was to analyze and optimize the production characteristics of PEA from L-Phe by *S. cerevisiae*. The impact of the bioconversion on kinetics and stoichiometry was examined according to the following steps:

- The kinetics of growth inhibition were determined in the presence of different exogenous PEA and ethanol concentrations.
- The impact of the bioconversion on the metabolism of *S. cerevisiae* was analyzed in chemostat experiments.
- PEA production was optimized for batch cultures applying the experimental knowledge from the first two steps.

2. Materials and methods

2.1. Strain and medium composition

The wild strain *S. cerevisiae* Giv 2009 was a gift of Givaudan Flavors Ltd. and was used for all experiments. Medium L, that was used in all experiments, contained the following compounds (Fluka, Buchs, Switzerland): 30 g/l glucose, 6 g/l $(NH_4)_2SO_4$, 1.92 g/l $(NH_4)_2HPO_4$, 0.87 g/l KCl, 0.45 g/l $MgSO_4 \cdot 7H_2O$, 0.28 g/l $CaCl_2 \cdot 2H_2O$, 22.5 mg/l EDTA, 6.8 mg/l $ZnSO_4 \cdot 7H_2O$, 1.2 mg/l $MnSO_4 \cdot H_2O$, 0.45 mg/l $CoCl_2 \cdot 6H_2O$, 0.45 mg/l $CuSO_4 \cdot 5H_2O$, 0.6 mg/l $Na_2MoO_4 \cdot 2H_2O$, 4.5 mg/l $FeSO_4 \cdot 7H_2O$, 1.5 mg/l H_3BO_3, 0.15 mg/l KI, 2.67 mg/l Ca-pentothenate, 66.67 mg/l *m*-inositol, 2.67 mg/l thiamin hydrochloride, 2.67 mg/l pyri-

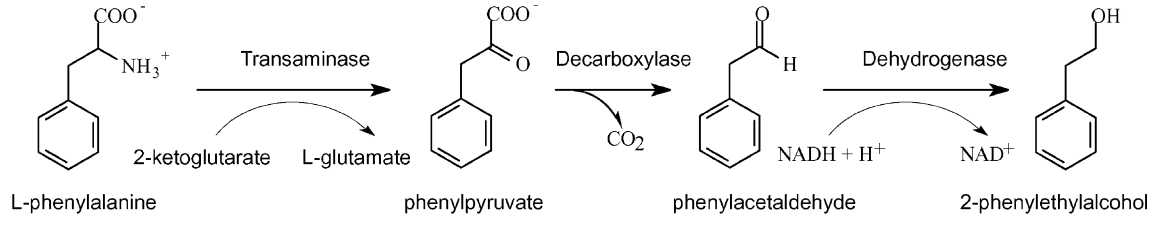

Fig. 1. The conversion of L-Phe into PEA via the Ehrlich pathway.

doxine hydrochloride, 2.67 mg/l nicotinic acid, 0.53 mg/l *p*-amino benzoic acid, 0.13 mg/l biotin, 0.05 ml/l polypropylene glycol P2000.

3. Analytical procedures

Optical density of biomass was measured with a spectrophotometer (CamSpec, Cambridge, UK) at a wavelength of 600 nm. Dry weight of biomass was determined in duplicate samples of each 8 ml each by centrifuging them for 10 min at 3500 rpm (ALC 4237R, Milan, Italy). The pellets were washed in distilled water by resuspension and centrifuged again at the same conditions. The wet pellets were dried for 48 h in an oven at 100 °C followed by weighing (type AE163, Mettler-Toledo, Greifensee, Switzerland). The main metabolites glucose, ethanol, acetic acid, acetaldehyde and glycerol of the reaction suspension were analyzed by HPLC (1100 series, Agilent Technologies, USA) containing a refractive-index (RI) detector. An ion exclusion chromatography column (Supelcogel H 300 mm, Supelco, USA) and a guard column (Superlguard C610H, Supelco, USA) were used at 50 °C. A 0.005 M sulfuric acid solution with ultrapure water was applied at a constant eluent flow of 0.8 ml/min. PEA and L-Phe were analyzed by HPLC. A flow of 1 ml/min was applied through a reversed-phase column (RP-18 endcapped, 250 mm, LiChroCART, 250-4, Merck, Darmstadt, Germany) that was protected by a guard column (LiChroCART 4-4, Merck). A DAD-detector was used at a fixed wavelength of 254 nm. A gradient, going from 100% of eluent A (ultrapure water containing 0.025 M HCl) to 100% of eluent B (80% HPLC-grade acetonitrile and 20% of a 0.025 M HCl solution of ultrapure water) within 23 min, was applied to separate the compounds.

4. Batch cultures

The yeast *S. cerevisiae* Giv 2009 was grown aerobically in a batch culture on 30 g/l of glucose and the defined medium L. All batch cultures were performed in a 3.6 l KLF fermenter (Bioengineering, Wald, Switzerland) at 30 °C and a stirring speed of 1200 rpm. The working volume was 2.5 l and the pH was adjusted to 4 by the addition of 2 M NaOH. The aeration rate was constant at 2.5 l/min (1 vvm). Dissolved oxygen tension was measured by a pO_2 probe (Bioengineering) and was always above 70%. Carbon dioxide, oxygen and ethanol were monitored online by an acoustic gas analyzer (Brüel & Kjær type 1311, Nærum, Denmark).

5. Fed-batch cultures

The same basic experimental set-up that was used for batch cultures was also applied for fed-batch cultures. In addition, an 2 l solution of 270 g/l of glucose was fed by a peristaltic pump (Preciflow lambda, Visperterminen, Switzerland) into the fermenter. A simple feed forward strategy was used to control the feed rate. According to the following equation the volumetric feed rate F was calculated explicitly from the running time t by choosing the specific growth rate μ [16]. The following parameters of the system needed to be determined beforehand: initial biomass concentration c_{X_0}, initial reaction volume V_0, initial glucose concentration c_{S_0} in the fermenter and glucose concentration in the feed c_S^{in}. In addition, a biomass yield from glucose under oxidative conditions $Y_{X/S}^{ox}$ of 0.3 g/g was used in the presence of the bioconversion of L-Phe to PEA (cf. Fig. 4a).

$$F = \left(\frac{\mu / Y_{X/S}^{ox}}{c_S^{in} - c_{S_0}} \right) V_0 c_{X_0} \exp(\mu t)$$

A normal batch culture on 30 g/l of glucose was done before the fed-batch was started. Thereby, a biomass concentration was achieved which was high enough to attain a reasonable feed rate F at the starting point of the fed-batch. The initial biomass concentration c_{X_0} and the initial L-Phe concentration c_{Phe_0} were adjusted at the beginning of the fed-batch by emptying the fermenter and feeding a concentrated L-Phe (25 g/l) solution to appropriate amounts.

6. Continuous cultures

All chemostat cultures were performed in a 1.6 l KLF fermenter (Bioengineering) with a working volume of 1 l and a stirring speed of 1200 rpm. Culture conditions, control of the fermenter environment, data acquisition and sampling procedures were done the same way as in batch cultures. The aeration rate was constant at 1 l/min (1 vvm). A peristaltic pump (Preciflow lambda), adjusted the feed rate to the chosen dilution rate. The volume of the culture suspension in the fermenter was controlled at 1 l by an overflow device. Four to six samples were taken during three residence times when a steady state was reached. For all steady states balances of carbon and degree of reduction closed within 5%. The dilution rates of accelerostats, which crossed the critical dilution rate from oxidative to oxido-reductive metabolism, were increased with a rate of 0.0075 h^{-2}.

7. Results and discussion

7.1. Inhibition kinetics of exogenous 2-phenylethanol and ethanol

The metabolic products ethanol and PEA are assumed to diffuse without any major resistance across the cell membrane [17]. Therefore, it can be expected that, if exogenously added, these alcohols have a similar impact on the cell as if they are produced endogenously in the cell. Ethanol has to be

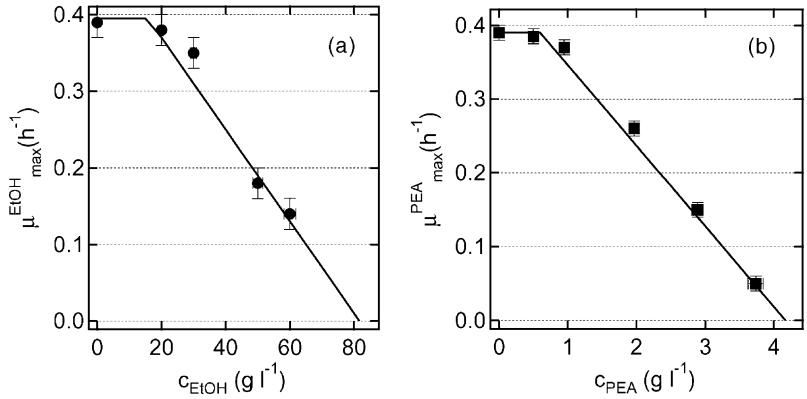

Fig. 2. Determination of the maximal growth rate μ_{max} from batch cultures at different initial ethanol (●) and PEA (■) concentrations. The linear inhibition model of Tables 1 and 2 are represented by the line (—).

considered inhibitory as well, because the Crabtree-positive yeast *S. cerevisiae* can produce ethanol even under aerobic conditions because of its limited respiratory capacity.

Batch cultures on glucose were done at several initial ethanol and PEA concentrations, which were added at the beginning of the culture. The maximum growth rates μ^i_{max} at different concentrations of the inhibitory product i were determined in the first third of the exponential growth phase by measuring the optical density. Fig. 2a shows the results of a series of batch cultivations for the determination of the ethanol inhibition. It can be concluded that the strain *S. cerevisiae* Giv 2009 has a similar ethanol tolerance as other *S. cerevisiae* strains found in literature [18–20]. The model of Holzberg et al. [18] describes well the experimental data for ethanol inhibition (Fig. 2a). This approach is based on linear growth inhibition by an inhibitory concentration c_i above a certain threshold value c_i^{inh}. This parameter and the second parameter k for the slope of the inhibition line were determined for ethanol as the inhibitory compound (Table 1). The parameter μ_{max} was determined by a batch culture without any exogenously added ethanol.

Another series of batch cultivations were done at different PEA concentrations (Fig. 2b). The alcohol was added exogenously to the culture suspension in the beginning of the batch cultures. Therefore, it needs to be emphasized that the presence of PEA did not result from the bioconversion of L-Phe since this precursor was not added to the culture. The linear model of Holzberg best fitted the experimental data. Concentrations of PEA below 0.6 g/l did not reveal any inhibition (Table 2). No growth was detected at a PEA concentration of 4 g/l. Thus, PEA is about 20 times more toxic than ethanol, and consequently also more toxic for *S. cerevisiae* than acetic acid, acetaldehyde or 1-butanol [21].

Table 1
Linear model of ethanol inhibition on the maximal specific growth rate of *S. cerevisiae*

$$\mu^{EtOH}_{max} = \begin{cases} \mu_{max}[1 - k_{EtOH}(c_{EtOH} - c^{inh}_{EtOH})] & \text{for } c_{EtOH} > c^{inh}_{EtOH} \\ \mu_{max} & \text{for } c_{EtOH} < c^{inh}_{EtOH} \end{cases}$$

$\mu_{max} = 0.39\ h^{-1}$
$k_{EtOH} = 0.015$ l/g
$c^{inh}_{EtOH} = 15$ g/l

Table 2
Linear model of PEA inhibition on the maximal specific growth rate of *S. cerevisiae*

$$\mu^{PEA}_{max} = \begin{cases} \mu_{max}[1 - k_{PEA}(c_{PEA} - c^{inh}_{PEA})] & \text{for } c_{PEA} > c^{inh}_{PEA} \\ \mu_{max} & \text{for } c_{PEA} < c^{inh}_{PEA} \end{cases}$$

$\mu_{max} = 0.39\ h^{-1}$
$k_{PEA} = 0.28$ l/g
$c^{inh}_{PEA} = 0.6$ g/l

8. Impact of 2-phenylethanol on the metabolism

The presence of PEA in the reaction suspension did not only change the growth rate of *S. cerevisiae* but also its metabolism and critical dilution rate D_{crit}. This phenomenon was investigated by carrying out continuous cultures at a constant dilution rate D of 0.13 h^{-1} in the presence of different PEA concentrations that were continuously added with the feed (Fig. 3). The yield of biomass from glucose $Y'_{X/S}$ was determined. Growth was completely oxidative (RQ = 1.05) up to a PEA concentration of 2.25 g/l in the reaction suspension. Glucose was always limiting and it raised from a residual concentration of 20 mg/l in the absence of exogenous PEA to 50 mg/l in the presence of 2.25 g/l of PEA (data not shown).

At a PEA concentration of 2.5 g/l ethanol formation of 0.2 g/l was detected (Fig. 3). The specific oxygen uptake rate q_{O_2} increased as well from 3.5 mmol/g/h without any PEA in the culture up to 7.3 mmol/g/h in the presence of 2.25 g/l of PEA (Fig. 3). It decreased sharply down to 3.7 mmol/g/h in the presence of 2.5 g/l of PEA. Simultaneously, PEA also

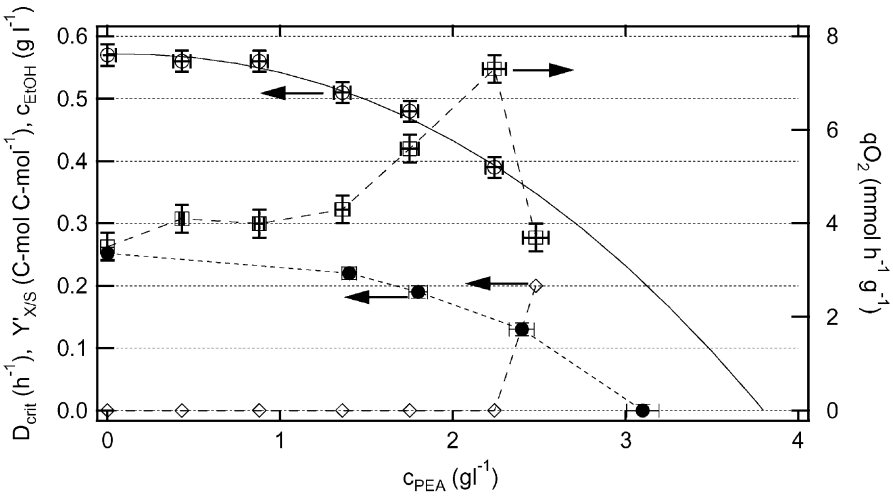

Fig. 3. Specific oxygen uptake q_{O_2} rates (□), biomass yields $Y'_{X/S}$ (○) and ethanol concentrations c_{EtOH} (◇) in aerated chemostat cultures ($D = 0.13\,h^{-1}$) at different exogenous PEA concentrations. Critical dilution rates D_{crit} (●) were determined by accelerostats at different exogenous PEA concentrations.

shifted the critical dilution rate D_{crit} to lower values (Fig. 3). It dropped from $0.25\,h^{-1}$ without any PEA to $0.14\,h^{-1}$ in the presence of 2.4 g/l PEA.

These experimental results from Fig. 3 yield the following main interpretations.

8.1. 2-Phenylethanol acts as metabolic uncoupler

Higher specific oxygen uptake rates q_{O_2}, and a lower biomass yield $Y'_{X/S}$ were noticed in the presence of increasing exogenous PEA concentration at a constant dilution rate. Therefore, under energy-limited conditions PEA uncoupled the anabolism from catabolism, as glucose was used less efficiently for biomass formation. The exact reason for this uncoupling remains to elucidated, but the following three energetic explanations need to be taken into consideration. PEA increases membrane permeability and thereby causes a dissipation of the protonmotive force across the plasma membrane, as it has been observed for other alcohols [22,23]. The P/O ratio may be lowered by inhibitory concentrations of PEA, which have been found to modify mitochondrial permeability and to inhibit thereby respiration [11,24]. As mentioned in the Section 1, PEA is also assumed to provoke inhibition of protein and RNA synthesis. This disorder in anabolism leads to a lower biomass yield per ATP generated $Y_{X/ATP}$.

In addition, a maximal specific oxygen uptake rate of 7.3 mmol/g/h in the presence of 2.25 g/l of PEA was even higher than the maximal respiratory capacity $q_{O_2}^{max}$ of 6.4 mmol/g/h, which was detected in chemostat cultures without any PEA at dilution rates above $0.25\,h^{-1}$ (Fig. 4d). This additional stimulation of the respiration of *S. cerevisiae* by metabolic uncouplers was also found in the presence of weak organic acids such as butyric and benzoic acid [25]. Thereby, the maximal specific oxygen uptake rate doubled up to 20 mmol/g/h when benzoic acid was present in the culture suspension [26]. It was found that the presence of benzoate coincided with an increase in the mitochondrial volume in the cell. This study proved very well that *S. cerevisiae* has not an intrinsic maximal respiration capacity $q_{O_2}^{max}$ but it is able to adapt its respiration capacity towards certain changes in the environment. Compared to alcohols the uncoupling impact of weak organic acids is more significant since they do more than simply permeabilize the membranes. In order to maintain the protonmotive force across the membranes, membrane-bound ATPase has to pump out of the cell all the protons from dissociated acids which entered the cell by diffusive influx of its undissiocated form due to the pH gradient between the medium and the cytoplasm [27].

As a consequence of the metabolic uncoupling, the maximal specific oxidative capacity $q_{O_2}^{max}$ was reached already at lower dilution rates in the presence of PEA. Thereby, the critical dilution rates D_{crit} were shifted to lower values (Fig. 3).

8.2. 2-Phenylethanol concentrations above 2.5 g/l reduce the respiratory capacity

In contrast to the stimulated oxygen uptake at PEA concentrations below 2.25 g/l, concentrations of the flavor above 2.5 g/l reduced the specific oxygen uptake q_{O_2} down to 3.7 mmol/g/h in the chemostat culture at a dilution rate of $0.13\,h^{-1}$ (Fig. 3). The metabolism was not completely oxidative anymore at this high PEA concentration and 0.2 g/l of ethanol were formed. However, the presence of this very low ethanol concentration was certainly not responsible for the reduced respiratory capacity as it can be seen from the ethanol inhibition in Fig. 2. Furthermore, no critical dilution rate was detected anymore at a PEA concentration of 3.1 g/l (Fig. 3). Therefore, PEA concentrations above 2.5 g/l clearly inhibited respiration of *S. cerevisiae*.

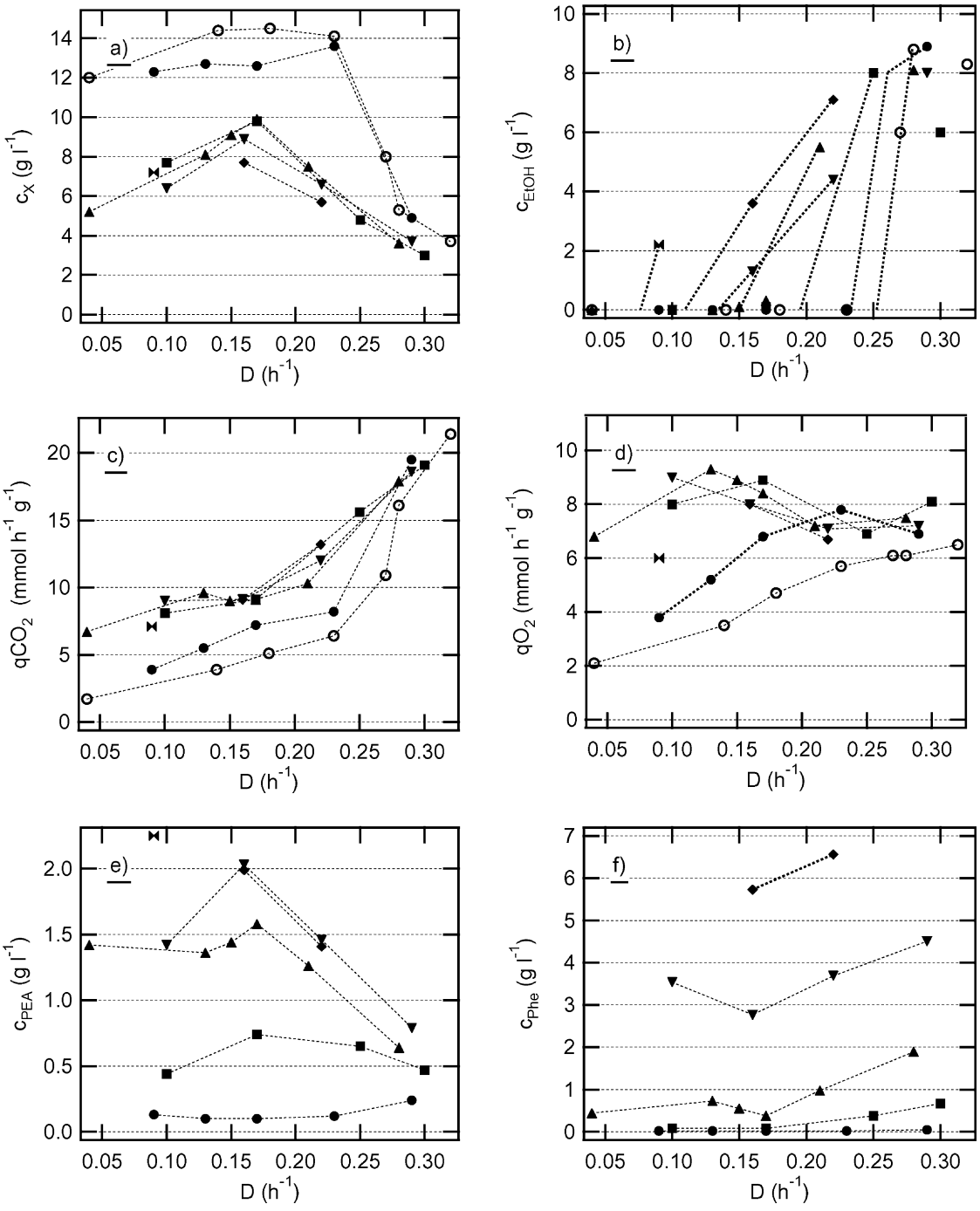

Fig. 4. Aerated chemostat cultures having 0 g/l (○), 0.5 g/l (●), 1.4 g/l (■), 2.9 g/l (▲), 5.8 g/l (▼), 8.4 g/l (◆) and 18.3 g/l (⋈) of L-Phe in the feed.

The same inhibitory phenomenon is also known for higher concentrations of ethanol [28,29].

9. Impact of the bioconversion on the metabolism

Although PEA diffuses without any important resistance across the cell membranes, additional inhibition impacts on cell growth will be investigated in the following when it is produced via the bioconversion of L-Phe. Continuous cultures on 30 g/l of glucose were done with different L-Phe concentrations in the feed. Oxidative, oxido-reductive and anaerobic cultures gave insight into the metabolic response of *S. cerevisiae* in the presence of the bioconversion of L-Phe to PEA. The metabolic uncoupling caused by the bioconversion is analyzed in a first part, whereas the yield of the bioconversion is discussed in a second part.

9.1. Bioconversion causes metabolic uncoupling

Aerated continuous culture experiments at different dilution rates D and different L-Phe concentrations in the feed (0.5, 1.4, 2.9, 5.8, 8.4 or 18.3 g/l) are summarized in Fig. 4. As in the above mentioned case of exogenously added PEA, *S. cerevisiae* revealed the same inhibitory effects when L-Phe was converted into PEA. The biomass yield $Y'_{X/S}$ dropped (Fig. 4a), the critical dilution rate D_{crit} was shifted to lower values (Fig. 4b) and uncoupling provoked an increase of the specific oxygen uptake rate q_{O_2} (Fig. 4d).

At a dilution rate of 0.13 h^{-1} for instance, 14.5 g/l of biomass were produced in the absence of the bioconversion. When 2.9 g/l of L-Phe was fed to the culture, 1.36 g/l of PEA

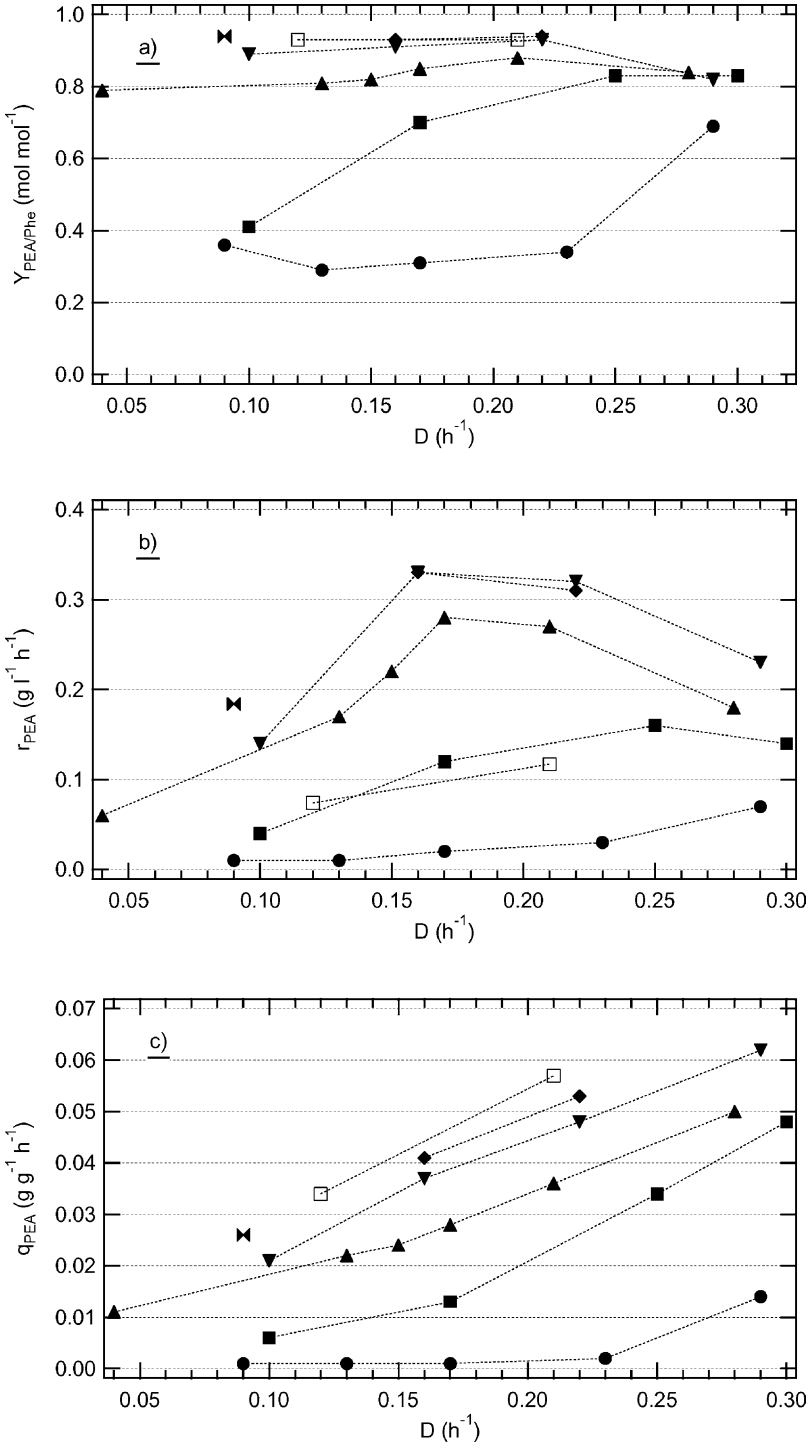

Fig. 5. Aerated chemostat cultures having 0.5 g/l (●), 1.4 g/l (■), 2.9 g/l (▲), 5.8 g/l (▼), 8.4 g/l (◆) and 18.3 g/l (⋈) of L-Phe in the feed. Furthermore, anaerobic continuous cultures (□) having 1.4 g/l of L-Phe in the feed are shown.

were formed and the biomass concentration was reduced to 8.1 g/l of dry cell weight (Fig. 4a). In the same case, the specific oxidative capacity increased from 3.5 mmol/g/h in the absence of the bioconversion to 9.3 mmol/g/h, when the bioconversion took place (Fig. 4d). Critical dilution rates were also shifted down to lower values in the presence of the bioconversion (Fig. 4b). In general, the metabolism of *S. cerevisiae* was significantly more uncoupled when a certain concentration of PEA is produced via the bioconversion from L-Phe than when the same amount of PEA was added exogenously.

9.2. Yields of the bioconversion and PEA production rates

L-Phe was never completely converted into PEA. However, considerable differences in the yield of produced PEA from consumed L-Phe $Y_{PEA/Phe}$ were found during continuous cultures with feed concentrations of 0.5, 1.4, 2.9, 5.8, 8.4 or 18.3 g/l of L-Phe (Fig. 5a). High L-Phe concentrations in the feed increased the $Y_{PEA/Phe}$ up to a value of 0.93 mol/mol. In addition, cultures with a L-Phe feed concentration of 0.5 and 1.4 g/l showed significantly lower PEA yields under purely oxidative conditions. However, their $Y_{PEA/Phe}$ values were raised under oxido-reductive conditions. The trend to higher PEA yields under reductive conditions was confirmed by anaerobic cultures that showed PEA yields of 0.93 mol/mol (Fig. 5a). Overall, it was found from the aerated chemostat cultures (Figs. 4e and 5a), that a minimal L-Phe concentration of 0.8 g/l was needed in the fermenter to get a minimal $Y_{PEA/Phe}$ of 0.8 mol/mol.

Highest PEA production rates ($r_{PEA} = 0.34$ g/l/h) occurred in the dilution rate range of 0.15–0.23 h^{-1} with a L-Phe concentration of 5.8 g/l or higher (Fig. 5b). Since PEA is a growth associated product its production rate decreased at higher dilution rates due to a lower biomass yield under oxido-reductive conditions. However, the highest PEA rates were reached when not all the L-Phe was converted and residual concentrations of the amino acid were measured in the reaction suspension (Fig. 4f). Anaerobic cultures with 1.4 g/l of L-Phe in the feed showed about the same productivity as the oxido-reductive cultures with the same L-Phe concentration in the feed. Even if anaerobic cultures had low biomass yields $Y_{X/S}$, a high PEA productivity was reached because of high PEA yields from L-Phe ($Y_{PEA/Phe} = 0.93$ mol/mol, Fig. 5a). The degradation of L-Phe via another way than the Ehrlich pathway is not known for *S. cerevisiae* in literature. However, it is known that other yeast strains degrade L-Phe via the cinnamate pathway [30,31]. Therefore, further studies need to be done to elucidate the degradation pathway of L-Phe that was found in this process for *S. cerevisiae*.

The production of PEA is growth related. The Ludeking–Piret equation $q_p = \alpha\mu + \beta$ describes the specific product formation q_p as a function of a growth-associated, $\alpha\mu$, and a non-growth-associated, β, term. Fig. 5c shows that if the experimental data of the continuous cultures for each L-Phe concentration in the feed are extrapolated to $D = 0\,h^{-1}$, their values go through the origin. It can be seen from Fig. 5c that the slope α rises with higher L-Phe concentrations in the feed. However, at low L-Phe concentrations of 0.5 and 1.4 g/l in the feed the slope changed at the respective critical dilution rate. This observation is based on the same phenomenon that also caused the decrease of the PEA yield under oxidative conditions, if the feed contained only low L-Phe concentrations (Fig. 5a). The maximal value $\alpha = Y_{PEA/X}$ of 0.37 g/g was attained under anaerobic conditions.

10. Additional observations caused by the bioconversion

The continuous cultures also provided additional information on (a) the morphology and (b) the formation of unpleasant odors at different dilution rates.

10.1. Morphology changes

In addition to these quantitative measurements of the metabolism, yeast morphology revealed some considerable changes as well when exposed to L-Phe under different metabolic conditions. Fig. 6 shows the changes of the yeast morphology when growth conditions were switched from oxidative to oxido-reductive growth ($D = 0.13\,h^{-1}$). *S. cerevisiae* showed an elongated shape under purely oxidative growth conditions when the bioconversion took place. This morphology change was not observed in the absence of the bioconversion. The predominant view in literature is that starvation for nitrogen is the signal for the switch from the yeast shape to a filamentous form [32]. Thereby, pseudohyphae are formed, which are defined as chains of regular-shaped, elongated cells in which polar budding predominates [33]. Oxygen limitation is also mentioned to trigger the formation of pseudohyphae in *S. cerevisiae* cultures [34]. However, other authors claim that there is a clear requirement of oxygen for the morphological change in *S. cerevisiae* to occur [35]. Dickinson [33] discovered that different fusel oils induce hyphal-like extensions and pseudohypal formation of yeasts. Dickinson found that especially isoamylalcohol formed these two elongation patterns, if the fusel oil was either added to the culture or when it was formed via the Ehrlich pathway from its precursor L-leucine. In addition, he found that the fusel oil PEA did not affect yeast morphology. This result corresponds well with our observation, because we did not detect any hyphal-like growth or any pseudohyphae, when PEA was added exogenously. However, as in the case of the bioconversion of L-leucine to isoamylalcohol, the transformation of L-Phe to PEA causes elongated cell growth under oxidative conditions. These pseudohypae disappeared completely when the cells grew under oxido-reductive conditions at high dilution rates (Fig. 6c). At the onset of ethanol forma-

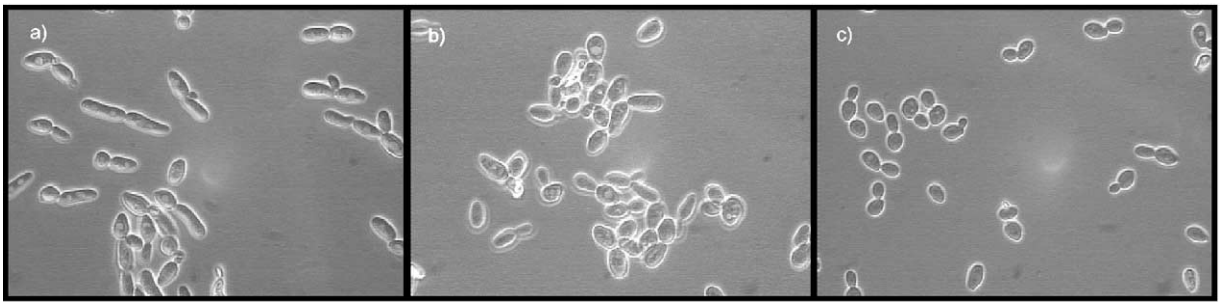

Fig. 6. Light microscope photos (1000× magnification) of continuous cultures of *S. cerevisiae* in the presence of 2.9 g/l of L-Phe. The pseudohyphae form of the yeast appeared under oxidative conditions at $D = 0.13\,h^{-1}$ (a). At the onset of ethanol formation ($D = 0.15\,h^{-1}$) the yeast was less elongated (b) and the "normal" form appeared at high dilution rates ($D = 0.28\,h^{-1}$) under oxido-reductive growth conditions (c).

tion (D_{crit}) *S. cerevisiae* started to change their morphology from a pseudohyphae to a yeast form (Fig. 6b). Literature describes this morphological change as a response to metabolic stress induced by nutrient imbalance [34], which corresponds to uncoupling effects mentioned beforehand.

10.2. Formation of unpleasant odor

An unpleasant, butyric acid-like odor was always formed when the bioconversion of L-Phe to PEA took place under completely oxidative conditions. However, the strength of this smell got reduced immediately when ethanol was formed at the critical dilution rate D_{crit} and disappeared completely under oxido-reductive conditions at higher dilution rates. GC–MS analysis confirmed the presence of unpleasant smelling compounds like isobutyric and isovaleric acid in trace concentrations (~4 mg/l) at dilution rates below D_{crit}. These substances were suspected to be formed because of cell lysis of some yeast under the unfavorable oxidative growth conditions in the presence of the bioconversion and the subsequent degradation of the cell debris.

11. Process considerations of the bioconversion

Results from batch cultures in the presence of L-Phe were compared with the results from chemostat cultures. In addition, based on the knowledge from the continuous and batch cultures, a fed-batch strategy on glucose was tested in order to avoid major ethanol formation and to achieve a higher final PEA concentration.

11.1. Batch process

A diauxic batch culture on 30 g/l of glucose was carried out with 6 g/l of the precursor L-Phe. Thereby, PEA production followed exactly the biomass production during the whole diauxic growth on glucose and ethanol (Fig. 7). A final PEA concentration of 2.35 g/l was achieved. The maximum growth rate μ of $0.38\,h^{-1}$ was not much lower during batch growth on glucose when less than 1 g/l of the inhibitory PEA was present in the culture than the reference batch culture ($\mu = 0.40\,h^{-1}$) without any L-Phe present in the medium. The yield of the bioconversion $Y_{PEA/Phe}$ of 0.91 mol/mol and

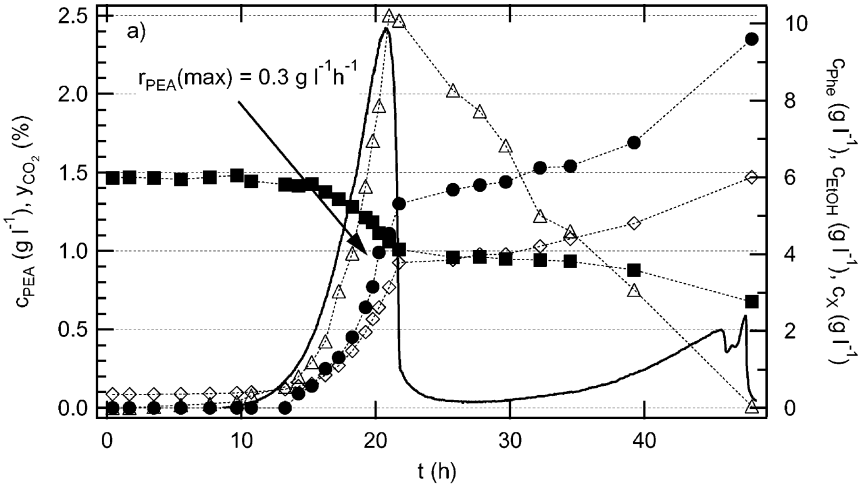

Fig. 7. Diauxic batch growth on 29 g/l of glucose in the presence of a starting concentration of 6 g/l of L-Phe. The concentrations of L-Phe (■), PEA (●), ethanol (△) and biomass (◇), and the online signal of the molar fraction of CO_2 in the off gas (—) are shown.

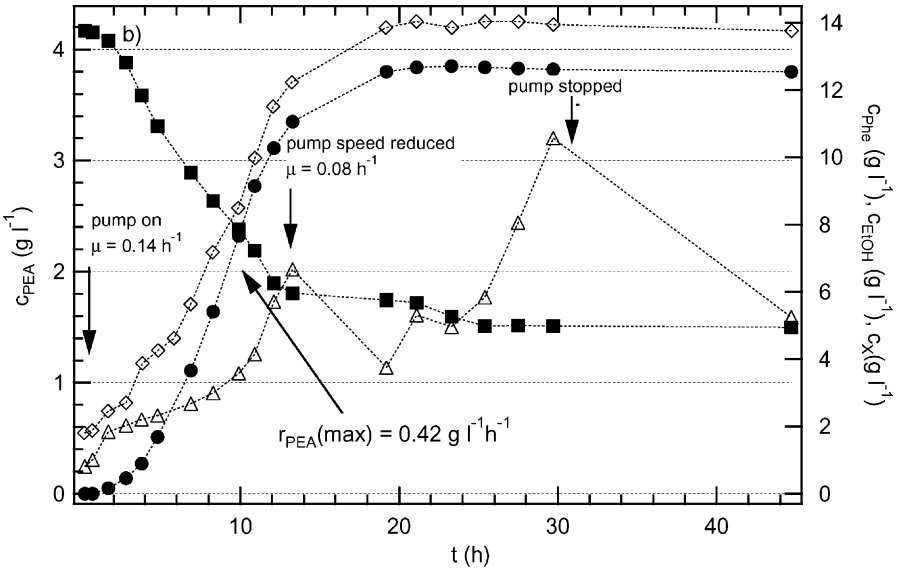

Fig. 8. Fed-batch culture using a feed of 270 g/l of glucose. L-Phe was added in the beginning at a concentration of 13.8 g/l. The concentrations of L-Phe (■), PEA (●), ethanol (△) and biomass (◇) are shown.

the yield of produced PEA per produced biomass $Y_{PEA/X}$ = 0.35 g/g were also in the range that was expected from the continuous cultures. A slightly higher yield $Y_{PEA/X}$ of 0.38 g/g was found during growth on ethanol.

When the bioconversion of L-Phe to PEA was done in a batch culture on 150 g/l glucose or a culture with several repetitive batchwise feeds of 30 g/l glucose (data not shown), growth was stopped at concentrations of 2.4 g/l PEA and 52 g/l of EtOH or at concentrations of 2.5 g/l of PEA and 40 g/l of EtOH. Hence, the co-presence of both inhibitory products stopped the fermentation at considerably lower concentrations compared to their independent toxic values (cf. Fig. 2). Since a batch fermentation with *S. cerevisiae* on glucose intrinsically produces ethanol, this operation mode never allows PEA production to reach 3.9 g/l due to the synergistic inhibitory effect of both toxic compounds. Thereby, the PEA concentration of 2.6 g/l was never exceeded in batchwise operation. Furthermore, it was found that the yeast did not recover from its exposure to the two toxic products. After additional 2 days the ethanol was stripped away and a PEA concentration of only 2.6 g/l remained in the reactor. Also, added glucose was not metabolized anymore.

11.2. Fed-batch process

In order to avoid major ethanol formation a fed-batch on glucose (c_S in the feed 270 g/l) was done by feeding the substrate exponentially to get a specific growth rate μ of 0.14 h^{-1} (Fig. 8). This growth rate was chosen to be 0.14 h^{-1} according to the critical dilution rates that were determined by continuous cultures in the presence of L-Phe (Fig. 4b). Ethanol formation increased rapidly when over 2 g/l of PEA were present in the culture suspension (Fig. 8). Therefore, the pump speed was reduced and the yeast was even able to consume ethanol until t = 20 h. Then the PEA concentration of 3.8 g/l was so high that ethanol was formed rapidly again due to PEA inhibition. Biomass and PEA did not increase any further, since glucose was just metabolized into ethanol. This final step of ethanol formation demonstrated as well that PEA production is strictly growth associated. Metabolic activity by itself did not enable the biotransformation of L-Phe to PEA. The maximum production rate of PEA q_{PEA} (max) was increased in the fed-batch up to 0.42 g/l/h (Fig. 8), compared to a q_{PEA} (max) value of 0.3 g/l/h in the batch culture (Fig. 7) and 0.33 g/l/h during the continuous cultures (Fig. 5b). The yield of the fed-batch bioconversion $Y_{PEA/Phe}$ was determined to be 0.83 mol/mol and the yield of produced PEA per formed biomass was 0.40 g/g. In addition, the maximum concentration of 3.8 g/l PEA achieved in the fed-batch culture agreed very well with the 3.9 g/l that was determined by extrapolation of the inhibitory kinetics of exogenous PEA. This result demonstrated well that PEA did not accumulate within the cells, and that it diffused freely across the cell membrane.

12. Conclusions

Table 3 summarizes the main process-relevant conclusions for the production of PEA from L-Phe and gives rough design guidelines. A fed-batch culture on glucose under oxidative growth conditions is necessary to attain the maximal possible PEA concentration of 3.8 g/l. In addition, high L-Phe concentrations c_{Phe} in the fermenter provide higher bioconversion yields $Y_{PEA/Phe}$. If the formation of unpleasant odors under completely oxidative growth conditions becomes disadvantageous to the PEA production process,

Table 3
Process-relevant conclusions and guidelines for the production of PEA from L-Phe by *S. cerevisiae*

Facts	Consequences
Synergistic inhibition of PEA and EtOH.	Fed-batch culture on glucose is preferred to batch culture in order to avoid ethanol production.
Growth association of bioconversion.	High biomass yields are needed for high PEA production. Therefore, aerobic cultures are preferred to anaerobic cultures because of their higher biomass yields $Y_{X/S}$.
Bioconversion uncouples catabolism from anabolism under oxidative conditions.	If one of the three following points becomes crucial to a PEA production process, an oxido-reductive culture may be superior to a completely oxidative culture. • The uncoupling considerably reduces the biomass yield under oxidative conditions. At very low growth rates the $Y_{X/S}$ of purely oxidative cultures can be even lower as under oxido-reductive conditions (cf. Fig. 4a). • An unpleasant odor is formed under purely oxidative conditions in the presence of the bioconversion. • Lower bioconversion yields $Y_{PEA/Phe}$ are achieved under oxidative conditions at low L-Phe concentrations c_{Phe} (cf. Fig. 5a).
Higher bioconversion yields $Y_{PEA/Phe}$ are attained at high L-Phe concentrations c_{Phe} in the fermenter.	Fed-batch cultures are preferred to continuous cultures because of their higher L-Phe conversions. This results from the fact that a certain fraction of L-Phe is lost in the outflow of the continuous cultures, because a minimal L-Phe concentration c_{Phe} is needed in the fermenter in order to achieve high bioconversion yields $Y_{PEA/Phe}$. On the other hand, batch cultures are performed with a high initial c_{Phe} in order to achieve a low c_{Phe} only at the very end of the culture.

the culture needs to be performed under oxido-reductive conditions where these off-flavors completely disappear. The formation of inhibitory ethanol becomes less important in continuous cultures, where it is not accumulated. However, continuous cultures must be performed at a sufficiently high residual L-Phe concentration in the fermenter in order to achieve sufficiently high bioconversion yields $Y_{PEA/Phe}$. Thus, a continuous set-up reduces the L-Phe conversion of the process.

To reduce the problem of ethanol formation, Crabtree negative strains such as *Kluyveromyces marxianus* or *H. anomala*, which are also known to produce PEA from L-Phe, should be tested. They probably are able to grow at higher growth rates without any ethanol formation or may grow even in a batch culture completely oxidatively. Another solution to circumvent the problem of ethanol formation would be to use glycerol as carbon and energy source.

In order to increase final PEA product concentrations or productivities even more, the intrinsic inhibitory impact of PEA needs to removed. This can be achieved by various in situ product recovery techniques (ISPR) that remove PEA from the fermentation suspension while it is produced [36]. Thereby, the concentration of PEA stays below a inhibitory level and the yeast is able to continue the production of PEA.

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