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Modeling of an integrated fermentation/membrane extraction process for the production of 2-phenylethanol and 2-phenylethylacetate

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ABSTRACT

An unstructured model for an integrated fermentation/membrane extraction process for the production of the aroma compounds 2-phenylethanol and 2-phenylethylacetate by *Kluyveromyces marxianus* CBS 600 was developed. The extent to which this model, based only on data from the conventional fermentation and separation processes, provided an estimation of the integrated process was evaluated. The effect of product inhibition on specific growth rate and on biomass yield by both aroma compounds was approximated by multivariate regression. Simulations of the respective submodels for fermentation and the separation process matched well with experimental results. With respect to the *in situ* product removal (ISPR) process, the effect of reduced product inhibition due to product removal on specific growth rate and biomass yield was predicted adequately by the model simulations. Overall product yields were increased considerably in this process (4.0 g/L 2-PE + 2-PEA vs. 1.4 g/L in conventional fermentation) and were even higher than predicted by the model. To describe the effect of product concentration on product formation itself, the model was extended using results from the conventional and the ISPR process, thus agreement between model and experimental data improved notably. Therefore, this model can be a useful tool for the development and optimization of an efficient integrated bioprocess.

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1. Introduction

Over recent years, there has been an increasing interest in products synthesized by biotechnological processes. However, bioprocesses are often characterized by low levels of productivity due to the accumulation of inhibitory products in the fermentation medium, which makes them economically unattractive [1,2]. A promising approach to overcome this drawback is the removal of inhibitory products from the vicinity of the cells as soon as they are synthesized [3]. Such *in situ* product removal (ISPR) forms part of the general concept of integrated processing, in which upstream, reaction and downstream processes are coordinated [1]. ISPR increases the yield and productivity of a process via three effects: (a) it minimizes product inhibition due to the accumulation of the product in the fermentation medium; (b) it minimizes product losses (e.g. by degradation, evaporation); and (c) it reduces the number of subsequent downstream processing steps [3]. Continuous removal can be achieved using techniques based on extraction, permeation, immobilization, adsorption and evaporation [1,2,4].

ISPR has been studied extensively on a laboratory scale [1]. However, only a few processes have been realized on an industrial scale, e.g. the BIOSTIL® process for ethanol production and a patented ISPR process for extractive lactic acid fermentation [1,5]. The low level of industrial implementation is a result of high investment costs and the long periods of development that are required for process design and optimization [5]. Thus, strategies are required that help to estimate the feasibility of ISPR, particularly in the very early stages of process development with little experimental effort. For this purpose, process models based only on information obtained from the separate subprocesses of an integrated bioprocess can be a suitable instrument. However, although several studies have dealt with modeling of ISPR processes [6–9], there is lack of information about the applicability of such models.

2-Phenylethanol (2-PE) and 2-phenylethylacetate (2-PEA) are high-value aroma compounds with a rose-like odor that are used widely in flavor and fragrance compositions [10,11]. For most applications natural flavors are preferred, but, due to limited natural resources for 2-PE and 2-PEA, biotechnological production of these compounds is essential. The most efficient approach is the bioconversion of L-phenylalanine (L-Phe) to 2-PE and 2-PEA by yeasts [11]. However, both aroma compounds exhibit a high inhibitory potential, which makes conventional bioconversion inefficient [10,11]. For this reason, there have been several attempts to develop methods of removing the synthesized compounds *in situ*, e.g. by pervaporation, *in situ* extraction and extraction with supercritical CO_2 [12–14]. Fabre et al. [15] have shown that membrane

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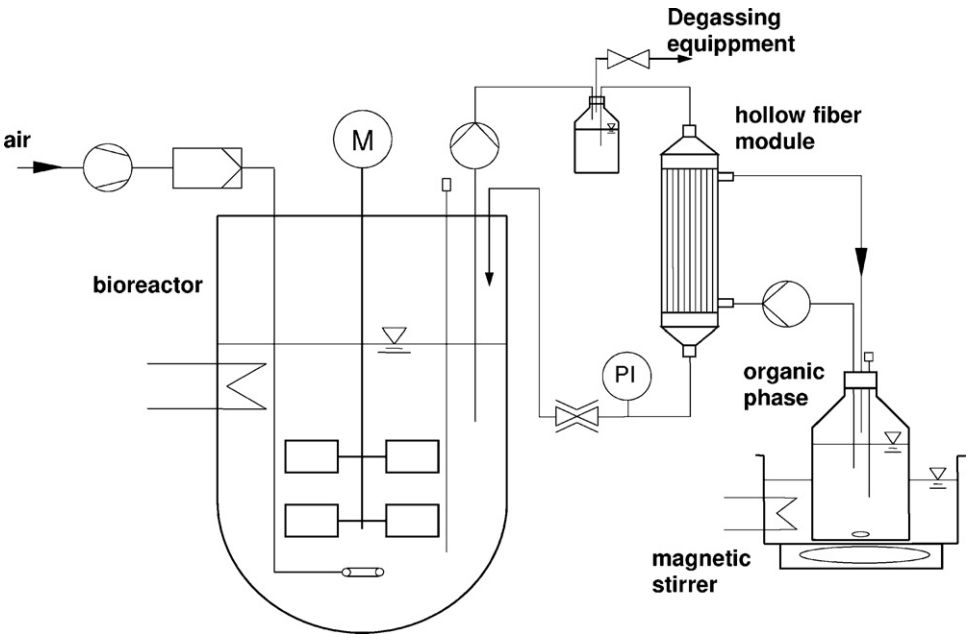

Fig. 1. Experimental setup of the integrated fermentation/membrane extraction process.

based solvent extraction is appropriate for the recovery of 2-PE from aqueous solutions, but it has not been used for *in situ* removal of 2-PE and 2-PEA. Thus, in the present study, synthesis of 2-PE and 2-PEA by the yeast *Kluyveromyces marxianus* was coupled with hollow fiber membrane extraction. The objective of this study was to investigate the extent to which a model that was based exclusively on results from the conventional fermentation and the separation process could be used to predict the integrated bioprocess. Therefore, the models for the individual subprocesses were developed and evaluated separately and subsequently combined to give an overall model for the integrated bioprocess.

2. Materials and methods

2.1. Strain and medium composition

The yeast *K. marxianus* CBS 600 was obtained from the Centraal Bureau voor Schimmelcultures (Utrecht, The Netherlands). The strain was stored on HPG-Agar at −18 °C covered with paraffinum. A synthetic medium based on the results of Etschmann et al. [16] was used for all cultivations and contained 20 g of glucose (monohydrate), 9 g of L-Phe, 1.24 g of KH_2PO_4, 0.16 g of K_2HPO_4, 21 mg of $Ca(OH)_2$, 13 mg of $NiSO_4{\cdot}6H_2O$, 1.02 g of $MgSO_4{\cdot}7H_2O$, 0.2 mg of KI, 0.4 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.56 mg of $FeSO_4{\cdot}7H_2O$, 52 mg of $MnSO_4{\cdot}H_2O$, 4 mg of $CuSO_4{\cdot}5H_2O$, 1.7 mg of $ZnSO_4{\cdot}7H_2O$, 0.03 mg of $CoSO_4{\cdot}7H_2O$, 0.02 mg of H_3BO_3, 0.02 mg of biotin, 3 mg of Ca-pantothenate, 2.4 mg of niacin, 3.2 mg of thiamine–HCl, 1.6 mg of *p*-amino benzoic acid, 1.6 mg of riboflavin, 16 mg of *myo*-inositol and 3.2 mg of pyridoxine–HCl per liter of medium.

2.2. Membrane extraction

Membrane extraction was carried out using a hollow fiber module (housing material: polyacryl) provided by Membrana GmbH (Wuppertal, Germany). It was equipped with 120 microporous, hydrophobic hollow fibers (d_i = 600 μm, d_o = 800 μm, total membrane area A_m = 0.0719 m^2) made of polypropylene (Accurel PP Q3/2, Membrana). For extraction experiments, 800 mL of an aqueous solution that contained 0.6 g/L 2-PE (Sigma–Aldrich, Germany) and 0.15 g/L 2-PEA (Sigma–Aldrich) were used as the aqueous phase. For the organic phase 400 mL of (Synopharm, Barsbüttel, Germany) was used. The aqueous phase was pumped through the lumen of the hollow fibers at a flow rate of 300 mL/min applying a transmembrane pressure difference of 0.5 bar, whereas the organic phase was pumped through the shell side of the module in countercurrent flow (100 mL/min). Both phases were recirculated to the reservoirs, stirred continuously and kept at 33 °C. Samples were taken periodically.

To determine the partition coefficients of 2-PE and 2-PEA five aroma solutions at different initial aroma concentrations [0.06 (0.02), 0.125 (0.04), 0.25 (0.08), 0.50 (0.15), 1.0 (0.30) g/L 2-PE (2-PEA)] were prepared. 25 mL of each aqueous aroma solution was mixed with 25 mL of Miglyol 812 N in a 100 mL separation funnel and stored at 33 °C for 24 h to obtain phase equilibrium. Subsequently, samples were taken from each phase.

2.3. Conventional fermentation and the integrated fermentation/membrane extraction process

The inocula for conventional and integrated fermentation were prepared by two steps of cultivation in 500-mL shaking flasks with four baffles at 33 °C and 175 rpm. In the first step of cultivation, 200 mL of sterile medium was inoculated with *K. marxianus* obtained from the HPG-Agar stock and then incubated for 14 h. In the next step, 180 mL of sterilized medium was inoculated with 20 mL of the culture from the first step and incubated for 10 h. 125 mL of this culture was used as inoculum for fermentation. All fermentation procedures were performed in a 2.0-L BIOSTAT A bioreactor (B. Braun, Melsungen, Germany) with a total working volume of 1250 mL at 33 °C and a stirring speed of 750 rpm. The bioreactor was aerated at 1 vvm and the pH was controlled at 4.0 by automatic addition of 0.25 M H_2SO_4 and 1 M NaOH. Samples were taken every hour.

During the integrated batch fermentation, the fermentation broth was pumped continuously through the lumen of the hollow fiber module (300 mL/min, 0.5 bar) and recycled to the bioreactor. The organic phase (417 mL of Miglyol) was pumped through the shell side of the module with a flow rate of 100 mL/min in recirculation mode (Fig. 1).

2.4. Analytical methods

Growth was monitored by measuring the optical density of the broth with a UV/Vis-spectrophotometer (Genesys 6; Thermo Electron, Erlangen, Germany) at 600 nm. Biomass dry weight was determined gravimetrically via membrane filtration (0.45 μm) and subsequent drying of the filters at 105 °C for 24 h. To determine the glucose concentration, an enzymatic test kit (D-Glucose; R-Biopharm, Darmstadt, Germany) was used. L-Phe was analyzed by HPLC (Alliance 2690 Separation Module; Waters, Eschborn, Germany) equipped with a reversed phase column (LiChroCart 125-4; Merck, Darmstadt, Germany) using a 1:1 water/ethanol mixture as the mobile phase with a flow rate of 1 mL/min at 40 °C. L-Phe was detected using a PDA-detector (996, Waters) at 210 nm. The concentrations of 2-PE and 2-PEA in the aqueous and the organic phase were quantified by gas chromatography (GC 6890N; Agilent Technologies, Böblingen, Germany) using an Optima-δ-6 column (30 m × 0.25 mm × 0.5 μm; Macherey-Nagel, Düren, Germany) and 0.9 mL/min nitrogen as carrier gas at a column temperature of 180 °C. The FID was operated at 250 °C and the injector temperature was 330 °C. The injection volume was 1 μL (split ratio 40:1). Prior to gas chromatographic analysis aqueous phase samples were extracted with ethylacetate and organic phase samples were extracted with a 3:1 ethanol/water mixture. The ethanol concentration was determined by headspace gas chromatography (gas flow: 0.9 mL/min; column: 180 °C; FID: 250 °C; injector: 280 °C/splitless; incubation: 5 min at 80 °C; injection volume: 1 mL).

2.5. Software

MATLAB & SIMULINK *Student Version, Release 13* (The MathWorks, Natick, MA, USA) was used for parameter optimization and numerical simulations. For the simulations the *ode 45* solver was used. Multivariate regression was carried out using the MATLAB function *lsqcurvefit*.

3. Calculation

In the present study, membrane extraction was carried out in a hollow fiber module, with both phases flowing continuously through the module with recirculation to the phase reservoirs. For laboratory modules the change in concentration during a single pass through the module can be neglected [17,18]. Assuming ideal mixing in the reservoirs, the mass balances for 2-PE and 2-PEA in the aqueous and organic phases are [17–19]:

$$\frac{dc_{a,i}}{dt} = -\frac{K_{a,i} \cdot A_m}{V_a} \cdot \left[c_{a,i}(t) - \frac{c_{o,i}}{H_i} \right] \quad (1)$$

$$\frac{dc_{o,i}}{dt} = \frac{K_{a,i} \cdot A_m}{V_o} \cdot \left[c_{a,i}(t) - \frac{c_{o,i}}{H_i} \right], \quad i = \text{2-PE, 2-PEA} \quad (2)$$

Thus, membrane extraction kinetics are characterized by two material-dependent kinetic parameters: The overall mass transfer coefficient K_a determines mass transfer velocity, the partition coefficient H defines the phase equilibrium and has an impact on the driving force of the process. Owing to the fact that, in this study, the partition coefficient was described as a function of the solute concentration, Eqs. (1) and (2) could not be solved analytically [18,19]. Therefore, numerical methods had to be applied to integrate these differential equations.

To describe growth of *K. marxianus* CBS 600 and product formation an unstructured model was applied. It was assumed that growth was an autocatalytic reaction (maintenance was neglected):

$$\frac{dc_x}{dt} = \mu(c_s, c_{2\text{-PE}}, c_{2\text{-PEA}}) \cdot c_x \quad (3)$$

where the specific growth rate μ depended on substrate and product concentrations. In this study, glucose is the main carbon source for growth (in the presence of glucose, L-Phe catabolism is suppressed). In addition, it has been observed that *K. marxianus* produces significant amounts of by-products, such as ethanol, that can be metabolized in a second growth phase [20]. Therefore, growth on ethanol was also included in the model. Substrate limitation of glucose and ethanol was modeled using Monod's equation, the repression of ethanol consumption by glucose was described according to Sonnleitner and Käppeli [21]. To describe the inhibitory effects of 2-PE and 2-PEA, Luong's model [22] was applied, as performed previously by Etschmann et al. [12] and Maltzahn [23]. Hence, the specific growth rate is:

$$\mu = (\mu_{Glc} + \mu_{EtOH}) \cdot \left(1 - \left(\frac{c_{2\text{-PE}}}{c_{2\text{-PE,max}}} \right)^{\alpha} \right) \cdot \left(1 - \left(\frac{c_{2\text{-PEA}}}{c_{2\text{-PEA,max}}} \right)^{\beta} \right) \quad (4)$$

$$\mu_{Glc} = \mu_{max,Glc} \cdot \frac{c_{s,Glc}}{c_{s,Glc} + K_{M,Glc}} \quad (5)$$

$$\mu_{EtOH} = \mu_{max,EtOH} \cdot \frac{c_{s,EtOH}}{c_{s,EtOH} + K_{M,EtOH}} \cdot \frac{K_{i,Glc}}{c_{s,Glc} + K_{I,Glc}} \quad (6)$$

With respect to the lag phase, it was implied that the specific growth rate was constant if $c_x < 2 \cdot c_{x0}$.

Considering the described effects, the mass balances for glucose and ethanol are:

$$\frac{dc_{Glc}}{dt} = -\frac{1}{Y_{x/s,Glc}} \cdot \mu_{Glc} \cdot c_x \quad (7)$$

$$\frac{dc_{EtOH}}{dt} = \left(Y_{p,EtOH/x} - \frac{1}{Y_{x/s,EtOH}} \cdot \mu_{EtOH} \right) \cdot c_x \quad (8)$$

When investigating the bioconversion of L-Phe to 2-PE by *Saccharomyces cerevisiae*, Stark [24] observed that the aroma concentration not only affects the specific growth rate but also the biomass yield and explained these findings by an uncoupling of anabolism and catabolism due to increased permeability of the cell membrane, reduced respiration and an inhibited synthesis of protein and RNA. Because this effect of product concentration on biomass yield was also observed for *K. marxianus* in this study, it was described by Jerusalimsky's model [25], which was modified to the following equation:

$$Y_{x/s,Glc} = Y_{x/s,Glc,max} \cdot \frac{1}{1 + c_{2\text{-PE}}/K_{i,Y_{xs},2\text{-PE}}} \cdot \frac{1}{1 + c_{2\text{-PEA}}/K_{i,Y_{xs},2\text{-PEA}}} \quad (9)$$

Due to the reduction of respiratory capacity by 2-PE and 2-PEA [10], a proportion of the glucose was used for ethanol formation (reductive metabolism). Thus, ethanol yield can be considered to be a function of aroma concentration.

If L-Phe is the only nitrogen source, it is metabolized mainly via the Ehrlich pathway. To a limited extent, L-Phe is also degraded via the cinnamate pathway (catabolism) and used for the production of L-Phe-tRNA for protein biosyntheses (anabolism) [20]. By combining anabolic and catabolic reactions in a simplified expression, L-Phe consumption can be described as follows:

$$\frac{dc_{L\text{-Phe}}}{dt} = \left(\frac{1}{Y_{x/s,L\text{-Phe;ehrlich}}} + \frac{1}{Y_{x/s,L\text{-Phe;ana\&cata}}} \right) \cdot \mu \cdot c_x \quad (10)$$

The end product of L-Phe consumption via the Ehrlich metabolism is 2-PE. Considering the esterification of 2-PE to 2-PEA, the mass balances for 2-PE and 2-PEA can be arranged:

$$\frac{dc_{2\text{-PE}}}{dt} = \left(\frac{m_{2\text{-PE/L-Phe}}}{Y_{x/s,L\text{-Phe;ehrlich}}} - \frac{1}{Y_{x/s,2\text{-PE}}} \right) \cdot \mu \cdot c_x \quad (11)$$

$$\frac{dc_{2\text{-PEA}}}{dt} = \frac{m_{2\text{-PEA/2-PE}}}{Y_{x/s,2\text{-PE}}} \cdot \mu \cdot c_x \quad (12)$$

where $m_{j/i} = M_j/M_i$ represents the stoichiometric coefficient for j and i.

In the model for the integrated bioprocess, product formation and transfer to the organic phase have to be considered. Therefore, the mass balances for the aroma compounds [Eqs. (11) and (12)] were combined with Eqs. (1) and (2) leading to:

$$\frac{dc_{a,2\text{-PE}}}{dt} = \left(\frac{m_{2\text{-PE/L-Phe}}}{Y_{x/s,L\text{-Phe;ehrlich}}} - \frac{1}{Y_{x/s,2\text{-PE}}} \right) \cdot \mu \cdot c_x - \frac{K_{a,2\text{-PE}} \cdot A_m}{V_a} \cdot \left[c_{a,2\text{-PE}}(t) - \frac{c_{o,2\text{-PE}}}{H_{2\text{-PE}}} \right] \quad (13)$$

$$\frac{dc_{o,2\text{-PE}}}{dt} = \frac{K_{a,2\text{-PE}} \cdot A_m}{V_o} \cdot \left[c_{a,2\text{-PE}}(t) - \frac{c_{o,2\text{-PE}}(t)}{H_{2\text{-PE}}} \right] \quad (14)$$

$$\frac{dc_{a,2\text{-PEA}}}{dt} = \frac{m_{2\text{-PEA/2-PE}}}{Y_{x/s,2\text{-PE}}} \cdot \mu \cdot c_x - \frac{K_{a,2\text{-PEA}} \cdot A_m}{V_a} \cdot \left[c_{a,2\text{-PEA}}(t) - \frac{c_{o,2\text{-PEA}}}{H_{2\text{-PEA}}} \right] \quad (15)$$

$$\frac{dc_{o,2\text{-PEA}}}{dt} = \frac{K_{a,2\text{-PEA}} \cdot A_m}{V_o} \cdot \left[c_{a,2\text{-PEA}}(t) - \frac{c_{o,2\text{-PEA}}(t)}{H_{2\text{-PEA}}} \right] \quad (16)$$

4. Results and discussion

4.1. Membrane extraction

The partition coefficients of 2-PE and 2-PEA were determined from the separation funnel experiments by linear regression leading to $H_{2\text{-PE}} = 7.7$ ($R^2 = 0.9993$) and $H_{2\text{-PEA}} = 361.0$ ($R^2 = 0.9965$), where $c_{o,i} = H_i \cdot c_{a,i}$.

The mass transfer coefficients were determined by iterative fitting of the integrated model for membrane extraction [derived from Eqs. (1) and (2)] to the concentrations in the aqueous phase which were obtained from the membrane extraction experiment [$K_{a,2\text{-PE}} = 2.08 \times 10^{-6}$ m/s ($R^2 = 0.99$) and $K_{a,2\text{-PEA}} = 1.48 \times 10^{-5}$ m/s ($R^2 = 1.00$)]. Fig. 2 compares the results of the membrane extraction experiment and the simulations model. It is obvious that the model reproduces the experimental data for both aroma compounds adequately. Further investigations (data not shown) demonstrated that the model was robust at varying process parameters (volume ratio, membrane area, initial aroma concentrations); the relative standard deviations of the calculated K_a values were 4.1% (2-PE) and 10.8% (2-PEA), whereas the coefficient of determination (R^2) was always greater than 0.99. Thus, it can be concluded that the applied model is able to predict accurately the removal of 2-PE and 2-PEA from aqueous media.

4.2. Conventional fermentation

K. marxianus CBS 600 was cultivated in a defined growth medium with L-Phe as the single nitrogen source. The experimental results were used to calculate the kinetic parameters for the bioprocess model. The yield coefficients $Y_{x/s,L\text{-Phe;ehrlich}}$, $Y_{x/s,L\text{-Phe;ana\&cata}}$ and $Y_{x/s,2\text{-PE}}$ were determined by stoichiometric balancing of L-Phe consumption and product formation, considering the stoichiometric coefficients $m_{2\text{-PE/L-Phe}}$ and $m_{2\text{-PEA/2-PE}}$. The Monod constants $K_{M,Glc}$ and $K_{M,EtOH}$ as well as $K_{i,Glc}$, $\mu_{max,EtOH}$ and $Y_{xs,EtOH}$ were taken from data reported in the literature for *S. cerevisiae* [21]. The specific growth rate during lag phase was estimated from biomass growth observed within the first 5 h of fermentation.

In the modeling of microbial kinetics product inhibition is described generally by a reduction in the specific growth rate. Luong's model has been used by Etschmann et al. [12] and Maltzahn [23] to model the effect of 2-PE and 2-PEA on growth. In these studies, inhibition kinetics of 2-PE and 2-PEA were developed separately in series of experiments that were carried out in shaking flasks that contained defined concentrations of the relevant aroma compound. However, the transferability of results from experiments in shaking flasks to fermentation in a reactor is limited. Furthermore, it is known that 2-PE and 2-PEA are interconverted by enzymatic reactions, leading to mixed inhibition effects. For this reason, in this study product inhibition kinetics were established on the basis of the results from the conventional batch fermentation. The specific growth rate was calculated according to the finite difference method ($\mu = \Delta c_x/\Delta t/c_x$) and correlated with the aroma concentrations $c_{2\text{-PE}}(t)$ and $c_{2\text{-PEA}}(t)$. For mathematical evaluation, data from the exponential growth phase on glucose were included because it can be assumed that the specific growth rate is only affected by the product concentration as long as glucose is not limiting growth. The model parameters were estimated by multivariate regression [Eq. (4)] ($R^2 = 0.98$). As a result the product inhibition parameters of 2-PE and 2-PEA, as well as the maximal specific growth rate were obtained. The functional correlation $Y_{x/s,Glc}(c_{2\text{-PE}}, c_{2\text{-PEA}})$ was determined in an analogous manner to the correlation between specific growth rate and product concentration, using the modified Jerusalimsky's model [Eq. (9)] ($R^2 = 0.48$[1]). The yield coefficient of ethanol $Y_{p,EtOH/x}$ was described by a linear equation of the first order:

$$Y_{p,EtOH/x} = A + B \cdot c_{2\text{-PE}} + C \cdot c_{2\text{-PEA}} \quad (17)$$

where it was presumed that $Y_{p,EtOH/x,min} = 0$ g/g and $Y_{p,EtOH/x,max} = 0.9$ g/g. The R^2 for the fit of Eq. (17) was 0.98. The results of the multivariate regressions to the data of specific growth rate, biomass yield on glucose and ethanol yield are depicted in Fig. 3.

Table 1
Kinetic parameters of the bioprocess model.

Parameter	Determination method
$m_{2\text{-PE/L-Phe}} = 0.74$ g/g	Stoichiometric value
$m_{2\text{-PEA/2-PE}} = 1.35$ g/g	Stoichiometric value
$Y_{x/s,L\text{-Phe,ehrlich}} = 1.5$ g/g	Mass balance
$Y_{x/s,L\text{-Phe,ana\&cata}} = 17.5$ g/g	Mass balance
$Y_{x/s,2\text{-PE}} = 8.4$ g/g	Mass balance
$Y_{x/s,EtOH} = 0.72$ g/g	Mass balance
$Y_{x/s,max} = 0.32$ g/g	Fitting, Eq. (9)
$K_{M,Glc} = 0.1$ g/L	Sonnleitner and Käppeli [21]
$K_{M,EtOH} = 0.1$ g/L	Sonnleitner and Käppeli [21]
$K_{i,Glc} = 0.1$ g/L	Sonnleitner and Käppeli [21]
$K_{i,Yx/s,2\text{-PE}} = 7.79$ g/L	Fitting, Eq. (9)
$K_{i,Yx/s,2\text{-PEA}} = 0.45$ g/L	Fitting, Eq. (9)
$\mu_{max,Glc} = 0.42\ h^{-1}$	Fitting, Eq. (4)
$\mu_{max,EtOH} = 0.17\ h^{-1}$	Sonnleitner and Käppeli [21]
$\mu_{Lag} = 0.13\ h^{-1}$	Estimated
$c_{2\text{-PE,max}} = 2.4$ g/L	Fitting, Eq. (4)
$\alpha = 1.29$	Fitting, Eq. (4)
$c_{2\text{-PEA,max}} = 0.5$ g/L	Fitting, Eq. (4)
$\beta = 2.0$	Fitting, Eq. (4)
$A = -0.4$ g/g	Fitting, Eq. (17)
$B = 0.04$ g/L	Fitting, Eq. (17)
$C = 4.2$ g/L	Fitting, Eq. (17)

The kinetic parameters that were determined as described above (shown in Table 1) were used for numerical simulations [Eqs. (3)–(12)] in order to compare the predictions of the model with the experimental data from the conventional batch fermentation (Fig. 4). The results shown in Fig. 4 indicated that the model reproduced the experimental data from the conventional bioprocess adequately. Biomass growth, substrate consumption, and the formation of ethanol and 2-PEA were described with a high level of accuracy. It can be concluded that biomass yield, ethanol yield, and specific growth rate, which are functions of the product concentrations, are represented well by the model for mixed product inhibition. In contrast to the model simulations, *K. marxianus* started metabolizing ethanol after 13 h, although glucose was not exhausted at this point. However, growth on ethanol contributes only little to overall growth (Fig. 4A and C). Comparison of the experimental data with the simulations of aroma formation and L-Phe depletion showed that the mass balance of L-Phe consumption [Eqs. (10)–(12)] could be confirmed in general. However, the production of 2-PE was over-estimated during the last phase of fermentation (hours 11–15, Fig. 4B). As a result a total product yield of 1.7 g/L (2-PE + 2-PEA) was expected according to the model simulation, whereas 1.4 g/L 2-PE + 2-PEA were obtained experimentally. This indicated that, in addition to biomass yield and specific growth rate, product formation itself was affected by product inhibition. Wittman et al. [20], who investigated the metabolic physiology of *K. marxianus*, observed similar characteristics. The observed effect might be attributed to a feedback inhibition of the Ehrlich metabolism by 2-PE, while 2-PE is still esterified

[1] The low R^2 arises from high scattering of the yield coefficient, which was calculated according to the finite difference method ($Y_{x/s,Glc} = \Delta c_x/\Delta c_s$). Anyway, the obtained correlation provided a trend for the effect of product inhibition on $Y_{x/s,Glc}$ (Fig. 2B) and was thus considered in the model.

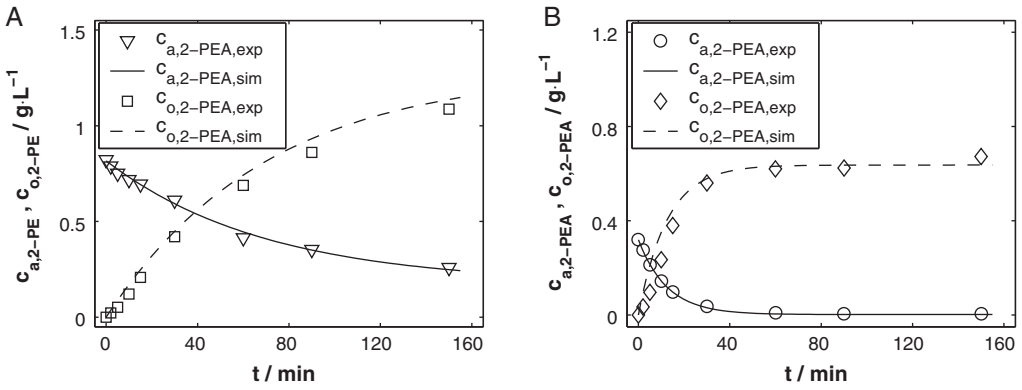

Fig. 2. Membrane extraction kinetics of (A) 2-PE and (B) 2-PEA. Eqs. (1) and (2) were used for modeling, including variable partition coefficients according to Eqs. (14) and (15). Process parameters: $A_m = 0.0719\,m^2$, $V_a = 800\,mL$, $V_o = 400\,mL$. Fitted parameters: $K_{a,2\text{-}PE} = 2.08 \times 10^{-6}$ m/s ($R^2 = 0.99$), $K_{a,2\text{-}PEA} = 1.48 \times 10^{-5}$ m/s ($R^2 = 1.00$).

to 2-PEA. Although this metabolic regulation was not included in the applied model, the results from the conventional fermentation showed that, in general, the model represented the experimental data adequately and appeared to be appropriate for predictive simulations.

4.3. Integrated fermentation/membrane extraction process

To evaluate the model developed for the integrated process [Eqs. (3)–(10) and (13)–(16)], batch fermentation of *K. marxianus* CBS 600 coupled with *in situ* membrane extraction of 2-PE and 2-PEA was carried out. The experimental data obtained were compared with the model simulations (Fig. 5).

Biomass growth and glucose consumption were predicted with a high level of accuracy (Fig. 5A and C). Biomass growth rate as well as biomass yield were described well by the process model. In the experimental integrated process, glucose was depleted after 13 h and the cells started to metabolize ethanol at this point, whereas in the conventional bioprocess glucose was still present after 15 h. This correlation was reproduced accurately by the simulation. However, ethanol production during growth on glucose was under-estimated by the model (Fig. 5A). Despite this, enhanced growth on ethanol due the removal of the aroma compounds was predicted correctly. The measured and simulated concentrations of 2-PEA in the aqueous phase were almost zero throughout the whole process (Fig. 5B). This was a result of the high mass transfer coefficient of 2-PEA, which led to the immediate removal of the substance after its formation. In contrast, 2-PE was still present in the fermentation broth throughout the process because its partition coefficient and mass transfer coefficient were lower than those of 2-PEA [Eq. (1)]. The model described the observed characteristics qualitatively. Furthermore, the model predicted higher product yields for the integrated process (2.7 g/L 2-PE + 2-PEA) than for the conventional process (1.7 g/L 2-PE + 2-PEA) due to increased biomass yield. However, the actual product yields (4.0 g/L) and levels of productivity (0.29 g/L h) were even higher than predicted (Fig. 5B and D).

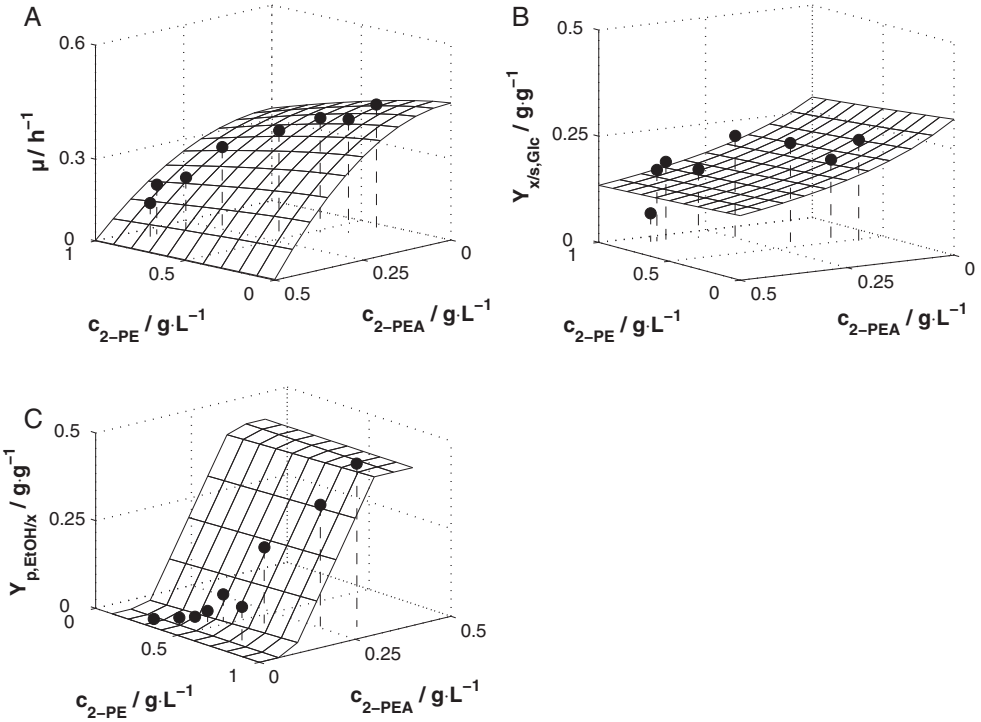

Fig. 3. Specific growth rate (A), biomass yield coefficient on glucose (B) and ethanol yield coefficient (C) as functions of the 2-PE and 2-PEA concentrations, approximated by the inhibition kinetics according to Luong (A), the modified Jerusalimsky model (B) and Eq. (17) (C). The obtained kinetic parameters are shown in Table 1.

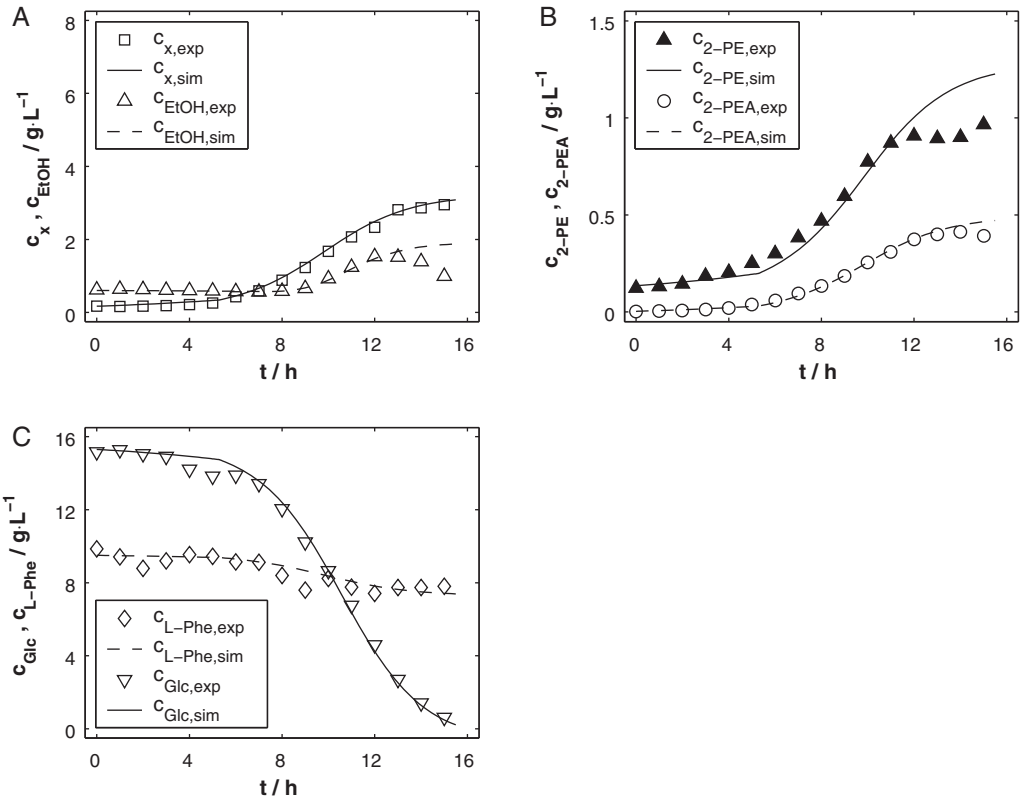

Fig. 4. Comparison of the model simulations and experimental results for the conventional batch fermentation using *K. marxianus* CBS 600. Parameters used for simulation are listed in Table 1.

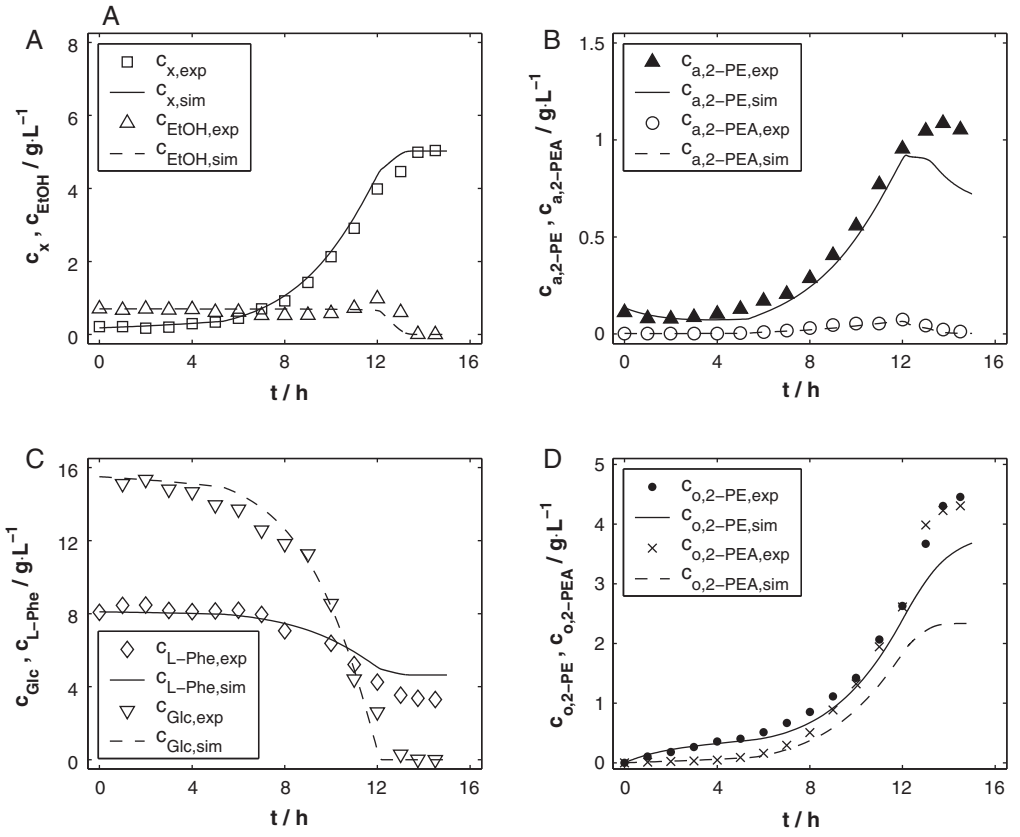

Fig. 5. Comparison of the model simulations and experimental data for the integrated fermentation/membrane extraction process using *K. marxianus* CBS 600. Kinetic parameters are shown in Table 1.

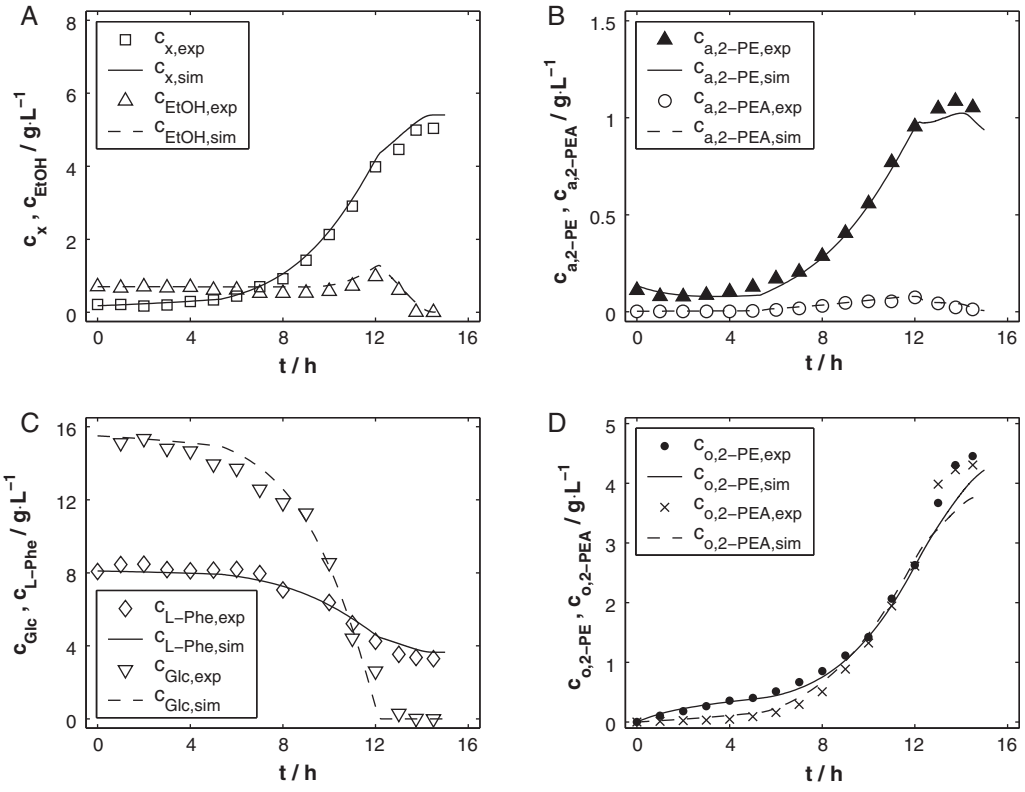

Fig. 6. Comparison of the simulations from the modified model and experimental data for the integrated fermentation/membrane extraction process.

Accordingly, the consumption of L-Phe in the model was lower than under experimental conditions (Fig. 5C). This effect could be attributed to the higher activity of the Ehrlich pathway under experimental conditions due to lower concentrations of 2-PE and particularly 2-PEA in the fermentation broth. Similar results were obtained by Maltzahn [23] and Etschmann et al. [12] when they coupled bioconversion with organophilic pervaporation. Maltzahn assumed a disturbance in the reaction equilibria of the enzymatic reactions for the conversion of 2-phenylacetaldehyde to 2-PE and 2-PE to 2-PEA due to product removal, which resulted in the enhanced metabolism of L-Phe via the Ehrlich pathway. To understand these regulatory mechanisms fully, it would be necessary to characterize the effect of product inhibition extensively, e.g. with the help of continuous cultures. However, such complex investigations are not feasible in the development of industrial processes. In particular, experimental effort must be minimized during early stages of process development. Therefore, it could be useful to integrate the observations from the ISPR into the model developed here. For instance, the effect of the concentration of 2-PE and 2-PEA on the yield of product and ethanol can be described by $Y_{X/S,L\text{-}Phe,ehrlich}$, $Y_{X/S,2\text{-}PE}$, and $Y_{p/x,EtOH}$ as functions of aroma concentration using the experimental data from the conventional and the integrated fermentation. For this purpose, multivariate regression was carried out (data not shown). Fig. 6 shows that the model predicted higher yields of the aroma products due to lower product concentrations in the fermentation medium particularly during the last 4 h of fermentation. Consequently, this led to a better description of the product curves in the aqueous as well as in the organic phase (Fig. 6B and D), and predicted total product yield (3.6 g/L 2-PE + 2-PEA) and productivity (0.25 g/L h 2-PE + 2-PEA) matched well with the experimental values (4.0 g/L and 0.29 g/L h, respectively). Hence, by this procedure, the quality of the model could be improved with only little effort.

5. Conclusion

The model developed here provides a basic understanding about the integrated fermentation/membrane extraction process. We demonstrated that the model can be used to give a rough estimate when based solely on the data from the subprocesses. However, such a model can be continuously adapted to new findings and thereby improved. The numerical simulations of the model could be used to estimate the feasibility of the process at early stages of process development with minimal experimental effort.

Notation

A	empirical constant (g/g)
A_m	membrane area (m^2)
B	empirical constant (g/L)
C	empirical constant (g/L)
c	concentration (g/L)
d_i	inner hollow fiber diameter (m)
d_o	outer hollow fiber diameter (m)
H	partition coefficient ((g/g)
K_a	overall mass transfer coefficient (m/s)
K_i	inhibition constant (g/L)
K_M	Monod constant (g/L)
$m_{j/i}$	stochiometric coefficient j/i
M	molecular weight (g/mol)
R^2	coefficient of determination
t	time (h)
V	volume (m^3)
$Y_{j/i}$	yield coefficient j/i (g/g)

Greek letters

α	inhibition constant of 2-PE
β	inhibition constant of 2-PEA
μ	specific growth rate (h^{-1})

Subscripts

a	aqueous phase
ana&cata	metabolism with anabolic and catabolic function
ehrlich	Ehrlich pathway
EtOH	ethanol
exp	experiment
Glc	glucose
L-Phe	L-phenylalanine
max	maximum
o	organic phase
2-PE	2-phenylethanol
2-PEA	2-phenylethylacetate
s	substrate
sim	simulation
x	biomass

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