Bioprocess Biosyst Eng 25 (2002) 85–96
DOI 10.1007/s00449-002-0279-8

Integrated L-phenylalanine separation in an *E. coli* fed-batch process: from laboratory to pilot scale

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Abstract Pilot-scale reactive-extraction technology for fully integrated L-phenylalanine (L-Phe) separation in *Escherichia coli* fed-batch fermentations was investigated in order to prevent an inhibition of microbial L-Phe production by-product accumulation. An optimal reactive-extraction system, consisting of an organic kerosene phase with the cation-selective carrier DEHPA (di-2-ethylhexyl phosphonic acid) and an aqueous stripping phase including sulphuric acid, was found particularly efficient. Using this system with two membrane contactors, mass-transfer coefficients of up to 288×10^{-7} cm s^{-1} for the aqueous/organic and 77×10^{-7} cm s^{-1} for the organic/stripping phase were derived from experimental data using a simple modelling approach. Concentration factors higher than 4 were achieved in the stripping phase as compared to the aqueous donor phase. Reactive extraction enabled a 98% cation portion of L-Phe in the stripping phase, leading to final product purity higher than 99% after L-Phe precipitation. A doubling of L-Phe/glucose yield was observed when kerosene/DEHPA was added to the fermentation solution in the bioreactor to experimentally simulate a fully integrated L-Phe separation process.

Received: 25 June 2001 / Accepted: 17 January 2002
Published online: 26 March 2002

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This work was funded by a BioRegio- initiative of German Federal Ministry of Education and Research (Grant No. 0311644). The authors are deeply indebted to Professor C. Wandrey, Research Center Juelich, Germany, for his support for this work and for the possibility of using the exclusive technical facilities of the Institute of Biotechnology. The authors would like to thank Dr. Dr. Georg Sprenger and Dr. Johannes Bongaerts for providing the L-Phe production strain, Ms. Heidi Haase for analytical support, Mr. Andreas Franz for technical support and Ms. Sandra Kielmann, Ms. Katja Silberbach and Ms. Nicole Kaftcik for their experimental work. Furthermore, the authors would like to thank Professor Nitsch, Institute of Technical Chemistry (Technical University Munich) for his support for basic mass-transfer experiments.

List of symbols

A_M	effective membrane area (m^2)
$c^{eq}_{Phe,aq}$	L-Phe equilibrium concentration in aqueous solution (mM)
$c_{Phe,aq,0}$	L-Phe start concentration in aqueous solution (mM)
$c^{eq}_{Cphe,org}$	carrier/L-Phe concentration at equilibrium in organic phase (mM)
$c_{Phe,D}$	L-Phe concentration in donor phase (g/l)
$c^{eq}_{Phe,D}$	L-Phe equilibrium concentration in donor phase (g/l)
$c^{eq}_{Phe,A}$	L-Phe equilibrium concentration in acceptor phase (g/l)
$(CH)_2$	carrier dimer concentration (mM)
d_i	inner diameter of extraction module (m)
d_h	hydraulic diameter (m)
E	extraction degree (–)
F^+	cation concentration of amino acids (mM)
F^0	start concentration of amino acid (mM)
F^1	final concentration after batch extraction (mM)
K	equilibrium constant (mM^{-1})
Re_{HF}	Reynolds number in hollow fibre module (–)
l_{eff}	effective module length (m)
N	number of hollow fibres (–)
$\dot{m}_D$	L-Phe mass transfer (g/s)
OD_{620}	optical density (–)
Q	stream (L/s)
V_D	volume (donor phase) (L)
V_{module}	module volume (organic phase) (L)
V_{aq}	volume (aqueous phase) (L)
V_{org}	volume (organic phase) (L)
$\bar{w}$	superficial velocity (average) (m/s)
w_{HF}	superficial velocity (inside hollow fibre) (m/s)
β_A	mass-transfer coefficient (organic/acceptor phase) (cm/s)
β_D	mass-transfer coefficient (donor/organic phase) (cm/s)
ν	viscosity (m^2/s)

Introduction

The aromatic amino acid L-phenylalanine (L-Phe) represents an important building block for the production of

the low calorie sweetener aspartame [1]. In comparison to the currently used chemo-enzymatic process approach based on a racemic mixture of D- and L-Phe, a microbial production of the pure enantiomer by fermentation could be advantageous for aspartame process optimisation.

By removing the wild-type genes *aroF* (encoding for DAHP (2-desoxy-arabino-heptulusonate 7-phosphate)-synthase), *tyrA* (encoding for chorismate mutase/prephenate dehydrogenase) and *pheA* (encoding for chorismate mutase/prephenate dehydratase) and introducing feedback-insensitive genes instead, an *Escherichia coli* production strain was obtained. Although $aroF^{fbr}$ no longer responds to L-tyrosine concentrations we, R. Jossek (Institute of Biotechnology, Jülich, Germany, unpublished results) and others (Backman et al., [2]) have observed that L-Phe concentrations well above physiological levels reduce the activity of *AroF*. Since one of the other DAHP-synthase encoding genes *aroG* is also subject to feed back inhibition by L-Phe, the levels of this enzyme activity will reduce rather drastically. DAHP synthase represents one of the most regulated enzymes of the microbial aromatic amino acid pathway as it catalyses the entrance reaction towards the shikimate pathway, taking erythrose 4-phosphate and phosphoenolpyruvate as substrates. Assuming that the L-Phe export out of *E. coli* cells is concentration driven, high extracellular product concentrations would thus lead to a significant reduction of microbial L-Phe synthesis and possibly to increased by-product formation. It was therefore the aim of this work to avoid this problem by preventing external L-Phe accumulation by the use of process engineering tools.

Because of the complexity of the composition of the fermentation medium, an L-Phe separation approach should be very selective in order to achieve high product purity. As product concentrations in fermentation solution are usually relatively low, product titres in an acceptor solution should be simultaneously increased during separation. In this way, smaller liquid volumes need to be handled in further purification steps. To prevent product accumulation during production, separation technology should be integrated into the fermentation process. This represents an ambitious goal, since any inhibitory influence of downstream processing on the fermentation process should obviously be prevented. In order to avoid loss of unseparated product or fermentation components, it was planned to fully integrate the downstream separation step into the fermentation process. Thus, the fermentation medium that was processed should be completely recycled, which represents another challenge since inhibitory effects of L-Phe separation would directly influence *E. coli* cultivation.

To characterise the L-Phe separation, some physicochemical properties should be considered: L-Phe represents an aromatic amino acid with a water solubility of approximately 30 g/L (under typical fermentation conditions: pH 6.7, T=36°C) and possesses an affinity to organic solvents. Depending on pH, L-Phe cations, zwitter ions or anions exist that provide a molecule charge useful for L-Phe separation. Our preliminary experiments using the principle of Donnan dialysis [3, 4] had shown that a purely charge-dependent amino acid separation was not sufficiently L-Phe selective. An additional selectivity barrier needed to be installed in the form of an organic phase that could be performed by reactive extraction. Compared to physical extraction, reactive extraction provides higher efficiencies owing to higher distribution coefficients [5, 6, 7] and it allows the separation of polar agents such as organic and amino acids in contrast to non-carrier-dependent extraction processes [8, 9]. Furthermore, the working temperatures of reactive-extraction and fermentation processes are similar.

In the past, the application of integrated reactive extraction in fermentation processes was mainly focused on the extraction of organic acids. For instance, lactic acid was separated on-line in *Lactobacillus delbrueckii* fermentations with the anion-specific carrier Alamine 336 in small- and lab-scale approaches [10, 11, 12, 13]. The separation of citric acid during continuous fermentations with *Aspergillus niger* was also described [14, 15]. Other examples such as the integrated separation and esterification of ethanol using *Saccharomyces cerevisiae* (together with an immobilised lipase) [16] were also reported.

In addition to separation approaches for organic acids, the applicability of reactive extraction for amino acid separation was studied previously [17, 18, 19, 20, 21, 22, 23, 24, 25], also focusing on L-Phe separation [26] and aiming at a detailed description of L-Phe mass-transfer kinetics [8, 27, 28, 29]. However, most of these studies used "aqueous model solutions" instead of complex fermentation media and focused their interest only on anion-selective carriers like Aliquat 336. Furthermore, process approaches consisting of fermentation and integrated reactive extraction for L-Phe separation have not thus far been investigated.

We hereby present a new approach that leads to a fully integrated L-Phe reactive-extraction process. As interactions between solvents and/or carriers of the organic phase with microorganisms in the aqueous fermentation solution cannot fully be avoided, special emphasis was put on biological constraints of the current process development. Besides biocompatibility, basic extraction parameters like pH, selectivity, extraction capacity and stability of the process were investigated.

Materials and methods

Carrier systems

Because of the zwitterionic character of L-Phe, cation- as well as anion-specific carriers were investigated. Only commercially available carriers were used to ensure that process scale-up would not be limited by carrier availability. Carriers were dissolved in organic solvents. To identify the optimal carrier/solvent composition for a fully integrated reactive-extraction process, the following boundary conditions need to be met:

1. Carriers and organic solvents should possess low water solubility.
2. The carriers should be readily soluble in the organic phase.
3. No tendency for emulsification should occur in the total aqueous/organic system.

4. Because of the application of reactive-extraction integrated in a bioprocess, sufficient biocompatibility should be achieved.
5. The handling of carriers and solvents should not imply unnecessary safety risks.
6. Carriers and solvents should be "cheap" in order to limit downstream processing costs.

As a result, the solvents kerosene, butyl stearate, decanol, octanol and xylene were used for preliminary experiments.

Cation-specific carriers

Among the group of cation-selective carriers DEHPA (di-2-ethylhexyl phosphoric acid, Bayer AG, Germany) and DNNSA ((di-nonylnapthalene sulphonic acid, King Industries, USA) were considered. DEHPA is a liquid cation exchanger that has already been described for off-line L-Phe extraction [17, 26]. It is assumed that the acid-anion DEHPA extracts the L-Phe cation into the organic phase [24]. DNNSA was used as an alternative liquid cation exchanger. Applications of cation-selective extractions have been reported for the separation of di-amino acids [19], indicating extraction degrees higher than 90% for low pH values.

Anion-specific carriers

To consider L-Phe extraction at pH values above 10, liquid cation exchangers such as quaternary or ternary ammonia salts were investigated. The quarternary ammonia salt tri-octyl-methyl ammonium chloride (Aliquat 336, Henkel, Germany) represents a standard liquid anion exchanger for the extraction of heavy metals on an industrial scale but also for enzymes [30], organic and amino acids [8, 9, 10, 11, 12, 13, 14, 15, 16, 27, 28, 29]. Furthermore, tri-*iso*-octyl-amine (Alamine 308, Henkel, Germany) was considered.

Table 1 gives an overview of the physicochemical properties of the carriers.

Batch extractions in separation funnels

The identification of the optimal reactive-extraction system was based on preliminary batch extraction experiments as well as on further biocompatibility tests. Separation funnels were used for batchwise extractions of L-Phe with different carriers (Alamine 308, Aliquat 336, D_2EHPA, DNNSA), solvents (kerosene, butylstearate, decanol, octanol, xylene) and counter ions ($(NH_4)_2SO_4$, NaOH, KOH, H_2SO_4, KCl). In this way, the most promising system was identified for use in further hollow fibre extraction experiments.

After being filled with equal volumes of aqueous and organic phase (the organic phase consisted of a mixture of solvent with 10% v/v carrier), funnels were shaken intensively for 2 min to ensure a good mixture. After this, at least 10 min were allowed for phase settling. When phase separation had been completed, the aqueous phase was analysed and the organic phase was used for back extraction. Back extraction was performed by mixing the preloaded organic phase with an equal volume of stripping phase in separation funnels. In the stripping phase, counter ions were added to enable the L-Phe unloading of the carrier. As a result, L-Phe was extracted into the stripping phase and the carrier was loaded with corresponding ions from the stripping phase. L-Phe titres in the aqueous phase were measured via HPLC analysis in order to estimate the amount of extracted L-Phe and to calculate the degree of extraction E according to

$$E = \frac{c^{eq}_{Phe,aq}}{c^{eq}_{Cphe,org}} 100 \quad (1)$$

$c^{eq}_{Phe,aq}$ and $c^{eq}_{Phe,aq}$ are the equilibrium concentrations of L-Phe in the aqueous and organic phase after extraction. While $c^{eq}_{Phe,aq}$ is accessible via HPLC measurement $c^{eq}_{Cphe,org}$ can be estimated using L-Phe mass balance with the aid of the aqueous L-Phe concentration $c_{Phe,aq,0}$ which was measured before extraction was started.

Principles of fully integrated reactive extraction

As indicated in Fig. 1, reactive extraction should be fully integrated into the fermentation process. L-Phe was separated from a cell-free fermentation solution that was obtained by biomass retention in a cross-flow ultrafiltration unit. Using hollow fibre modules to install supported liquid membrane (SLM)-based L-Phe transfer, the aqueous solution was brought into contact with an organic phase. An ion exchange took place at the aqueous/organic interface. If cation-specific carriers are used in the organic phase, a proton is transferred into the aqueous phase while at the same time L-Phe$^+$ is loaded onto the carrier. If anion-specific carriers are used, anions are exchanged by analogy. In Fig. 1 carriers are indicated by X and counter ions (e.g., protons) by C. It was assumed that the ion-exchange process occurs dispersion free. However, because of the solubility of carriers in aqueous solution, a

Table 1. Physicochemical properties of carriers

	D_2EHPA Di-2-ethylhexyl phosphonic acid	DNNSA Di-nonylnapthalene sulphonic acid	Aliquat 336 Tri-octyl-methyl ammonium chloride	Alamine 308 Tri-*iso*-octyl-amine
Supplier	Bayer	King Industries	Henkel	Henkel
M (g/mol)	322	495	404	353
Density (kg/L)	0.97	0.87	0.88	0.80
Flashpoint (°C)	198	–1,1	110	190
Solubility in H_2O (20°C) (g/L)	<1	<1	<1	<1
Affinity/selectivity	cations	cations	anions	ionic

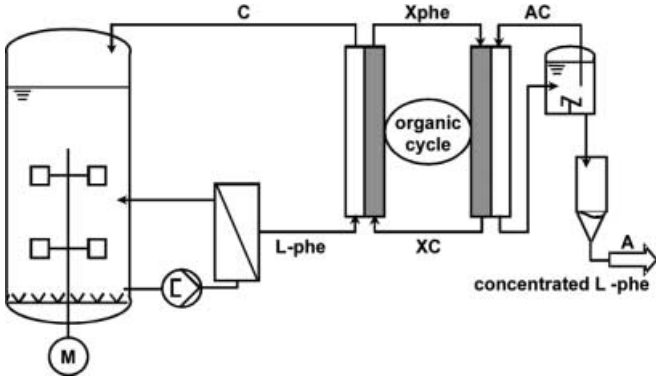

Fig. 1. Principle of fully integrated reactive extraction separating L-Phe from a cell-free aqueous fermentation solution via carriers X of an organic phase into an aqueous stripping phase consisting of the counter ions C and and acceptor A. pH-dependent charges of L-Phe were not considered

diffusive carrier and solvent transport into aqueous phase must be taken into account. After transport of carrier/L-Phe complex to the organic/aqueous interface, L-Phe is released into the stripping phase while the carrier is loaded with the counter ion again and regenerated for the next extraction cycle. As the driving force for the extraction process is not the L-Phe concentration, but the stripping phase concentration of counter ions (which could be freely chosen), a concentration of L-Phe against the gradient of the amino acid is possible. Thus, a significant reduction of liquid volumes could be achieved for further product purification, for instance, by precipitation.

Fermentation

Biological system

The genetically engineered L-Phe production strain *E. coli* W3110–4(pF20), a derivative of *E. coli* K12, was used as a model system. W3110–4 contains a chromosomal deletion Δ (*pheA tyrA aroF*) indicating that the genes *pheA* (coding for chorismate mutase/prephenate dehydratase), *tyrA* (coding for chorismate mutase/prephenate dehydrogenase) and *aroF* (coding for the tyrosine sensitive DAHP synthase (2-desoxy-D-arabino-heptusonate 7-phosphate) are removed. Tyrosine ($aroF^{fbr}$) and phenylalanine ($pheA^{fbr}$) feedback-resistant genes were inserted using plasmid pF20 (based on pJF119EH [31]) carrying $aroF^{fbr}$ and $pheA^{fbr}$ in order to allow L-Phe production. The *tyrA* deletion makes the production strain tyrosine-auxotrophic. The vector also possesses an ampicillin resistance gene and gene expression $aroF^{fbr}$ and $pheA^{fbr}$ is IPTG-inducible.

Pre-cultivation

Cryocultures were stored at –80°C in Luria–Bertani (LB) medium containing 50% glycerol. For pre-cultivation, the same mineral medium was used as for cultivation except for the following changes: 0.3 g/L $MgSO_4 \cdot 7\ H_2O$, 0.1 g/L NaCl, 0.0075 g/L thiamine×HCl, 0.08 g/L L-tyrosine, 5.0 g/L glucose and additionally 12 g/L K_2HPO_4 (final pH 7.2). A volume of 250 ml medium was filled in 1,000 ml shake flasks, 1.0 ml from the feedstock was inoculated and the bacteria were cultivated for 16 h at 37°C on a reciprocal shaker at 145 rpm.

Cultivation

The composition of the synthetic medium for the main culture was as follows: 3.0 g/L $MgSO_4 \cdot 7\ H_2O$, 0.015 g/L $CaCl \cdot H_2O$, 3.0 g/L KH_2PO_4, 1.0 g/L NaCL, 5.0 g/L $(NH_4)_2\ SO_4$, 0.075/0.1 g/L $FeSO_4 \cdot 7\ H_2O$/Na-citrate, 0.075 g/L thiamine, 0.3 g/L L-tyrosine, 0.1 g/L ampicilline, 15 g/L glucose and 1.5 ml/L trace element solution containing 2.0 g/L $Al_2(SO_4)_3 \cdot 18\ H_2O$, 0.75 g/L $CoSO_4 \cdot 2\ H_2O$, 2.5 g/L $CuSO_4 \cdot 5\ H_2O$, 0.5 g/L H_3BO_3, 24 g/L $MnSO_4 \cdot H_2O$, 3.0 g/L $Na_2MoO_4 \cdot 2\ H_2O$, 2.5 g/L $NiSO_4 \cdot 6\ H_2O$ and 15.0 g/L $ZnSO_4 \cdot 7\ H_2O$. After sterilisation of the bioreactor and peripherals, the medium was directly filtered into the bioreactor via a dead-end microfiltration unit (0.2 μm cut-off) and adjusted to pH 6.5 by 25% ammonia water.

Fermentation in pH-controlled shaking flasks

Biocompatibility tests were carried out in pH-controlled shaking flasks using the FedbatchPro equipment of DASGIP mbH, Jülich, Germany. Twelve shaking flasks of 1 L volume were used in parallel with the pH set to 6.5 at 37°C and a working volume of 100 ml. Cultures were inoculated at 10% of the working volume. The maximum soluble amount of the different carriers was added to the shaking flasks in order to investigate their effect on cell growth, which was followed by measurements of optical density.

Fed-batch fermentation

Lab-scale fed-batch fermentations were carried out in order to simulate carrier and/or solvent effects on microbial behaviour. For the experiments a 7.5 L bioreactor (5 L working volume, Bioengineering, Switzerland) was used together with the pre-cultivation procedure as described above. The volume of inoculum was 400 ml, the stirrer speed was 250 rpm and the aeration rate was 1 vvm. L-Phe production was started by IPTG induction. The bioreactor was combined with an ultrafiltration unit using a hollow fibre module with 500 kDa cut-off, 0.042 m^2 membrane area and 1 mm inner fibre diameter (Schleicher & Schuell, Germany). The ultrafiltration unit was installed in order to simulate the effects of biomass retention, which have to be considered in an integrated reactive-extraction process (see Fig. 1). Cell-free permeate was pumped back into the reactor. A mixture of 10% v/v DEHPA in kerosene was added (150 mg/L) to investigate the combined effects of DEHPA and kerosene.

Analysis

Bioreactor

The lab-scale bioreactor was equipped with the standard controlling technique using PID controllers for pH, dissolved oxygen (DO), stirrer speed, aeration and temperature control. Ammonia was used for titration. Additionally, exhaust gas analysis was applied using NIR for CO_2 measurement and paramagnetic sensors for O_2 (Binos, Rosemount, Germany). Input liquid flows as

glucose feed were controlled gravimetrically. Air flow was measured by Brooks sensors (Brooks Instruments B.V., The Netherlands). All on-line data were processed by MEDUSA, a process-control software that was developed in-house.

Amino acids in aqueous solution

Reversed phase HPLC was used for the measurement of L-Phe in the aqueous phases and the fermentation broth. Pre-column derivation of amino acids was performed with *ortho*-phtal dialdehyde (OPA) and mercapto-ethanol. Further HPLC (Sycam, Germany) analysis was done using a reversed phase column (Lichrospher 100 RP 18–5 EC, Merck, Germany) and a fluorescence detector (RF-535, Shimadzu, Germany).

Elementary analysis

Purities of the aqueous acceptor solution and dried crystals (after precipitation) were investigated using elementary analysis. At the beginning, GC-MS scans were performed to identify dominating anion and cation impurities. Hence, Na, Mg, Al, Si, K, Mn, Fe, Ni, Cu, Zn and Mo were identified also taking the anion SO_4^{2-} into account. Inductively coupled plasma–mass spectroscopy (ICP–MS) was applied by Dr. Wesseling Laboratorien GmbH (Bochum, Germany) to quantify the ion concentrations in samples. The analytic procedure conformed to DIN EN 45 001.

Optical density and dry biomass

Optical density was measured with a spectrophotometer (Shimazu UV-160, Germany) at 620 nm after appropriate dilution with double-distilled water as the reference. Cell dry weight (CDW) was measured by filtration of 2.5 to 10 ml of fermentation suspension through a preweighed microfilter (0.2 μm cut-off, Schleicher & Schuell, Germany). After drying the filter for 24 h at 80°C and weighing, the cell dry mass was calculated.

Table 2. Geometry of hollow fibre modules used for lab- and pilot-scale reactive-extraction experiments

	5PCM-106 X10 240ID Lab scale	4X28 ×30 240ID Pilot scale
Module length	20 cm	100 cm
Diameter	2.5 cm	10 cm
Temperature range	1–60°C	1–60°C
Effective area	0.23 m^2	18.6 m^2
Spec. area	4,000 m^2/m^3	3,641 m^2/m^3
Max TMP	4.14 bar	4.14 bar
Max operating pressure	5 bar	5 bar
Number of fibres	2,100	31,800
Length of fibres	16 cm	71 cm
Membrane	polypropylene	polypropylene
Porosity	30%	40%
Pore size	0.05 μm	0.03 μm
Fiber inner diameter	240 μm	244 μm
Fiber outer diameter	300 μm	300 μm
Membrane thickness	30 μm	28 μm

Reactive-extraction units

Process development and scale-up were based on two different reactive-extraction units. For lab-scale investigations an effective membrane area of 0.23 m^2 was installed while 18.6 m^2 was used for the pilot-scale facility. The geometric data of the Liqui-Cell (Hoechst-Celanese Co., Charlotte, USA) hollow fibre modules are given in Table 2.

The experimental set-up for the laboratory and pilot scales was similar. Two modules were used, one for extraction, the other for back extraction. The L-Phe containing the aqueous phase and the stripping phase was circulated using separate storage tanks and pumps. In lab-scale experiments LiquiFlo Serie V051.12 (Verder, Germany) pumps were used. Pilot-scale pumping was performed by LiquiFlo Serie 37RX (Verder, Germany) and Watson Marlow 506 (Falmouth, UK). Aqueous phases were pumped through the hydrophobic hollow fibres. Correspondingly, the organic phase circulated in the opposite direction between both modules using the outer fibre space of the modules.

To avoid emulsion formation by breakthrough of the organic phase into the aqueous phases, the dispersion-free operation of the plant was ensured by approximately 1 bar overpressure to the aqueous phases.

Theory

Hydrodynamics in hollow fibre modules

In general, Reynolds numbers (*Re*) could be used to describe hydrodynamic conditions inside hollow fibre modules according to

$$Re_{HF} = \frac{w_{HF} d_i}{v} \qquad (2)$$

In Eq. (2) the superficial velocity speed w_{HF}, fluid viscosity v and a characteristic diameter d are used. In the case of the *Re* definition for flux inside the fibres, the inner diameter d_{HF} should be taken.

For the *Re* definition of flux outside fibres, the work of several groups should be considered [32, 33, 34, 35, 36], who have taken into account the module geometry as indicated in Fig. 2.

As a result, the following equation was used for the definition of the Reynolds number

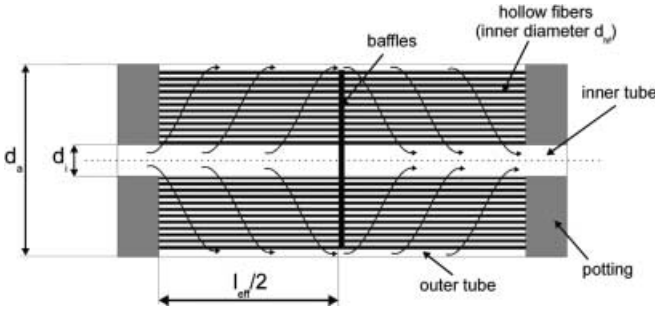

Fig. 2. Hollow fibre extraction module used for reactive-extraction experiments. The aqueous phase is pumped through the hollow fibres while the organic phase is outside the hollow fibres

$$Re = \frac{\bar{w} d_h}{\nu} = \frac{2Q\,(d_a + d_i)\ln(d_a/d_i)}{\pi \nu \quad l_{eff} N d_{HF}} \qquad (3)$$

Here, an average superficial velocity $\bar{w}$ together with a hydraulic diameter d_h were introduced. These parameters were estimated taking into account the stream Q, the number of fibres N and geometric values d_a, d_i, l_{eff} and d_{HF}, as shown in Fig. 2.

In pilot-scale experiments using the technical set-up as explained above, flow rates of 250–500 L/h, 60–156 L/h and 250–680 L/h were realised in the aqueous donor, the organic and the aqueous stripping phase, respectively. In this way, *Re* numbers of 11.6–23.3, 2.2–5.7 and 11.6–31.5 were achieved in these phases. As turbulent flow is assumed for *Re* numbers >2,300, the hydrodynamics inside the module could be described as laminar tube flow.

Calculation of mass-transfer coefficients

Coefficients for the description of mass transfer from the aqueous donor phase into the organic phase (β_D) and from the organic phase into the aqueous stripping phase (β_A) were estimated by experimental results using circulating streams in all phases. As indicated in Fig. 3, mass balances could be formulated for the donor tank and module 1 as well as for the stripping tank and module 2. For simplification, it was assumed that concentration gradients inside the modules could be neglected as described previously [27]. Thus L-Phe mass transfer from aqueous donor to organic phase could be formulated as

$$\dot{m}_D = \beta_D A_M \left(c_{phe,D} - c^{eq}_{Phe,D} \right) \qquad (4)$$

with A_M as the effective membrane area, $c_{Phe,D}$ as the L-Phe concentration in the aqueous phase and $c^{eq}_{Phe,D}$ as the equilibrium concentration in aqueous phase. Balancing concentration changes in the donor tank now lead to the simple differential equation

$$V_D \frac{dc_{Phe,D}}{dt} = \beta_D A_M \left(c_{Phe,D} - c^{eq}_{Phe,D} \right) \qquad (5)$$

taking into account the tank volume V_D, which was constant with $V_D \gg V_{module}$. By analogy, a corresponding equation was derived for mass transfer form organic to stripping phase. Using MicroMath Scientist (Salt Lake City, USA) parameters β_D, β_A, $c^{eq}_{Phe,D}$ and $c^{eq}_{Phe,A}$ were estimated using experimental results. It should be stressed that for modelling simplification $c^{eq}_{Phe,D}$ and $c^{eq}_{Phe,A}$ were considered as constant and not as time-varying equilibrium concentrations. Hence, resulting mass-transfer coefficients should be regarded as rough estimations rather than exact values.

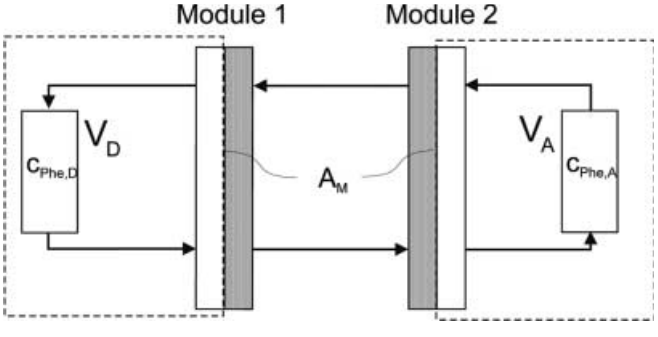

Fig. 3. Principle of the experimental set-up for reactive-extraction mass-transfer experiments. Extraction was balanced in module 1 considering donor volume V_D, L-Phe concentration $c_{Phe,D}$ and the effective membrane area A_M. By analogy, balancing of back extraction was performed

Heterogeneous reaction at the phase boundary layer

Owing to the relatively low solubility of carriers in aqueous phase (see Table 1), L-Phe transfer is assumed to take place at the aqueous/organic interface. If the cation-specific reactive-extraction process is focused (such as DEHPA), the extraction procedure could be described as follows [17, 24]:

$$F^+_{aq} + 2(CH)_{2,org} \Leftrightarrow FC(CH)_{3,org} + H^+_{aq} \qquad (6)$$

It is assumed that cation-specific carriers occur as dimers $(CH)_2$. Thus, altogether four carrier molecules CH are used to extract one F^+ (L-Phe$^+$). Using reaction equation (Eq. 6), an equilibrium constant K was derived:

$$K = \frac{\left[FC(CH)_{3,org}\right] \cdot \left[H^+_{aq}\right]}{\left[F^+_{aq}\right] \cdot \left[(CH)_2\right]^2} \qquad (7)$$

As indicated, a significant influence of pH with respect to L-Phe extraction should be taken into account. For further analysis, the equilibrium constant K was derived from experimental data, estimating the amount of carrier/L-Phe complex in the organic phase by simple mass balance.

$$\left[FC(CH)_{3,org}\right] = \left(\left[F^0\right]_{aq} - \left[F^1\right]_{aq} \right) \cdot \frac{V_{aq}}{V_{org}} \qquad (8)$$

Here F^0 and F^1 represent the start and equilibrium concentrations of L-Phe before (0) and after (1) extraction using the aqueous phase V_{aq} and the organic phase V_{org}.

Results and discussion

At the beginning of reactive-extraction process development, the key components of the system had to be identified. Preliminary studies were carried out to find the most promising carriers by batch experiments. After this, solvents and counter ions were investigated leading to a final reactive-extraction system. Additional experiments were carried out in order to identify the equilibrium and mass-transfer characteristics and to study this system under fermentation conditions.

Carrier identification

The identification of suitable carriers was based on two experimental series. Batch extractions in separation funnels were used to study carrier quality for L-Phe extraction. Batch fermentation experiments were carried out in order to test carrier toxicity using *E. coli* strains. While the extraction degree *E* (see Eq. 1) was calculated to qualify extraction results, carrier toxicity was judged by growth performance of the cells after carrier addition.

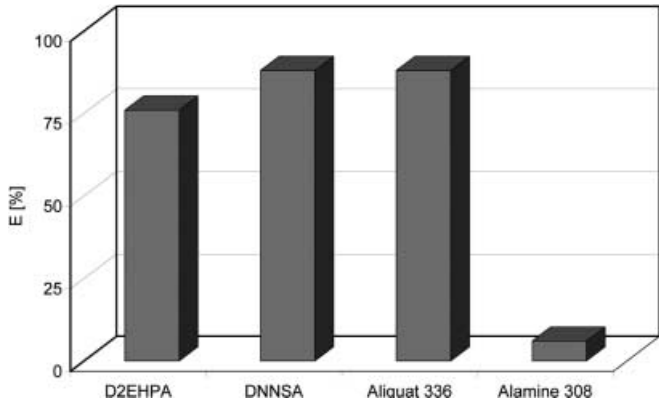

Fig. 4. Comparison of extraction degree *E* using dierent cation- and anion-selective carriers for L-Phe separation

Batch extractions in separation funnels

Figure 4 shows a comparison of extraction results using different carriers under appropriate pH conditions. pH conditions were chosen in order to ensure a sufficient availability of L-Phe cations or anions. For cation-specific L-Phe$^+$ extraction with DEHPA and DNNSA, a pH below 3 was set. Anion-specific L-Phe$^-$ extraction with Aliquat 336 and Alamine 308 was performed at a pH above 9. In this way, L-Phe ions were extracted out of aqueous solution, at the same time leading to the generation of new L-Phe ions after equilibration thanks to dissociation in the aqueous phase. A start concentration of 10 g/L L-Phe in the aqueous phase was chosen in each experiment. In order to prevent carrier-limited L-Phe extraction out of the organic kerosene phase, 10% v/v carrier in kerosene was used. In the case of Aliquat 336 this concentration was reduced to 5% v/v carrier in kerosene.

Alamine 308 is not suited for L-Phe separation because of the very low degree of extraction. Although only relatively low amounts of Aliquat 336 were used, a high extraction degree of 88% was achieved. Extraction results using DEHPA and DNNSA were similar. Thus, Aliquat 336, DEHPA and DNNSA were selected. Subsequent experiments were carried out in order to determine the best carrier, taking biological constraints into account.

Batch fermentations

Batch fermentation experiments were carried out in pH-controlled shaking flasks. To simulate the carrier effect on microbial growth, carriers (15 µL/L) were added after 5 h cultivation according to their maximum solubility in water. As indicated in Fig. 5, the addition of the quaternary ammonia salt Aliquat 336 was followed by an immediate growth stop. Compared to the reference fermentation without carrier addition, no significant growth effect could be observed after the addition of DEHPA, DNNSA or Alamin 308. These carriers apparently are tolerated by *E. coli*.

As a conclusion from the batch experiments with separation funnels and shaking flasks, the anion-specific carriers Alamine 308 and Aliquat 336 were excluded from further investigations – the first owing to insufficient extraction capability, the second because of *E. coli* toxicity. Additional extraction experiments in separation funnels were carried out focusing instead on the back extraction of L-Phe. It was found that back extraction from the DNNSA organic phase was not complete (data not shown). L-Phe was not fully removed from DNNSA, which was indicated by an incomplete L-Phe balance. Furthermore, handling of DNNSA represents a high safety risk compared to DEHPA, since the DNNSA flash point is –1.1°C. Thus, DEHPA was selected for reactive-extraction process development.

Solvent identification

The identification of an ideal solvent was carried out with the aid of batch separation funnel experiments as well as fermentation experiments on a lab scale. The latter were performed in order to qualify the solvent and carrier effect on microbial L-Phe production.

Separation funnel experiments

Using 10% v/v DEHPA for extraction experiments, different solvents were studied for the extraction of 10 g/L L-Phe in separation funnels. As indicated in Fig. 6,

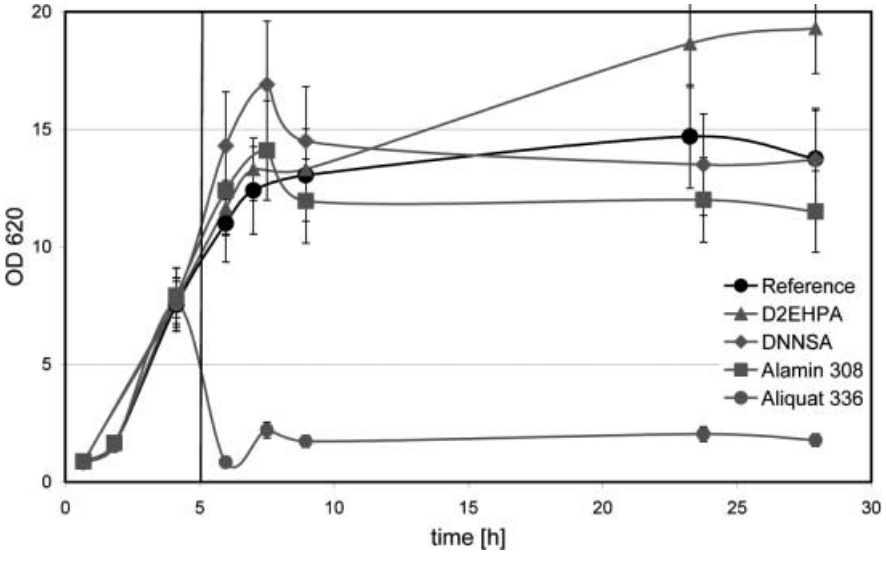

Fig. 5. Carrier toxicity test in pH-controlled shaking flasks comparing optical density (OD620) growth curves after addition of dierent cation- and anion-selective carriers

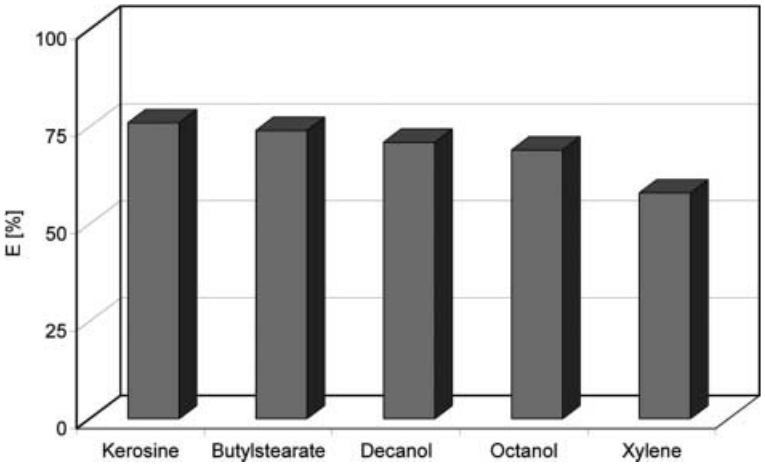

Fig. 6. L-Phe extraction degree *E* using 10%v/v DEHPA in dierent solvents

kerosene achieved the highest extraction degree. Kerosene represents a C12–C16 fraction of mineral oil and it is relatively cheap and readily available on the market. It possesses a low water solubility (5–10 mg/L). Hence, with respect to the formerly mentioned criteria, kerosene was regarded as a promising extraction solvent.

Lab-scale fermentations

After the identification of carrier DEHPA and solvent kerosene, the extraction system was tested in fed-batch fermentations with L-Phe producing *E. coli* strains. As described above, fermentations were carried out in a 7.5 L bioreactor that was equipped with an ultrafiltration unit. Fermentation broth was pumped through the filtration loop where a cell-free solution was drained off via a filtration membrane. Both cell-free permeate and cell-containing retentate were pumped back into the bioreactor. A mixture of 10% v/v DEHPA/kerosene was continuously added to the permeate stream in order to simulate a DEHPA/kerosene effect caused by a reactive-extraction unit that was not yet available. In total, 3.2 ml of DEHPA/kerosene mixture was added. As indicated in Figs. 7 and 8, significant changes of microbial L-Phe production were observed after the start of DEHPA/kerosene addition after approximately 9 h process time compared to the reference fermentation.

In the case of DEHPA/kerosene addition, considerably higher L-Phe titres were achieved than without carrier/solvent addition. If a reactive-extraction unit is experimentally simulated, L-Phe titres are almost doubled in the fermentation medium. As other process conditions in two fermentations were comparable, this effect could only be caused by the carrier and/or the solvent. To elucidate the phenomenon, biomass- (CDW)-specific L-Phe production rates were compared (see Fig. 8).

As can be seen, high L-Phe production rates >0.03 $g_{L\text{-}Phe}/g_{CDW}$ h were achieved in fermentation with DEHPA/kerosene addition for approximately 15 h. This high level of L-Phe production could only be maintained in reference fermentation for 2 h. Obviously, the addition of DEHPA

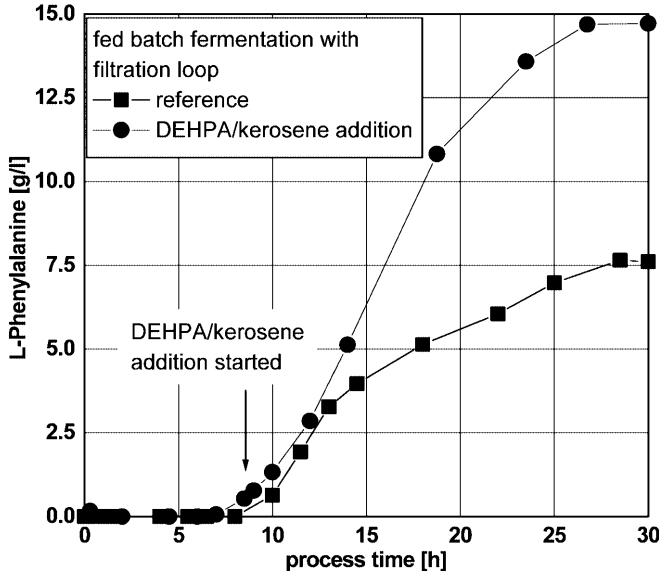

Fig. 7. Course of L-phenylalanine titres in fed-batch lab-scale fermentations with integrated filtration unit, with and without DEHPA/kerosene addition

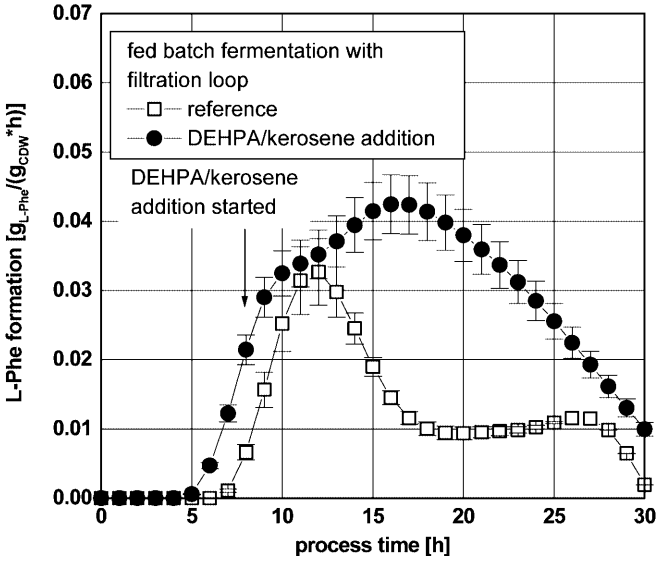

Fig. 8. Course of CDW-specific L-phenylalanine production rates in lab-scale fed-batch fermentations with integrated filtration unit, with and without DEHPA/kerosene addition

and kerosene sustained L-Phe production. Further investigations have shown that this effect is mainly caused by DEHPA alone even without the addition of kerosene (data not shown). It is assumed that the cell-membrane integrity is changed in the presence of DEHPA. As a consequence, L-Phe export could be facilitated and potential L-Phe inhibitions of aromatic amino acid pathway activities may be reduced. In total, the effect led to a doubling of final L-Phe/glucose selectivity in the case of DEHPA/kerosene addition compared to the reference fermentation.

The conclusion may therefore be drawn that a fully integrated reactive-extraction unit does not inhibit microbial L-Phe production. On the contrary, a significant increase of L-Phe/glucose selectivity caused by a prolonged high-level L-Phe production phase could be measured. This assumption was experimentally proven in the accompanying paper [37].

Counter ion identification

The most efficient counter ion system was identified by qualifying batch extraction results. The organic phase consisted of 10% v/v D_2EHPA in kerosene, which was preloaded by an extraction from a 10 g/L L-Phe solution. Two counter ion levels (1 and 2 molar) were investigated. In the case of KCl, only 1 M was used owing to the appearance of emulsions at 2 M. Figure 9 gives an overview of the total extraction degrees that were achieved using different counter ion systems. Overall extraction degrees were calculated referring to the start concentration of L-Phe in the aqueous donor phase.

Back extraction of L-Phe from DEHPA is based on cation exchange. Thus, cations like $(NH_4)^+$, Na^+, K^+, H^+ should be used for extraction. Experiments with NaOH and KOH showed a clouding of the aqueous phase that was caused by microemulsions produced by the entrainment of carriers into water. Despite the high extraction degrees of NaOH and KOH, these systems were not preferred for further investigations. On account of the unstable boundaries between aqueous and organic phases, NaOH or KOH could cause severe problems in a pilot process scale-up. When 1 molar KCl was used, no clouding could be observed. However the extraction degree was relatively low. As relatively high extraction degrees without clouding were measured with H_2SO_4, these counter ions were used for further experiments.

Extraction equilibrium

Extraction equilibrium studies were carried out using separation funnels. Different start pHs at 2, 5.9, 7.3, 9.5 and 12 were chosen in order to investigate cation-selective L-Phe extraction with 10% v/v DEHPA/kerosene. Start pH was installed by NaOH titration. 10 g/L L-Phe was added at the beginning. As indicated in Fig. 10, only a negligible amount of L-Phe cations should exist at this pH.

To our surprise, a significant L-Phe extraction was observed, even at the start pH of 12. To investigate this effect, 10% v/v DEHPA/kerosene was brought into contact with an aqueous phase of pH 12 with an equal volume. The same extraction conditions were therefore chosen, although no L-Phe was added to the aqueous phase. After phase mixing and settling, a final pH of 4.9 was measured. It is assumed that the dissociation of phosphoric acid of the DEHPA dimer was responsible for the pH drop. As a consequence, cationic L-Phe species are now present in the aqueous phase, which could be extracted by DEHPA.

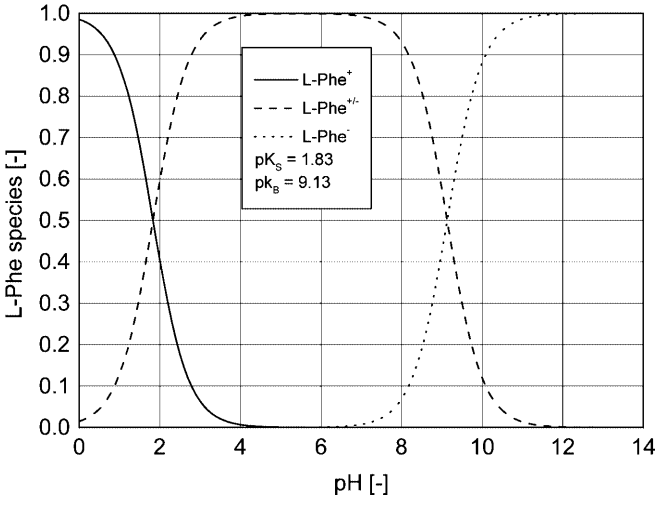

Fig. 10. pH-dependent dissociation of L-Phe at room temperature (T=22°C)

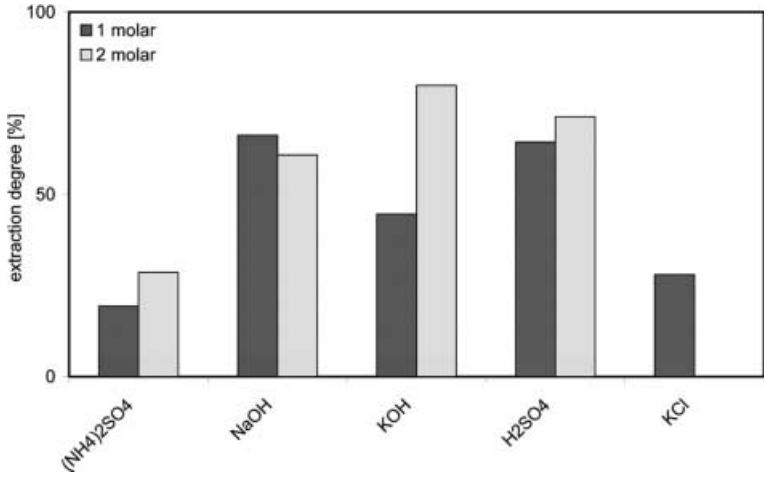

Fig. 9. Identification of optimal counter ions for the back extraction of L-Phe from the cation-specific carrier DEHPA in kerosene

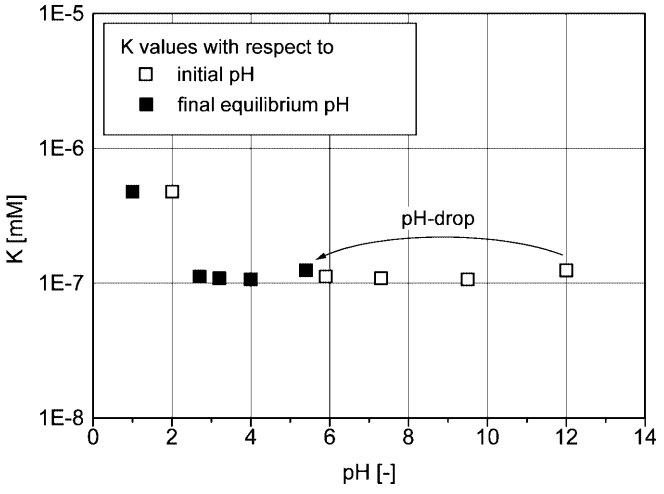

Fig. 11. Estimation of equilibrium constant for L-Phe extraction using DEHPA/kerosene in the organic phase. pH drop in aqueous phase owing to dissociation of phosphoric acid is indicated

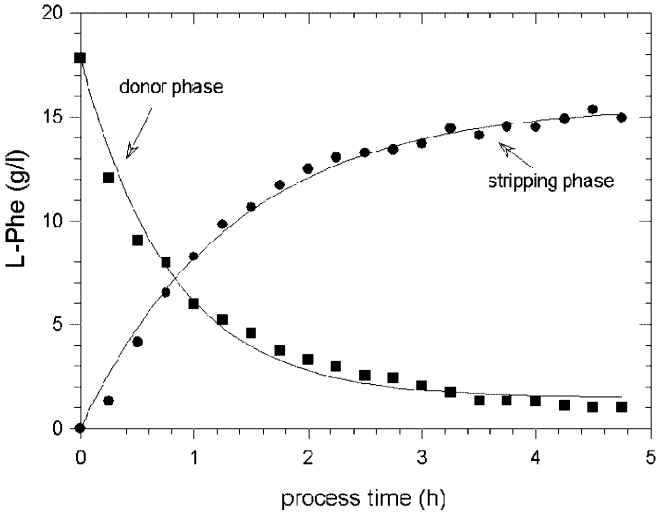

Fig. 12. Fitting example of L-Phe decline in donor phase and L-Phe increase in stripping phase for experiment 7 of Table 3

Using Eq. (7) for the estimation of the equilibrium constant K, Fig. 11 gives an overview of experimental results. Equilibrium conditions were used for K value calculation. It should be stressed that a significant pH drop occurred in every experiment, so that L-Phe cations are available in the aqueous solution.

Mass transfer in hollow fibre modules

After preliminary experiments in separation funnels and small-scale hollow fibre modules, mass-transfer studies were carried out on a pilot scale. A set-up was chosen to make sure that experimental results represent a system that could be used together with a 300-L fed-batch microbial process [37]. An experimental installation was realised, consisting of two pilot-scale hollow fibre modules (see Table 2), providing an effective membrane area of 18.6 m^2 for extraction and back extraction of L-Phe. For all experiments, the DEHPA/kerosene system was used together with sulphuric acid as the proton donor in the stripping phase.

Following the experimental procedure as described above, mass-transfer experiments with circulating donor, organic and stripping phases were carried out. Different levels of liquid flows were realised together with different levels of DEHPA and sulphuric acid concentrations. Thus, five experimental parameters were studied in 10 experiments and liquid volumes ranging from 5 to 9 L were used. Experimental conditions and estimated parameters are presented in Table 3.

As shown, regression coefficients indicated a good approximation between measured and simulated L-Phe concentrations in the donor and stripping phase in all experiments. As an example, fitting results for experiment 7 are given in Fig. 12.

As already mentioned, the assumption of constant equilibrium concentrations in the aqueous donor and the aqueous stripping phase represents a strong simplification of real conditions where rather time-varying equilibrium concentrations occur. Hence, estimated equilibrium concentration values should be regarded as model parameters which, in case of the aqueous stripping phase, are accurately estimated. When mass-transfer coefficients were investigated, a significant correlation to experimental conditions could be seen (see Fig. 13). For instance, a

Table 3. Experimental results of mass-transfer experiments

No.	Re_D (–)	Re_O (–)	Re_A (–)	c_{DEHPA} (v/v%)	c_{acid} (M)	$\beta_D \pm \Delta$ (cm/s×10^{-7})	$\beta_A \pm \Delta$ (cm/s×10^{-7})	$c^{eq,s}_{Phe,D} \pm \Delta$ (10^{-2})	$c^{eq,s}_{Phe,A} \pm \Delta$ (–)	$r_{Phe,D}$ (–)	$r_{Phe;A}$ (–)
1	11.6	2.2	11.6	20	1	72 ± 7.5	36 ± 4.7	5.8 ± 1.6	1.1 ± 0.07	0.999	0.991
2	11.6	5.7	23.3	10	1	79 ± 3.5	58 ± 4.4	0	1.2 ± 0.04	0.997	0.994
3	11.6	5.7	11.6	20	2	80 ± 6.9	54 ± 3.9	0.5 ± 1.1	1 ± 0.03	0.999	0.997
4	11.6	2.2	11.6	30	1	96 ± 4.4	50 ± 2.5	2.5 ± 0.6	1 ± 0.02	0.998	0.999
5	11.6	5.7	31.5	10	1	117 ± 6.7	67 ± 4.4	8.3 ± 1.6	1.1 ± 0.03	0.997	0.997
6	11.6	2.2	11.6	20	2	134 ± 11.7	61 ± 5.3	4.2 ± 1	1.1 ± 0.03	0.993	0.995
7	11.6	2.2	23.3	10	1	150 ± 7.1	67 ± 3.6	8.2 ± 0.9	1 ± 0.02	0.992	0.999
8	11.6	2.2	11.6	10	1	160 ± 8.1	72 ± 4.4	5.7 ± 1	1.1 ± 0.02	0.999	0.990
9	23.2	5.7	11.6	20	2	239 ± 51.1	42 ± 8	9.7 ± 1.3	1.1 ± 0.09	0.925	0.994
10	11.6	5.7	11.6	30	2	289 ± 30.6	77 ± 5.3	4.3 ± 0.6	1.1 ± 0.02	0.989	0.998

Reynolds numbers for aqueous donor (Re_D), organic (Re_O) and aqueous stripping phase (Re_A) are given together with DEHPA and H_2SO_4 (c_{acid}) concentrations. Absolute errors (Δ) for mass-transfer coefficients β_D and β_A and standardised equilibrium concentrations $c^{eq,s}_{Phe,D}$ and $c^{eq,s}_{Phe,A}$ were added. To qualify goodness of fit regression coefficients for donor $r_{Phe,D}$ and stripping phase $r_{Phe,A}$ are presented

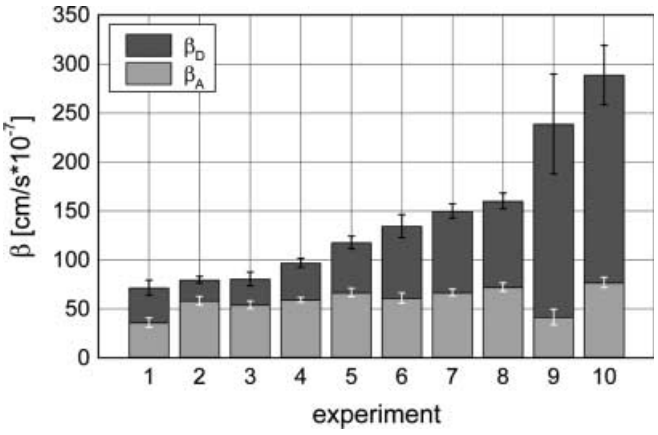

Fig. 13. Ranking of estimated mass-transfer coecients β_D and β_A with respect to Table 3

comparison of experiments 2 and 4 (also 9 and 10) indicates that increasing DEHPA concentrations lead to increasing β_D. If experiments 1 and 3 are compared, a significant improvement of β_A is apparent, while the sulphuric acid concentration was doubled. Because of technical constraints, Re_D was kept constant for most experiments. However, an increase of β_D and β_A could be observed in experiments 2 and 5 when Re_A was increased. This result was unexpected, but it indicates, that total mass transfer from donor to stripping phase is limited by the organic to stripping phase L-Phe transfer. Small changes of β_A coincide with bigger changes of β_D. This statement is supported by the analysis of the Re_O effect. If experiments 3 and 6 or 2 and 7 are compared, an increase of both mass-transfer coefficients becomes apparent together with a decrease of Re_O. As a possible reason, we assume that L-Phe back extraction was so slow that loaded carrier/L-Phe complexes were transported to the extraction module (1) at high organic phase cycling rates. Thus, extractive mass transfer was limited by the availability of "free" carriers. However, this working hypothesis should be critically investigated with the aid of detailed mass-transfer models.

L-Phe separation from fermentation solution

The selective separation of L-Phe from cell-free supernatant was studied using the reactive-extraction set-up on a pilot scale. A filtered fermentation solution (42 L) containing 25 g/L L-Phe at a fermentation pH of 6.5 was connected with the extraction unit. To study the concentration potential of reactive extraction, the stripping phase volume was only a sixth of donor volume and 10% v/v DEHPA in kerosene was used. As a result, the total amount of 588 g L-Phe was extracted in 13.5 h with an averaged permeability of 10 g_{Phe}/m^2 h. A L-Phe concentration four times higher was achieved in the stripping phase compared to the donor phase. An elementary analysis of the acceptor phase gave a 98% cation portion of L-Phe in the stripping phase. After precipitation of the stripping phase (titration to the isoelectrical point with NaOH), the dry product had a purity of more than 99%.

Conclusions

Reactive-extraction technology for the separation of L-Phe out of fermentation solution was studied. A suitable extraction system consisting of DEHPA/kerosene in the organic and H_2SO_4 in the stripping phase could be devised. Equilibrium investigations indicated that cation-selective L-Phe extraction is possible even at high pH-start values, provided that DEHPA mixing into the aqueous donor phase is allowed. Mass-transfer studies showed that coefficients up to 288×10^{-7} cm s^{-1} could be determined making DEHPA-based L-Phe transfer comparable to the results of [28] and [30] that identified $112–116 \times 10^{-7}$ cm s^{-1} using Aliquat 336 in hollow fibre modules.

Furthermore, it was found that no inhibiting effects of DEHPA/kerosene input on fermentation performance occurred during an experimentally simulated integrated process approach. On the contrary, significant L-Phe/glucose selectivity increase could be observed in the presence of DEHPA/kerosene.

Furthermore, experiments for fully integrated reactive extraction have been carried out on a pilot scale together with a 300-L bioreactor fed-batch set-up [36, 37].

This contribution therefore represents one of the rare examples of a fully integrated separation unit in a fermentation processes. Experimental results show that this process approach is not only necessary in order to prevent product inhibition but also it improves total process performance. The authors consider this is worth mentioning as downstream processing is usually regarded as a necessary subsequent step and not as an integrated step improving the process.

References

1. Ager DJ, Pantaleone DP, Henderson SA, Katrizka AR, Prakash I, Walters DE (1998) Synthetische nicht nutritive Süßstoffe. Angew Chem 110:1901–1916
2. Backman K, O'Connor J, Venkatasubramanian K (1990) Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann NY Acad Sci 89(5):16–24
3. Miyoshi H (1996) Donnan dialysis with ion-exchange membranes. I. Theoretical equation. Sep Sci Technol 31:2117–2129
4. Miyoshi H (1996) Donnan dialysis with ion-exchange membranes. II. Diffusion coefficients using same valence ions. Sep Sci Technol 31:2183–2194
5. Schlichting E, Halwachs W, Schügerl K (1987) Reactive extraction of salicylic acid and d/l phenylalanine in a stirred cell. Chem Eng Comm 151:193–205
6. Abbasian K, Degener W, Schügerl K (1989) Reactive extraction of primary and secondary metabolites. Ber Bunsenges Phys Chem 93:967–980
7. Schügerl K, Hänsel R, Schlichting E, Halwachs W (1986) Reaktivextraktion. Ver-Dt-Ingr Ber 607:779–803
8. Hänsel R, Halwachs W, Schügerl K (1986) Reactive extraction of d,l-phenylalanine with trioctylmethylammoniumchloride (TOMAC) as a carrier – III. Equilibrium and mass-transfer investigations. Chem Eng Sci 41:1811–1815
9. Schügerl K (1994) Solvent extraction in biotechnology. Springer, Berlin, Heidelberg, New York
10. Seevaratnam S, Holst O, Hjörleifsdottir S, Mattiasson B (1991) Extractive bioconversion for lactic acid production using solid sorbent and organic solvent. Bioproc Eng 6:35–41
11. Ye K, Jin S, Shimizu K (1996) Performance of lactic acid fermentation by multistage extractive fermentation. J Ferment Bioeng 81:240–246
12. Yabannavar VM, Wang DIC (1991) Extractive fermentation for lactic acid production. Biotechnol Bioeng 37:1095–1100

13. Honda H, Toyama Y, Takahashi H, Nakazeko T, Kobayashi T (1995) Effective lactic acid production by two-stage extractive fermentation. J Ferment Bioeng 79:589–593
14. Wieczorek S, Brauer H (1998) Continuous production of citric acid with recirculation of the fermentation broth after product recovery. Part 1: Continuous production of citric acid. Bioproc Eng 18:1–5
15. Wieczorek S, Brauer H (1998) Continuous production of citric acid with recirculation of the fermentation broth after product recovery. Part 2: Product recovery and recirculation of the fermentation broth. Bioproc Eng 18:75–77
16. Oliveira AC, Rosa MF, Cabral JMS, Aires-Barros MR (1997) Immobilization of *Saccharomyces cerevisae* cells and *Rhizomucor mihei* lipase for the production and extractive biocatalysis of ethanol. Bioproc Eng 16:349–353
17. Teramoto M, Yamashiro T, Inoue A, Matsuyama H, Miyake Y (1991) Extraction of amino acids by liquid membranes containing di(2-ethyl)phosphoric acid as a carrier. J Membr Sci 58:11–32
18. Yagodin GA, Yurtov EV, Golubkov AS (1986) Liquid membrane extraction of amino acids. In International solvent extraction conference, Preprints, vol 3, DECHEMA 86
19. Lukhezo M, Kelly NA, Reuben BG, Dunne LJ, Verall MS (1996) Reactive solvent extraction of amino acids. Proc ISEC 96:87–92
20. Thien MP, Hatton TA, Wang DIC (1986) Separation of amino acids from fermentation broth using liquid emulsion membranes. In International solvent extraction conference, Preprints, vol 3, DECHEMA 86
21. Uddin MS, Hidajat K, Lim B-G, Ching C-B (1992) Interfacial mass transfer in stripping of phenylalanine in a liquid–liquid extraction process. J Chem Tech Biotechnol 53:353–357
22. Deblay P, Minier M, Renon H (1990) Separation of l-valine from fermentation broths using a supported liquid membrane. Biotechnol Bioeng 35:123–131
23. Adarkar JA, Sawant SB, Joshi JB, Pangarkar VG (1997) Extraction of amino acids using immobilized liquid membrane. Biotechnol Prog 13:493–495
24. Wieczorek P, Jönsson JA, Mathiasson L (1997) Concentration of amino acids using supported liquid membranes with di-2-ethylhexyl phosphoric acid as carrier. Anal Chim Acta 346:191–197
25. Dzygiel P, Wieczorek P, Jonsson JA, Milewska M, Kafarski P (1999) Separation of amino acid enantiomers using supported liquid membrane extraction with chiral phosphates and phosphonates. Tetrahedron 55:9923–9932
26. Boyadzhiev L, Atanassova I (1994) Extraction of phenylalanine from dilute solutions by rotating film pertraction. Proc Biochem 29:237–243
27. Escalante H, Ortiz MI, Irabien, JA (1996) Concentration of l-phenylalanine by non-dispersive extraction in hollow fibre modules. ISEC 96, Melbourne, Australia, pp 1493–1498
28. Escalante H, Alonso AI, Ortiz I, Irabien A (1998) Separation of l-phenylalanine by nondispersive extraction and backextraction. Equilibrium and kinetic parameters. Sep Sci Technol 33:119–139
29. Scarpello JT, Stuckey DC (2000) The reactive extraction of phenylalanine with Aliquat 336: Buffer co-extraction equilibrium and mass transfer kinetics. Biotechnol Bioeng 69:469–477
30. Cardoso JP (1997) Purification of penicillin G amidase using quaternary ammonium salts and effect on the activity of the immobilised enzymes. Bioproc Eng 16:209–218
31. Fürste JP, Pansegrau W, Frank R, Blöcker H, Scholz P, Bagdasarian M, and Lanka E (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. Gene 48:119–131
32. Coulson JM, Richardson JF (1977) Chemical engineering, vol I. Pergamon Press, Oxford
33. Schöner P, Plucinski P, Nitsch W, Daiminger U (1998) Mass transfer in the shell side of cross flow hollow fiber modules. Chem Eng Sci 53:2319–2326
34. Grassmann P (1983) Physikalische Grundlagen der Verfahrenstechnik. Salle und Sauerländer, Frankfurt
35. Coulson JM, Richardson JM (1986) Chemical engineering, vol II. Pergamon Press, Oxford
36. Maaß D, Takors R, Paschold H, Weuster-Botz D, Wandrey C (2000) Verfahren zur Abtrennung organischer Substanzen aus einem wäßrigen Gemisch. German Patent Application DE 199 19 490.4–44 (and WO/66253)
37. Gerigk MR, Maass D, Kreutzer A, Sprenger G, Wubbolts M, Takors R (2002) Enhanced pilot-scale fed-batch l-phenylalanine production with recombinant *E. coli* by fully integrated reactive extraction. Bioproc Biosyst Eng DOI 10.1007/s00449-002-0280-2