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ORIGINAL PAPER

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# Fully integrated L-phenylalanine separation and concentration using reactive-extraction with liquid-liquid centrifuges in a fed-batch process with *E. coli*

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**Abstract** A novel in situ product recovery (ISPR) approach for the (fully) integrated separation of L-phenylalanine (L-phe) from a 20 l fed-batch process with the recombinant L-tyrosine auxotrophic strain *E. coli* F-4/pF81 is presented. The strain was rationally constructed for the production of the aromatic amino acid. Glucose and tyrosine control is used. A reactive extraction system consisting of kerosene, the cation-selective carrier $D_2$EHPA and sulphuric acid, all circulating in liquid-liquid centrifuges, is applied for the on-line L-phe separation from cell- and protein-free permeate. Permeate is drained off from the bioreactor bypass. Using the novel ISPR approach, a significantly extended product formation period at 0.25 mmol/(g*h) together with a reduced by-product formation and a 28% relative glucose/L-phe yield increase is observed. Thus, the ISPR approach is superior to the reference non-ISPR process and even offers extraction rates approximately three times higher than the published membrane-based process.

**Keywords** L-phenylalanine (L-phe) · Reactive extraction · Liquid-liquid centrifuge · *E. coli*

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## Introduction

Regarding the worldwide production of 11,000 tons in 1998 and 14,000 (estimated) tons in 2002 [1], the aromatic amino acid L-phenylalanine (L-phe) can be considered to be one of the most important commercially produced amino acids representing a necessary building block for the production of the low-calorie sweetener aspartame [2, 3].

Although chemo-enzymatic routes like the resolution of *N*-acetyl-D, L-phe or the stereo- and enantioselective addition of ammonia to *trans*-cinnamic acid offer a technical scale access for L-phe production, fermentation processes based on glucose-consuming mutants seem to be commercially superior [4]. Therefore, numerous studies have already been performed focusing, for instance, on L-phe production with isolated enzymes [5, 6, 7, 8], immobilized cells [9] or resting cells [10]. Fermentation processes have also been investigated using *Corynebacterium glutamicum* [11, 12, 13] or, most importantly, *Escherichia coli* [14, 15, 16, 17, 18, 19].

Among these processes, the approach of Maass et al. [18] and Gerigk et al. [15] is the only one which considers a fully integrated L-phe separation from a fed-batch fermentation. The in situ product removal (ISPR) process was inspired by the—perhaps unexpected—findings of our own in vitro studies (Jossek 1991 personal communication) and of Backman et al. [14] that *aroF* (coding for 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP-) synthase, *AroF*) — one of the three isoenzymes catalyzing the main entrance reaction of the aromatic amino acid pathway—is already significantly inhibited in the presence of 3 g/l L-phe. The inhibition is assumed to occur readily in an L-phe producing strain which secretes L-phe into the supernatant by diffusion. Backman et al. [14] succeeded to circumvent *AroF* feedback inhibition by using *E. coli* L-phe producers with feedback resistant $AroF^{fbr}$ which were obtained after strain selection based on toxic amino acids analogues. Because such genes/strains were not available and because L-phe

nevertheless needs to be separated and purified from the fermentation supernatant, the following study aimed at circumventing the L-phe inhibition problem with process engineering tools.

Previous findings [20] revealed that the wild-type enzyme $AroF^{wt}$ is superior to $AroF^{fbr,tyr}$, an L-tyrosine feedback resistant mutant. Thus, even the production strain *E. coli* F-4/pF81 possessed $AroF^{wt}$ which necessitated a tyrosine control [20].

Reactive extraction has already been applied for the off-line separation of amino acids [21, 22, 23, 24, 25] and the on-line separation of lactic acid produced by *Lactobacillus delbrueckii* [26, 27] and citric acid produced by *Aspergillus niger* [28]. The ISPR approach of Maass et al. [18] and Gerigk et al. [15] used reactive extraction for the cation-selective L-phe separation based on hollow fibre modules, which were installed to separate the aqueous fermentation and acceptor solutions from the organic phase consisting of kerosene and the cation-selective carrier di-2-ethylhexyl phophonic acid ($D_2EHPA$). The approach enabled a selective separation and concentration of L-phe. Presumably because inhibiting effects of accumulating L-phe were avoided, a significant increase of the final L-phe/glucose yield was achieved.

In the comprehensive reviews of Freeman et al. [29], Schügerl [30], and Stark and von Stockar [31] the necessity is outlined concerning the need for ISPR approaches to be as simple, as robust and as safe as possible to encourage their use. Although the aforementioned approach succeeded in separating L-phe and minimized the downstream procedure to three steps — namely extraction, back-extraction and product purification via precipitation from the acceptor solution—some technical drawbacks were observed which could potentially hamper the scale-up and technical realization of the ISPR approach. Such drawbacks include:

i. The interface stability between the aqueous and organic phases inside the pores of the hollow fiber modules is strongly dependent on the trans-membrane pressure difference. Thus, pressure peaks occurring during filling or operation of the modules could cause phase instabilities and breakthroughs, which finally could terminate the process.
ii. Due to strongly laminar conditions inside the fibers, the mass transfer is rather low, thus requiring large module areas or causing incomplete L-phe separation as was seen by Gerigk et al. [15] when only 2 kg of the total 7 kg L-phe could be separated on-line. Simulation studies of Takors [32] revealed that the membrane area needs to be increased more than threefold to allow complete on-line L-phe separation, thus reducing the attractiveness of the membrane-based ISPR investment.

Nevertheless, the reactive extraction ISPR approach is still attractive (also commercially) because, as already mentioned, it has been shown to be applicable during fed-batch L-phe production enabling a three-step downstream processing procedure. However, further improvements should focus on the handling of the separation devices and on the L-phe permeability. Therefore, liquid-liquid centrifuges have been considered as an alternative solution for ISPR process development.

Liquid-liquid centrifuges are well-known for phase separation and have even been applied for the off-line recovery of penicillin from fermentation supernatant [33, 34]. Compared to the previously used hollow fiber membrane contactors, liquid-liquid centrifuges offer the advantage of being more robust under pressure changes, which could easily occur during fermentation. This facilitates their handling and also their scale-up quality [35]. Because of the turbulent conditions inside the centrifuges, increased mass transfer rates could also be assumed. However, this advantage could easily be reversed if large amounts of solvent or carrier permeate into the aqueous solution to be recycled into the bioreactor. Because of their toxicity, a severe process inhibition of microbial metabolism could result.

Considering both the benefits and risks of integrating centrifugal extractors in a microbial production process, this article describes the development of a novel ISPR approach using centrifugal reactive-extraction for on-line L-phe separation. A fully integrated ISPR approach is aimed at avoiding loss of unseparated product or fermentation components. For fermentation experiments, the newly constructed L-phe producer *E. coli* F-4/pF81 was used. First, basic extraction experiments using the centrifuges will be presented. Second, the integration of the technology in the fed-batch process for L-phe production will be shown and then the performance of the total ISPR process will be discussed in detail.

## Materials and methods

### Biological system

A newly constructed L-phe production strain (F-4/pF81) based on *E. coli* K-12 W3110 (alias LJ110) was used [36]. The genotype was *E. coli* LJ110 Δ(*pheA tyrA aroF*) /pJF119EH *aroF* $pheA^{fbr}$ *aroB aroL*. The genes *pheA* (encodes chorismate mutase / prephenate dehydratase), *tyrA* (encodes chorismate mutase / prephenate dehydrogenase) and *aroF* (encodes tyrosine feedback-inhibited DAHP synthase) were deleted in the chromosome (Bongaerts 1998 personal communication). The strain was tyrosine auxotrophic to prevent carbon flux into tyrosine synthesis. Plasmid pF81 is based upon pJF119EH [37] and carries the *E. coli* genes *aroF*, $pheA^{fbr}$ (resistant to feedback inhibition by L-phe), *aroB* (encoding 3-dehydroquinate synthase) and *aroL* (encoding shikimate kinase II) in an artificial operon under control of the IPTG-inducible $P_{tac}$-promoter (Degner et al. 1999 personal communication). This plasmid-encoded gene overexpression was used to avoid an unwanted accumulation of pathway intermediates upstream of the enzymes encoded by the genes. A plasmid-encoded ampicillin resistance was used as a selection marker for plasmid-containing cells.

### Culture medium

The synthetic fermentation medium contained $MgSO_4$*7 $H_2O$ (3.0 g/l), $CaCl_2$*2$H_2O$ (0.015 g/l), $KH_2PO_4$ (3.0 g/l), NaCl (1.0 g/l), $(NH_4)_2SO_4$ (5.0 g/l), $FeSO_4$*7 $H_2O$/Na-citrate (0.1125/1.5 g/l), trace element solution (1.5 ml/l), thiamine*HCl (0.075 g/l), L-tyrosine (0.3 g/l), glucose (15 g/l) and ampicillin (0.1 g/l) in deionized water, adjusted to pH 6.5 with 25% $NH_4OH$.

The precultivation medium contained $MgSO_4$*7 $H_2O$ (0.3 g/l), $CaCl_2$*2 $H_2O$ (0.015 g/l), $KH_2PO_4$ (3.0 g/l), $K_2HPO_4$ (12 g/l), NaCl (1 g/l), (NH4)2SO4 (5.0 g/l), $FeSO_4$*7 H2O/Na-citrate (0.075 g/l/1.0 g/l), trace element solution (1.5 ml/l), thiamine*HCl (0.0075 g/l), L-tyrosine (0.08 g/l), glucose (5.0 g/l) and ampicillin (0.1 g/l) in deionized water (final pH 7.2).

The trace element solution contained $Al_2(SO_4)_3$*18 $H_2O$ (2.0 g/l), $CoSO_4$*7 $H_2O$ (0.75 g/l), $CuSO_4$*5 $H_2O$ (2.5 g/l), $H_3BO_3$ (0.5 g/l), $MnSO_4$*1 $H_2O$ (24 g/l), $Na_2MoO_4$*2 $H_2O$ (3.0 g/l), $NiSO_4$*6 $H_2O$ (2.5 g/l) and $ZnSO_4$*7 $H_2O$ (15 g/l), pH 1–2 in deionized water.

### Precultivation

Stock cultures were stored in a Luria-Bertani (LB) medium containing 50% glycerol at −80°C. 250 ml of precultivation medium was inoculated with 500 µl of stock culture and incubated for 15 h at 37°C and 150 rpm in a shake flask incubator up to an $OD_{620}$ of 3–4.

### Fed-batch cultivation

Fed-batch fermentations were performed in a 20 l bioreactor (ISF 200; Infors, Switzerland). After sterilization of the bioreactor and peripheral equipment, pH and DO sensors and exhaust gas analyzer were calibrated. 6.75 l of fermentation medium was filter-sterilized into the bioreactor (0.2 µm, Sartobran; Sartorius, Germany) and adjusted to pH 6.5 by ammonia. The fermentation was started by inoculating the preculture (10% of the 7.5 l fermentation volume). Temperature was controlled at 37°C, pH was kept at 6.5 by ammonia titration and the dissolved oxygen was controlled at 40%. Off-gas was analyzed. Glucose (700 g/l) and tyrosine (25 g/l in 5% $NH_4OH$) were added after the initially supplied substrates were consumed ($OD_{620}$ 12–15) (Fig. 3). Tyrosine had to be added because of the tyrosine auxotrophy of the production strain. Tyrosine was added in limiting amounts to prevent an inhibition of the tyrosine-sensitive DAHP synthase encoded by $AroF^{wt}$ [20]. Therefore, the growth rate was controlled via the addition of tyrosine. When an $OD_{620} \approx 80$ was reached the tyrosine supply was reduced to a minimum of 125 mg/h sufficient for maintenance. Glucose was controlled at a set point of 5 g/l in order to prevent acetic acid formation (which was shown to occur at higher concentrations) or limitations which would result in lower product formation rates [20]. The production of L-phe was induced with 100 µM IPTG at an $OD_{620}$ 8–12. Fermentation time was 50 h.

### On-line and off-line analysis

Oxygen and carbon dioxide were measured in the exhaust gas (BINOS 100 2M; Rosemount, Germany). Dissolved oxygen (DO) was determined with an amperometric electrode (Mettler Toledo, Germany). Temperature, pressure, air flow rate and agitation speed were measured. Glucose samples were taken on-line via a sterilized dialysis probe in the bioreactor and transferred to the Process TRACE system (TRACE, Germany) for enzymatic measurement of glucose. The measured values were shown on the display.

Samples for off-line analysis were taken every 1.5–2 h during the main parts of the fermentation process. Optical density (OD) was measured by a spectrophotometer (Shimadzu UV-160, Germany) at 620 nm. Dry weight was determined by filtering of a 2.5–10 ml sample (depending on optical density) through a 0.2 µm weighted filter, drying the filter for 24 h at 80°C and then weighing. Glucose was measured by the enzyme-based Accutrend system (Roche Diagnostics, Mannheim, Germany). HPLC was used for the analysis of amino acids and organic acids (Sycam, Germany). Amino acids were measured in a reversed phase column (Lichrospher 100 RP 18–5 EC; Merck, Germany) and a fluorescence detector (RF-535; Shimadzu, Germany) after derivatization with *ortho*-phthaldialdehyde (OPA) and mercapto-ethanol. Organic acids were measured with an ion-exclusion column (Aminex-HPX-87H; BioRad, Germany) and a spectrophotometric detector at 215 nm (S3300; Sycam).

### Process control

The standard process parameters were indicated and controlled by Infors (Switzerland) devices. pH was controlled via an implemented PID controller and adjusted to the set point 6.5 by addition of 25% $NH_4OH$ via an automatic feeding system (Sartorius, Germany). The dissolved oxygen concentration DO was controlled and kept at 40% by adjusting the stirring rate with the aid of a PID controller provided by the Infors system. Data acquisition was performed by LabView (National Instruments, USA). MEDUSA (IBT software) and the Process TRACE System were connected for glucose measurement. Signals from on-line glucose measurement by Process TRACE were sent to MEDUSA via LabView. Implemented in MEDUSA was a glucose control system consisting of a (semi-continuous) Kalman filter and a minimal variance controller [38]. This system was used to keep the glucose at a constant preset level during fermentation. L-tyrosine was added in limiting amounts via a preset feed profile. The feed profile was adapted to the increasing demand for tyrosine during the growth phase. By reducing the tyrosine feed to a minimum amount sufficient for maintenance metabolism, growth was stopped when an $OD_{620}$ of 80 was reached. Glucose and tyrosine were added using self-controlled feeding systems (Sartorius, Germany).

### Reactive extraction theory

To prevent inhibition of product formation by the product itself and to extend the production phase, L-phe has to be removed from the process as was already shown by [18] using reactive extraction in an ISPR approach. It was suggested that the cation exchanger $D_2EHPA$ should be used in the organic solvent kerosene for extraction and sulphuric acid in water for back-extraction. The principle of reactive extraction is depicted in Fig. 1. Cell-free permeate, including L-phe cations, anions and zwitterions, enters the extraction unit. Because the carrier possesses a low aqueous solubility ( < 1 g/l), L-phe transfer is assumed to take place very close to the aqueous/organic interface. The carrier is loaded with L-phe cations releasing a proton into the aqueous phase. The organic phase is pumped to the back-extraction unit where high sulphuric acid concentrations are found in the bulk of the liquid acceptor behind the organic/aqueous interface. Another cation exchange takes place resulting in an L-phe cation diffusion from the interface into the acceptor where it can be concentrated. The carrier is recovered by proton uptake at the interface and recycled into the extraction unit where it is used again.

### Reactive extraction devices

Two liquid-liquid centrifuges (Model V2; CINC, Brakel, Germany) were used for reactive extraction of L-phe. The rotation speed of the centrifuges was adjusted by the system's control units and the volumetric flow by peristaltic pumps (Watson & Marlow, Germany). The volume of this model is ≈140 ml, rotor speed is 2,000 to 5,900 rpm and maximum flow rate is 2 l/min. One centrifuge was

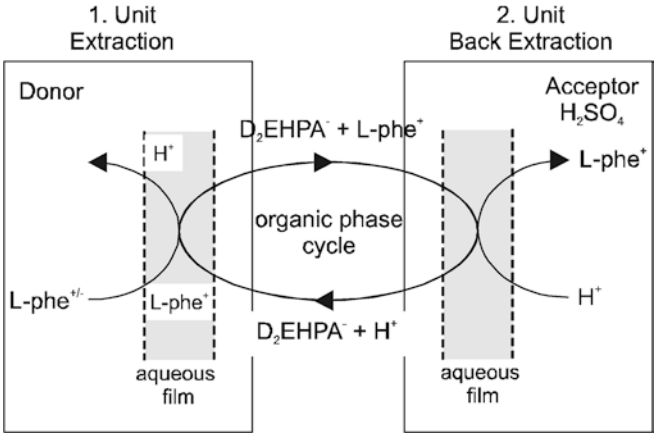


**Fig. 1** Principle of reactive extraction. Although turbulent conditions exist in the mixing chamber of the centrifuges, the reactive extraction is assumed to take place in a thin aqueous film around droplets of organic, carrier-containing solvents. L-phe cations permeate into the aqueous film where the loading of the cation-selective carrier $D_2EHPA$ takes place near the aqueous/organic interface. Loaded carriers are then transported to the back-extraction centrifuge where the carrier releases the L-phe into the acceptor. Protons originating from sulphuric acid are loaded on the carrier instead, again in the aqueous film near the aqueous organic interface. Afterwards, the proton loaded carrier is recycled to the extraction centrifuge

used for extraction of L-phe with the carrier $D_2EHPA$ (cation exchanger) in kerosene. A second centrifuge was used for back-extraction with the acceptor $H_2SO_4$ in water.

A liquid-liquid centrifuge is depicted in Fig. 2. Heavy and light phases enter the centrifuge tangentially and flow down to the bottom through a fissure between the rotating cylinder and the inner wall of the centrifuge. Mixing of both phases and extraction of L-phe takes place in the space between the rotor and support container. By continuous addition of new liquid, the fluid inside the centrifuge is transported into the inner part of the rotor where phase separation takes place on the basis of the different liquid densities. The heavy phase leaves the centrifuge at the outer, the light phase at the inner outlet.

Experiments for characterization of the centrifuges for reactive extraction were performed with aqueous model solutions of L-phe in 0.9% NaCl as well as with fermentation supernatant which was cell-free or cell- and protein-free. Heavy (aqueous) and light (organic) phases were circulated through the running centrifuges for 4–6 h with peristaltic pumps. Samples were taken at the inlet and outlet of the heavy phase of each centrifuge and HPLC measurements were performed as described above.

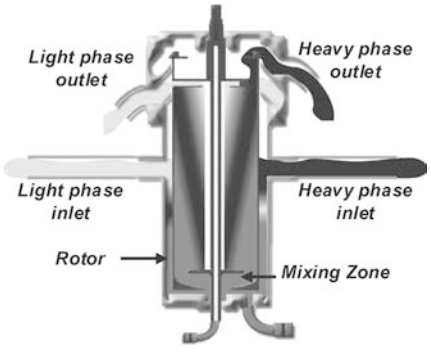


**Fig. 2** Scheme of the liquid-liquid centrifuge used (CINC, Brakel, Germany) showing the inlets and outlets of light (organic) and heavy (aqueous) phases

### Setup of integrated reactive extraction

A bypass was set up in order to produce cell- and protein-free permeate during the fed-batch fermentation process. Fermentation broth was circulated through a bypass with a cross-flow hollow fibre ultrafiltration module (3,600 $cm^2$, 500 kDa cut off; Schleicher & Schuell, Germany) for the generation of cell-free permeate with a peristaltic pump. To avoid oxygen limitation in the bypass, a pump rate of 6 l/min (residence time ≈3.6 s) was necessary. Volumetric flow out of the module was ≈2–4.8 l/h, depending on the process time. 1 M NaOH was used for sterilization of the bypass. For the production of protein-free permeate, cell-free permeate was pumped through an ultrafiltration unit consisting of five cassettes (Ultran-Lab, 10 kDa cut off; Schleicher & Schuell) at a flow rate of up to 4 l/min. Protein- and cell-free permeate was continuously pumped into the reactive extraction unit. A volumetric flow rate of 1.5–3.5 l/h, depending on the process time, was provided by this system. After reactive extraction, the processed fermentation solution (L-phe reduced) was recycled into the bioreactor.

### Fed-batch cultivation with integrated reactive extraction

The fermentation process was started as described above. An initial volume of 9 or 10 l (or 7.5 or 8.5 l, depending on the planned duration of the extraction period) was used to fill the bioreactor or the bioreactor together with the bypass volume. The drain off of cell-free (and protein-free) permeate via the bypass was started during the production phase after growth was stopped. The starting time depended on the experiment. Reactive extraction and fed-batch fermentation were run in parallel for several hours, both until the end of fermentation or reactive extraction and bypass were stopped after several hours, while the fermentation lasted 50 h.

## Results and discussion

### Fed-batch fermentation process

Fed-batch fermentations for L-phe production were performed with glucose control at 5 g/l to prevent acetate formation caused by overflow metabolism at high glucose concentrations, as well as to avoid carbon limitations resulting in reduced L-phe formation rates [15]. The L-tyrosine limited feeding was successfully applied to avoid an unwanted feedback inhibition of the wild-type entrance enzyme of the aromatic amino acid pathway, namely, DAHP synthase *AroF*. Figure 4 shows the setup of the fed-batch process.

A typical fermentation curve using the L-phe producer *E. coli* F-4/pF81 is shown in Fig. 3. As indicated, the glucose concentration was controlled at a set point of $5 \pm 0.5$ g/l after the initial batch phase. The biomass increased exponentially to 30 $g_{CDW}$/l until the growth rate dropped to maintenance metabolism because a significantly reduced tyrosine supply was installed after 14 h. L-phe production was induced with IPTG after 6 h process time, which was subsequently followed by the feeding of the substrates glucose and tyrosine as indicated in Fig. 3. L-tyrosine was only detectable in the

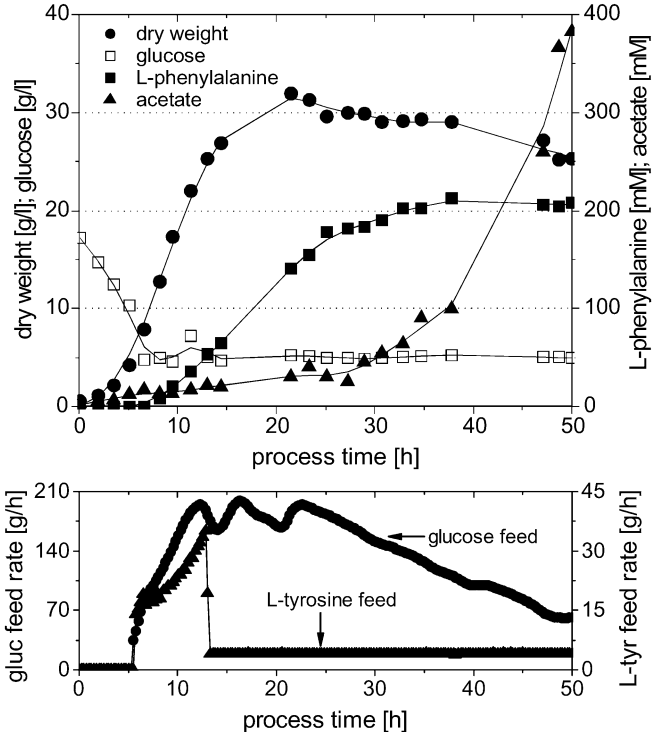


**Fig. 3** Curves of cell dry weight, glucose, L-phe and acetate concentrations during the fed-batch reference fermentation for L-phe production using *E. coli* F-4/pF81 in a 20-l lab-scale bioreactor. The feed-profiles for glucose and tyrosine as a result of glucose and tyrosine control are also given

initial feeding phase (data not shown). A biomass-coupled increase of L-phe was observed during the exponential phase which slowed down when 180 mmol/l was reached. The final L-phe concentration was 206 mmol/l (34 g/l) after 50 h process time. Because of the glucose control, acetate production was very low [20]. However, significant acetate formation started after L-phe production decreased, which led to a final concentration of 380 mmol/l. It is noteworthy that glucose and oxygen availability remained constant during the whole production period thus indicating that acetate formation was obviously not a consequence of 'classical' overflow metabolism.

## Reactive extraction with liquid-liquid centrifuges

To characterize the reactive extraction in the centrifuges, off-line experiments with aqueous model solution were performed. 10 g/l L-phe solution, 10% $D_2EHPA$ in kerosene and 1 M $H_2SO_4$ were used to study the effects of different volumetric flow rates and rotor speeds on the L-phe extraction. For the sake of brevity, only basic findings are listed here:

i. Rotor speed: in the typical range from 2,400 to 3,600 rpm, the rotor speed had almost no influence on the extraction performance.
ii. Volumetric flow rates: extraction and back-extraction mass transfer was improved by increasing volumetric flow rates of the aqueous donor and of the organic phase. Technical, upper limits were found at 8 l/h aqueous flow rate.
iii. System stability: the system was less stable when unequal flow rates of light and heavy phase were used. Flow rates below 4 l/h showed the best system stability. As expected, the system was more stable when L-phe was separated from salt solution (9 g/l NaCl) or from fermentation supernatant instead of from pure water. In the latter case, emulsions tended to occur readily. The fermentation supernatant had to be filtered to remove cell debris and proteins, otherwise unwanted protein precipitation occurred at the aqueous/organic interface in the centrifuge. As a consequence, the centrifuges were operated such that the organic/aqueous interface was radially driven into the centre of the centrifuge thus enabling a small fraction of the heavy phase to leave the device via the light phase outlet.
iv. Extraction components: different concentrations of L-phe (from 60 to 120 mM), carrier (from 10 to 20%) and sulphuric acid (from 1 to 2 M) were investigated. The carrier concentration was identified as the most dominating component. A six fold L-phe concentration of (initially) 15 g/l in the aqueous phase to 90 g/l in the acceptor (leaving 4 g/l L-phe back in the donor phase) was achieved with 1 M $H_2S0_4$

## Fed-batch fermentation process with integrated product separation

The total ISPR experimental set-up can be seen in Fig. 4. While running the fermentation process, fermentation suspension was recycled through the bypass which retained the cells with an ultrafiltration module. Cell-free permeate flowed into a buffer tank and from there into an additional ultrafiltration unit for protein retention. The set-up of two subsequent filtration units was chosen to minimize the cell-containing by-pass volume thus avoiding unwanted oxygen or pH gradients. Nevertheless, the ultrafiltration area had to be large enough to allow 1.5–3.5 l/h to permeate to the extraction devices. The L-phe-reduced aqueous donor phase leaving the centrifuge was recycled into the bioreactor. Back-extraction of L-phe took place in the second centrifuge.

In general, fed-batch fermentations started without running the bypass. L-phe production was induced after a 7 h process time (Fig. 5). Cell growth was stopped by a limited tyrosine supply after 14–15 h. After 18 h a product concentration of about 65 mmol/l was achieved, and bypass recycling together with reactive-extraction was started simultaneously. Based on the preliminary experiments, the reactive-extraction conditions with

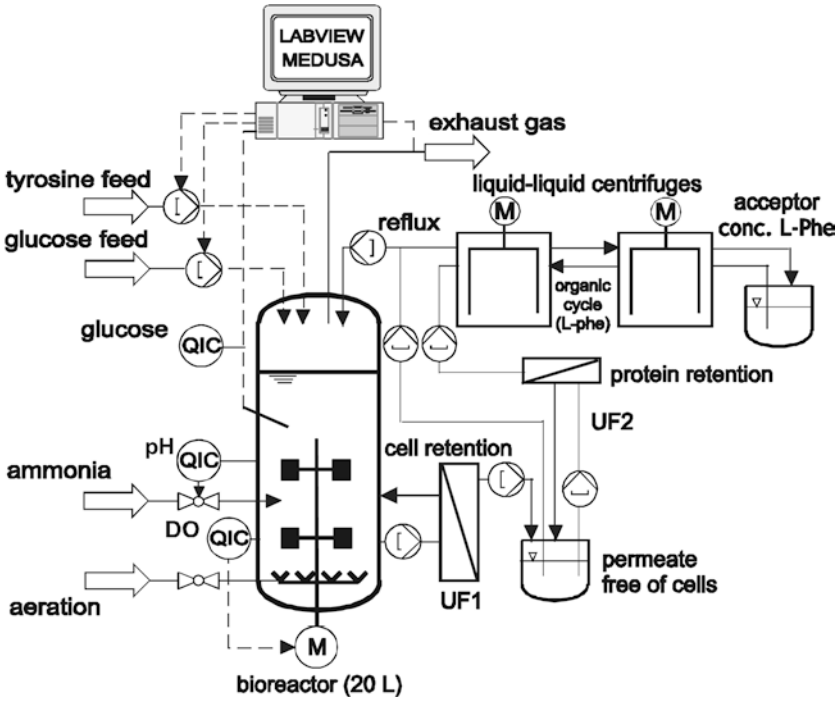


**Fig. 4** Experimental set-up of the total ISPR process using two liquid-liquid centrifuges for reactive extraction of L-phe in cell- and protein-free permeate drained off from the ultrafiltration module 1 (UF1, 500 kD) installed in the bypass of the 20-l bioreactor. As indicated, glucose and tyrosine are controlled, as well as pH, dissolved oxygen (DO), temperature (not indicated) and pressure (not indicated). Via the reflux, the reactive extraction unit is fully integrated in the production process. Reference fermentations are carried out solely using the fermentation equipment as shown

respect to the best phase stability were installed including a rotor speed of 2400 rpm, organic flow rate of 2 l/h, acceptor flow rate of 2 l/h, and a permeate flow rate of 1.5–3.5 l/h. In the case of the permeate stream, the flow depended on the time-variant filtration permeability. As indicated in Fig. 5, production and extraction worked at the same time. While the L-phe concentration in the bioreactor remained constant for 20 h and even decreased during the last 12 h of the process time, the L-phe level in the reflux of the centrifuge was about half of the bioreactor concentration, thus indicating the extraction capacity. In the 5 l acceptor tank, L-phe titers steadily increased to 360 mmol/l (60 g/l), which is more than ten-fold higher than the concentration in the bioreactor. Relative to the bioreactor working volume, a maximum L-phe concentration of about 180 mmol/l (30 g/l) would have been achieved. Although this value is slightly lower than the maximum titer of the reference fermentation (Fig. 3), the benefits of the on-line reactive extraction should be considered, leading to highly concentrated L-phe in the acceptor which can easily be further processed by precipitation [18].

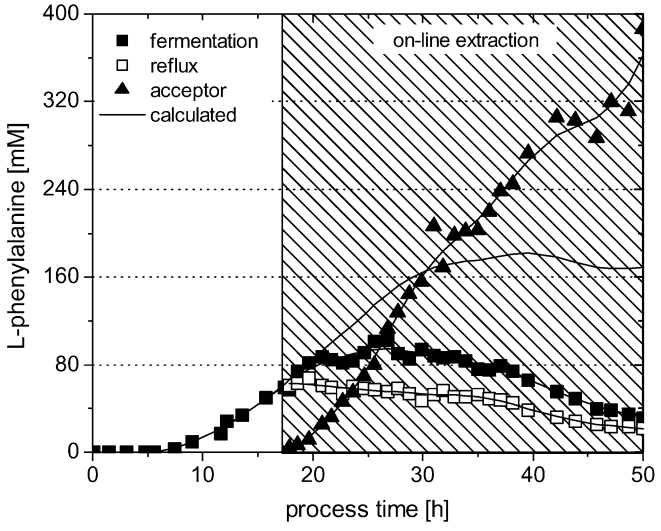


**Fig. 5** Curves of L-phe are shown in the acceptor phase, in the fermentation suspension and in the reflux during a 32-h ISPR experiment with integrated reactive extraction. Additionally, the calculated L-phe course is given regarding the total amount of separated and accumulated L-phe with respect to the working volume of the bioreactor

Most strikingly, the fully integrated centrifugal reactive-extraction showed its general applicability by allowing stable operation conditions for more than 32 h. Obviously, the primary assumed inhibiting effects of the solvent and/or of the carrier were not dominant. During the first 20 h, the L-phe separation rate kept pace with its microbial formation rate, indicating that cellular metabolism was not hampered. However, after 38 h, indications were observed that the metabolism activity declined, thus reducing the L-phe formation rates (data not shown).

From this we concluded that reactive extraction based on liquid-liquid centrifuges is promising and that the duration of on-line extraction should be a parameter for total process optimization.

Thus, a shorter on-line extraction period was studied. Additionally, the flow rates of the organic and the acceptor phase were set to 3.5 and ≈2 l/h to increase L-phe separation rates and to stabilize the extraction system. The resulting L-phe concentration curves are shown in Fig. 6 which indicate that L-phe production

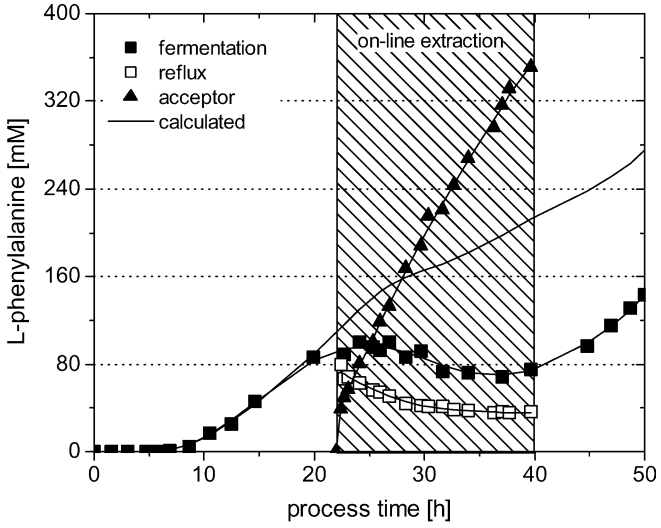


**Fig. 6** Curves of L-phe are shown in the acceptor phase, in the fermentation suspension and in the reflux during an 18 h ISPR experiment with integrated reactive extraction. Additionally the calculated L-phe course is presented regarding the total amount of separated and accumulated L-phe with respect to the working volume of the bioreactor

started after 7 h. Compared to the preceding experiment, reactive-extraction was actuated later (after 22 h process time) because the pre-chosen threshold value of 90 mmol/l was also achieved later owing to a slightly lower biomass concentration. As shown, the on-line L-phe separation was running for 18 h. During this period, the L-phe level in the bioreactor slightly decreased and the reflux contained about half the amount of L-phe in the reactor. 350 mmol/l (58 g/l) L-phe was concentrated in the acceptor, which is almost nine times higher than the level in the fermentation suspension. After 40 h, the extraction was stopped whereas the fermentation continued until 50 h. This procedure was performed to test the metabolic activity of the cells after reactive-extraction. As shown in Fig. 6, product formation still took place thus increasing the L-phe level to 143 mmol/l (23.5 g/l). Therefore, we concluded that the cells were still active and that they were not inhibited, for example, by too high kerosene or carrier concentrations. Relative to the final working volume in the bioreactor, a maximum titer of 275 mmol/l (45.5 g/l) was achieved.

To qualify the ISPR process, the space-time yield (STY) based on the calculated L-phe concentrations (Fig. 6) is given in Fig. 7. As shown, STY values from 5 to 11.5 mmol/(l*h) were achieved during the production phase. The initial drop of the production STY (immediately after the reactive-extraction start) is similar to previous findings described by Gerigk et al. [15] and, strictly speaking, not yet fully understood. Because any gradients of dissolved oxygen, concentration or pH inside the bypass circulation can be ruled out due to low bypass residence times of 3 to 4 s, alternative reasons such as increasingly inhibiting carrier/kerosene levels in the fermentation supernatant or rising plasmid instability can be considered as a working hypothesis. This

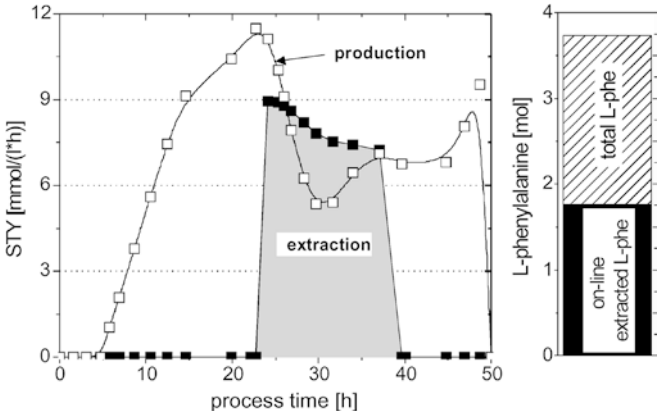


**Fig. 7** Space time yield (STY) during the 18-h ISPR process considering L-phe formation and on-line L-phe extraction. The total amount of L-phe produced and separated during the experiment is shown on the *right*

negative effect is more or less compensated during the course of separation. In general, L-phe extraction rates from 7 to 9 mmol/(l*h) were maintained during the whole separation period, thus keeping the L-phe level in the suspension almost constant. In total, 3.73 mol L-phe (616 g) was produced and approximately half (1.76 mole which represents 290 g) could be extracted on-line. The rest was left in the suspension for off-line separation after fed-batch production (Fig. 7).

Besides L-phe separation analysis, differences between the experiments with and without integrated reactive extraction can also be seen regarding the acetate formation (data not shown). In the reference fermentation, acetate production had already begun after 30 h when L-phe formation decreased. Similar results were obtained in the fermentation using long-lasting (32 h) reactive extraction, when acetate formation began after 38 h process time, again correlated to the decline of L-phe formation. Therefore, and because, glucose and oxygen supply remained constant during the whole production period, we regard acetate formation as a cellular response to the diminishing ability to convert glucose into L-phe which, for instance, can be caused by decreasing DAHP synthase activity. The latter was observed experimentally [20] and can also be a consequence of L-phe feedback inhibition [32]. Interestingly, almost no acetate production was found during the 18 h long reactive extraction experiment. As already shown, no cessation of L-phe formation was observed which consequently resulted in low acetate titers, thus outlining an additional benefit of the integrated reactive extraction approach.

Clear differences can be seen in comparing the production rates of a reference fermentation without integrated L-phe separation with those of the ISPR approach using integrated reactive extraction for 18 h (Fig. 8). During the ISPR period, the production rate was maintained at a constant level of about 0.25 mmol/(g*h) until the end of the experiment, whereas it decreased to zero after 35 h in the reference

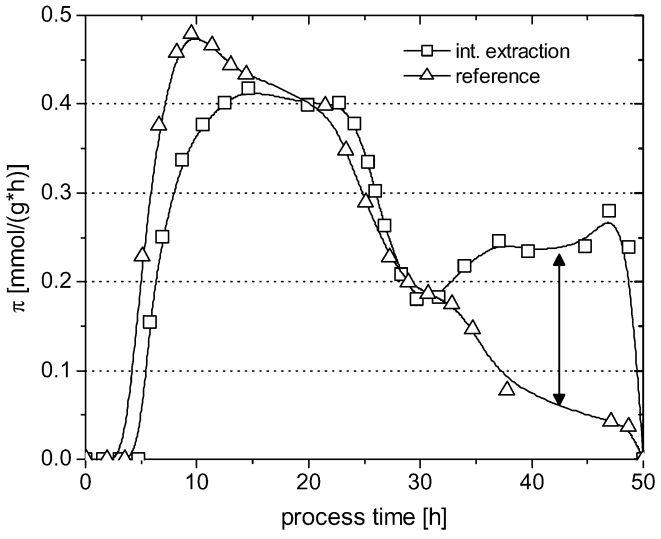


**Fig. 8** Comparison of biomass-specific product formation rates in the reference fed-batch fermentation without integrated reactive extraction and in the ISPR process considering the 18 h lasting reactive extraction

fermentation. As a consequence, the integral, molar L-phe/glucose yield enhanced from 14.5% in the reference fermentation to 18.6% in the ISPR approach, which represents a 28% relative yield rise. This relative yield improvement is comparable to the results of Gerigk et al. [15] using another *E. coli* production strain in their ISPR approach. From this, we concluded that the on-line separation of L-phe obviously avoids an inhibitory product accumulation (with respect to the DAHP synthase activity). However, this phenomenological finding cannot be substantiated by, e.g., *in vivo* enzyme activity measurements or the quantitative analysis of the potentially beneficial effect of $D_2$EHPA addition, as proposed by Maass [39]. The relevant studies have not yet been completed.

## Conclusion

A novel ISPR process approach for the on-line separation and concentration of L-phe from a running fed-batch fermentation using the recombinant strain *E. coli* F-4/pF81 is presented. Using liquid-liquid centrifuges for reactive extraction with kerosene, the cation selective carrier $D_2$EHPA and sulphuric acid in the acceptor, this system represents the first approach known so far for fully integrated reactive extraction based on liquid-liquid centrifuges to separate bio-products from a microbial production process.

It was shown that the ISPR approach (using reactive extraction for 18 h) is superior to the reference fermentation without product separation because of: (i) the significantly extended high-level product formation period of 0.25 mmol/(g*h) up to the end of the process, (ii) the prevention of unwanted by-product accumulation (acetate), and (iii) the consequently increased integral L-phe/glucose yield. It was observed, that the integration of liquid-liquid centrifuges was only successful with cell- and protein-free permeate. An optimum ISPR-duration of 18–20 h must be considered to avoid too high kerosene and carrier permeation into the bioreactor which can cause microbial metabolism inhibition.

Compared to earlier publications of Gerigk et al. [15] and Maass et al. [18] using hollow fiber modules, some basic differences in the system performance become apparent. While both approaches enable an on-line concentration of L-phe, the liquid-liquid centrifuges support higher L-phe concentrations in the acceptor (up to 360 mM), which significantly reduces the amount of liquid to be handled during further product purification, e.g., during precipitation. This approach allows the online separation of almost half of the total L-phe produced during the fed-batch process, which is higher than the previously described value. If volumetric extraction rates are compared, the ISPR approach using liquid-liquid centrifuges also offers some advantages. Here, 1.19–1.47 $g_{L\text{-phe}}$/(l*h) is extracted, while the membrane-based extraction only achieved approximately 1/3 of this, i.e., 0.3–0.5 $g_{L\text{-phe}}$/(l*h).

Summarizing, ISPR reactive extraction using liquid-liquid centrifuges possesses some inherent advantages compared to the application of hollow fiber modules which include:

i. higher robustness, i.e., reactive extraction system stability is not at risk when pressure changes/peaks occur (as it can happen while using hollow fiber modules for reactive extraction),
ii. 'easy' handling, because preparation and operation of the centrifuges is simpler compared to the hollow fiber modules,
iii. higher profitability, because a three-fold higher product permeability is achieved, thus reducing the investment costs compared to the relatively expensive membrane-based approach which has the inherent additional disadvantage of limited membrane lifetime,
iv. and facilitated system scale-up, because centrifugal extractors are available in different, optimized scale-up sizes (actually the smallest type was used here) while the necessary increase of membrane area would mainly be achieved by the parallel installation of various modules, thus raising the technical complexity manifold.

If this ISPR approach is compared to the best L-phe process results published so far [14, 40], the ISPR process (product titer: 45.5 g/l (calculated), 60 g/l (acceptor phase); L-phe/glucose yield: 18.6 mol%; space-time yield: 0.91 g/(l*h)) is superior to the data of Konstantinov et al. [40] (product titer 46 g/l; L-phe/glucose yield: 17.4 mol%; space-time yield: 0.85 g/(l*h)) while it does not yet achieve the results of Backman et al. [14] who

used a specially designed feedback resistant mutant (product titer: 50 g/l; L-phe/glucose yield: 25 mol%; space-time yield: 1.39 g/(l*h)). However, it is noteworthy, that only by application of the ISPR process, *E. coli* F-4/pF81 achieved competitive results and, at the same time, offers an effective downstream processing technology which was typically neglected during all previous non-ISPR studies. Hence, ISPR processes using liquid-liquid centrifuges for reactive extraction represent an interesting tool for the on-line (cation) separation of inhibitory products.

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