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# Microbial De-emulsification: A Highly Efficient Procedure for the Extractive Workup of Whole-Cell Biotransformations

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**Abstract:**

**Formation of stable emulsions with the organic solvent is a general complication in the extractive workup of aqueous whole-cell biotransformations. This hold-up has been overcome by biocatalytic lysis of emulsifying agents present in the medium through addition of living microorganisms. Of these, *Bacillus subtilis* and *Rhodococcus erythropolis* exhibited the most powerful de-emulsifying activity. As exemplified by microbial treatment of cell-free biotransformation media of *Saccharomyces cerevisiae* and *Lactobacillus kefiri*, phase separation time ($t_p$) was drastically reduced from one week to 20 s without significantly affecting product integrity. This practicable ready-to-use method is appropriate to both fungal and bacterial biocatalysts. The highly efficient de-emulsification power and the considerably short phase separation time of this technique allow for cost-effective continuous extractions on a large-scale, for example with mixer−settler units.**

## Introduction

The economic attractiveness of commercial whole-cell biocatalysis mainly depends on a highly efficient, cost-effective downstream process. Extractive workup of the aqueous phase is an often highly time-consuming process, and therefore, the most important cost factor in industrial biocatalysis. Complications arise from the formation of highly stable gels and slimes which may be stable for weeks unless the solvent is allowed to evaporate.[1−6]

This phenomenon is typical for the whole-cell biocatalytic production of fine chemicals, where cell densities often exceed those of conventional fermentation protocols by a factor of 10−100.[7]

On a laboratory scale the unfavorable gel and slime formation is typically solved by centrifugation of the organic solvent/water mixtures.[8] For large-scale operations primarily steam distillation and membrane filtration come into use. However, steam distillation is suited solely for thermostable products. Further, thermal decomposition of biological material causes impurities of the raw product, which then requires a second purification step. Since in most cases a thermal purification procedure (distillation) comes into use, stereopurity may be affected, especially of high-boiling products. These problems are circumvented by membrane filtration technology which often furnishes the raw material in satisfactory purity. However, since three membrane filtration steps—microfiltration, ultrafiltration, and nanofiltration—are necessary, until emulsifying agents are completely removed, this technology requires replacement by less cost-intensive alternatives in order to run a biocatalytic production competitively.[9−12]

It was therefore a major breakthrough when bioemulsifiers responsible for gel formation during extractive work-ups were identified.[13] Finally substantial progress had been made with the invention of hydrolases as de-emulsifying agents.[14,15] They act by cleaving emulsifying biomolecules, whereupon these lose their amphiphilic properties.

Enzymatic de-emulsification bears several advantages over existing methodologies. With proteases, phase separation is complete after 30 min instead of hours or weeks. Not only is the extractive workup of whole-cell biotransformations greatly facilitated, but emulsification problems of biotransformations with isolated enzymes which use technical enzyme preparations are also solved by this technology.

However, although substantial improvements have been achieved, this method is not suited for continuous extractions

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in mixer−settler units where phase separation is required to be complete after 60 s max.[16,17]

The gel and slime formation effect is caused by bioemulsifiers, surface-active substances, secreted by the microorganisms into the medium.[13,18] They are classified into (i) glycolipids, (ii) lipopeptides and lipoproteins, (iii) glycoproteins, and (iv) fatty acids, neutral lipids, and phospholipids.[19−21] The strategy behind enzymatic treatment of bioemulsifiers is to cleave the amphiphilic backbone into the hydrophobic and the hydrophilic structural unit. For these purposes protease treatment has furnished the best results thus far, but also glycosylases gave good phase separation times ($t_p$), and fatty acids precipitated after lipase treatment.[14]

This indicates that mixtures of bioemulsifiers rather than one type are responsible for the observed phenomenon, predominantly glycoproteins and lipoproteins. Composition of these mixtures is highly dependent on the substrate used and on the reaction conditions of a bioconversion. In addition, since the substrate itself may stimulate microorganisms to increasingly secrete bioemulsifiers into the medium, compositions of bioemulsifier mixtures in biotransformations are difficult to predict.

A generally practicable, reliable de-emulsification strategy reasonably needs to work independently from the composition of a biotransformation broth. Furthermore, since the so far best $t_p$ did not fall below 60 s, "de-emulsified" broths obviously still contain emulsifying biomolecules of unknown nature.

In this contribution a highly efficient process will be presented, where phase separation is reliably complete after 20 s. The new technology uses living microorganisms as de-emulsifying agents and does not suffer from formation of unfavorable emulsions. Our study was conducted with the whole-cell biotransformation systems *Saccharomyces cerevisiae* and *Lactobacillus kefiri* in order to exemplarily demonstrate that this methodology is easily applicable for both fungal and bacterial cultures.

## Results and Discussion

**Microbial Cleavage of Bioemulsifiers.** Biotransformation broths contain mixtures of bioemulsifiers rather than one type, and compositions of these mixtures are highly dependent on both the substrate used and on the reaction conditions. Therefore, enzyme combinations were tested in order to degrade biosurfactants more efficiently, but no ameliorating effect was noticeable. For these reasons living microorganisms were investigated as potential de-emulsifiers. Living cells constitute multi-enzyme systems—a property that is mindfully made use of in whole-cell biotransformations for the production of compounds of value. Here microbes were conceived as multipotent de-emulsifying whole-cell biocatalysts, since their extracellular enzyme equipment would allow for a broad spectrum of biomolecules being degraded.

Favorably in the forefront of microbial de-emulsification, cell vitality and maximum tolerated dose are determined in order to exclude detrimental effects of substrate and products on the microbial de-emulsifier and to realize optimal de-emulsification conditions where necessary.

In our experiments phase separation time ($t_p$) of a microbially de-emulsified sample was compared with that of an untreated sample. $t_p$ served as a reproducibly quantifiable measure for the efficacy of the enzyme-catalysed lysis of bioemulsifying agents.

The investigations straightforwardly demonstrate the de-emulsifying effect of microorganisms on phase separation and gel formation, respectively. The results for cell-free media of *Saccharomyces cerevisiae* are summarized in Table 1 and of *Lactobacillus kefiri* in Table 2.

The best performance was obtained by treatment with *Bacillus subtilis* under micro-aerobic condition. $t_p$ of biotransformation broths treated with this whole-cell de-emulsifier amounted to the so far unequalled 20 s. Under aerobic and micro-aerobic de-emulsification conditions *Rhodococcus* sp. ($t_p$ = 30 s) showed the best performance. In addition to incubation with *Escherichia coli* TG1, *Pseudomonas* sp. and *Candida* sp. phase separation time below 60 s was obtained. Phase separation times >60 s are incompatible with continuous extraction in mixer−settler units.

*Rhodococcus erythropolis* DSM 43297 showed the best performance under aerobic conditions ($t_p$ = 20 s). Besides biotransformation broths treated with *Rhodococcus erythropolis* DSM 43297, *Rhodococcus erythropolis* DSM 743 and *Saccharomyces cerevisiae* VW1A no phase separation time below 60 s was obtained. Under micro-aerobic de-emulsification conditions only *Rhodococcus erythropolis* DSM 743 ($t_p$ = 40 s) gave a reduced phase separation time.

The de-emulsification experiments were executed after biotransformations of exemplary substrates **1**−**7** (Figure 1). In all cases except for ethyl 2-chloro-acetoacetate **(6)** phase separation times below 60 s were obtained. As pointed out earlier, microorganisms may tend to secrete bioemulsifiers into the medium. From the fact that **6** is an alkylating agent, it appears reasonable that xenobiotic cell stress is involved in microbial bioemulsifier production during whole-cell biotransformations.

The effect of whole-cell de-emulsification on extraction efficiency was investigated exemplarily with the biocatalytic synthesis of chiral $\beta$-hydroxy esters **8**−**10** (Figure 2), for retention up to 20% of these products in the aqueous phase is a typical complication found in extractions of untreated cell-free media. In contrast the beneficial effect of microbial treatment of the aqueous phase on product extractability was evident. After treatment with *Rhodococcus erythropolis* DSM 43297 or *Bacillus subtilis*, products were fully extractable (≥99%).

Apart from $t_p$, completeness of de-emulsification greatly affects the outcome of the extractive workup procedure. Where a phase is opaque or slimy, phase separation is insufficient, and undesired constituents of the aqueous phase

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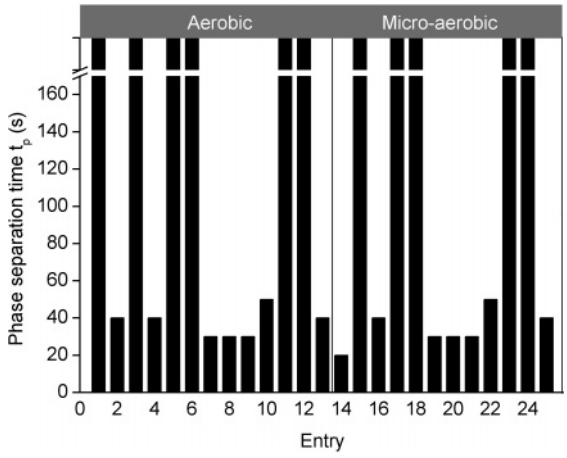
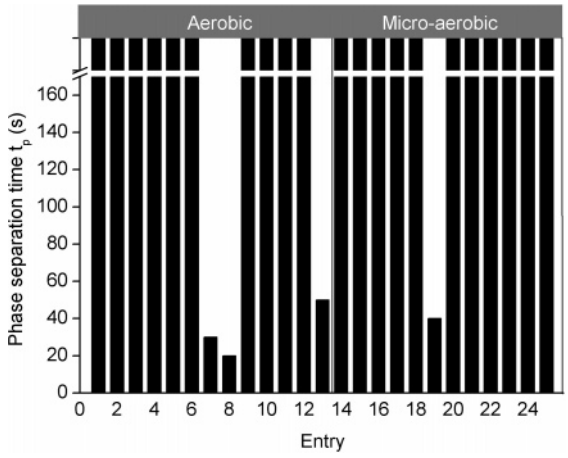
**Table 1.** Results of the microbial cleavage of the bioemulsifier of *Saccharomyces cerevisiae* in cell-free medium ($t_i$ = 48 h[a], pH = 6.6, $T$ = 30 °C)




| entry | microorganism | $t_p$ (s)[b] | observation |
|---|---|---|---|
| 1 | control[c] | >600 | stable, colorless gel |
| | Aerobic | | |
| 2 | *Bacillus subtilis* | 40 | few bubbles at liquid−liquid interface |
| 3 | *Escherichia coli* TG1[d] | >600 | stable, colorless gel |
| 4 | *Pediococcus acidilactici* 519 | 40 | few bubbles at liquid−liquid interface |
| 5 | *Pseudomonas aureofaciens* ACN | >600 | stable, colorless gel |
| 6 | *Pseudomonas fluorescens* BL 915 | >600 | stable, colorless gel |
| 7 | *Rhodococcus erythropolis* DSM 743 | 30 | few bubbles at liquid−liquid interface |
| 8 | *Rhodococcus erythropolis* DSM 43297 | 30 | few bubbles at liquid−liquid interface |
| 9 | *Rhodococcus* sp. GK1 | 30 | few bubbles at liquid−liquid interface |
| 10 | *Lactobacillus kefiri* DSM 20587 | 50 | few bubbles at liquid−liquid interface |
| 11 | *Candida parapsilosis* DSM 70125 | >600 | stable, colorless gel |
| 12 | *Candida boidinii* ATCC 26175 | >600 | stable, colorless gel |
| 13 | *Saccharomyces cerevisiae* VW1A | 40 | few bubbles at liquid−liquid interface |
| | Micro-aerobic | | |
| 14 | *Bacillus subtilis* | 20 | sharp liquid−liquid interface |
| 15 | *Escherichia coli* TG1[d] | >600 | stable, colorless gel |
| 16 | *Pediococcus acidilactici* 519 | 40 | few bubbles at liquid−liquid interface |
| 17 | *Pseudomonas aureofaciens* ACN | >600 | stable, colorless gel |
| 18 | *Pseudomonas fluorescens* BL 915 | >600 | stable, colorless gel |
| 19 | *Rhodococcus erythropolis* DSM 743 | 30 | few bubbles at liquid−liquid interface |
| 20 | *Rhodococcus erythropolis* DSM 43297 | 30 | few bubbles at liquid−liquid interface |
| 21 | *Rhodococcus* sp. GK1 | 30 | few bubbles at liquid−liquid interface |
| 22 | *Lactobacillus kefiri* DSM 20587 | 50 | few bubbles at liquid−liquid interface |
| 23 | *Candida parapsilosis* DSM 70125 | >600 | stable, colorless gel |
| 24 | *Candida boidinii* ATCC 26175 | >600 | stable, colorless gel |
| 25 | *Saccharomyces cerevisiae* VW1A | 40 | Few bubbles at liquid−liquid interface |

[a] $t_i$ - incubation time. [b] $t_p$ - phase separation time. [c] Without microorganism added. [d] $T$ = 37 °C (see Experimental Section).

**Table 2.** Results of the microbial cleavage of the bioemulsifier of *Lactobacillus kefiri* in cell-free medium ($t_i$ = 48 h[a], pH = 6.6, $T$ = 30 °C)




| entry | microorganism | $t_p$ (s)[b] | observation |
|---|---|---|---|
| 1 | control[c] | >600 | stable, colorless gel |
| | Aerobic | | |
| 2 | *Bacillus subtilis* | >600 | stable, colorless gel |
| 3 | *Escherichia coli* TG1[d] | >600 | stable, colorless gel |
| 4 | *Pediococcus acidilactici* 519 | >600 | stable, colorless gel |
| 5 | *Pseudomonas aureofaciens* ACN | >600 | stable, colorless gel |
| 6 | *Pseudomonas fluorescens* BL 915 | >600 | stable, colorless gel |
| 7 | *Rhodococcus erythropolis* DSM 743 | 30 | few bubbles at liquid−liquid interface |
| 8 | *Rhodococcus erythropolis* DSM 43297 | 20 | few bubbles at liquid−liquid interface |
| 9 | *Rhodococcus* sp. GK1 | >600 | stable, colorless gel |
| 10 | *Lactobacillus kefiri* DSM 20587 | >600 | stable, colorless gel |
| 11 | *Candida parapsilosis* DSM 70125 | >600 | stable, colorless gel |
| 12 | *Candida boidinii* ATCC 26175 | >600 | stable, colorless gel |
| 13 | *Saccharomyces cerevisiae* VW1A | 50 | few bubbles at liquid−liquid interface |
| | Micro-aerobic | | |
| 14 | *Bacillus subtilis* | >600 | stable, colorless gel |
| 15 | *Escherichia coli* TG1[d] | >600 | stable, colorless gel |
| 16 | *Pediococcus acidilactici* 519 | >600 | stable, colorless gel |
| 17 | *Pseudomonas aureofaciens* ACN | >600 | stable, colorless gel |
| 18 | *Pseudomonas fluorescens* BL 915 | >600 | stable, colorless gel |
| 19 | *Rhodococcus erythropolis* DSM 743 | 40 | few bubbles at liquid−liquid interface |
| 20 | *Rhodococcus erythropolis* DSM 43297 | >600 | stable, colorless gel |
| 21 | *Rhodococcus* sp. GK1 | >600 | stable, colorless gel |
| 22 | *Lactobacillus kefiri* DSM 20587 | >600 | stable, colorless gel |
| 23 | *Candida parapsilosis* DSM 70125 | >600 | stable, colorless gel |
| 24 | *Candida boidinii* ATCC 26175 | >600 | stable, colorless gel |
| 25 | *Saccharomyces cerevisiae* VW1A | >600 | stable, colorless gel |

[a] $t_i$ - incubation time. [b] $t_p$ - phase separation time. [c] Without microorganism added. [d] $T$ = 37 °C (see Experimental Section).

may have entered the organic phase, which necessitates an additional product purification step after extraction. Other-

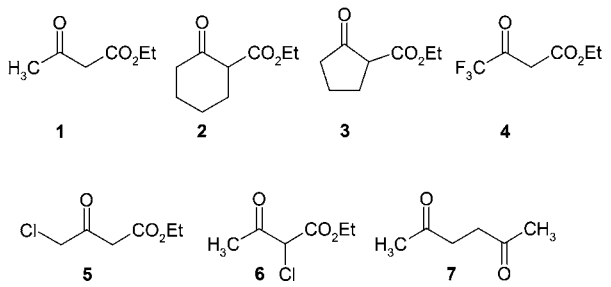


***Figure 1.*** **Cell-free media of biotransformations of substrates 1−7 were tested for microbial de-emulsification.**

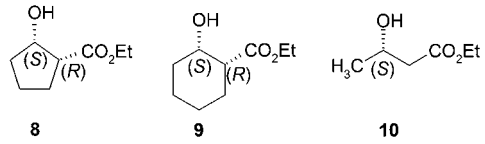


***Figure 2.*** **Products tested for extractability.**

wise, material of value may be retained in the aqueous phase and extraction will be incomplete. Conversely, little slime or few bubbles at the liquid−liquid interface can be tolerated. We experienced no disadvantageous effects in these cases. Further, thanks to the low cell density of the microbial de-emulsifier these cells themselves were observed to form no emulsifying agents.

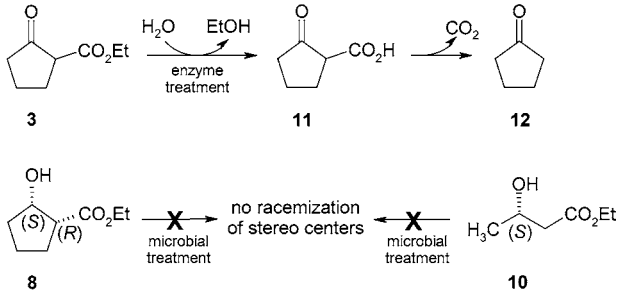
As a consequence of microbial de-emulsification, products were found completely extractable from the biotransformation broth. No significant transfer of biological material into the organic phase occurred, and raw material purity was ≥97%. These effects were independent of the solvent used.

Commercial-scale applications profit from the applicability of frozen microbial de-emulsifiers. The selected microorganism can be grown on a large scale and is fit for storage at −25 °C for weeks without loss of activity, which is clearly another great advantage of this methodology. Cells should not be grown differently from what is described in the Experimental Section; otherwise the cells may enter the stationary phase, where they are less active as de-emulsifiers. Moreover, they can begin to sporulate, or even surfactant production may set in as is observed, for example, with *Rhodococcus* sp. Forthcoming activities will be directed towards increasing de-emulsifier cell-density.

An incubation time of $t_i = 48$ h appears acceptable in light of this workup variant being more cost effective than classical methodology. In addition, from a regulatory point of view the same regulations apply for nonpathogenic microbial de-emulsifiers that apply for nonpathogenic whole-cell biocatalysts.

**Product Integrity.** Apart from phase separation time ($t_p$), product stability is a central criterion. As the whole-cell multi-enzyme system exhibits extracellular hydrolytic activity,[22−24] there is also the need to check product integrity with respect to ester hydrolysis and racemization. The extent of microbial hydrolysis was determined for the representative

***Scheme 1.*** **Test for effects on product integrity by microbial treatment of culture medium**



model substrate ethyl 2-oxocyclopentanoate (**3**). Effects on reoxidation of product and product stereopurity were checked exemplarily with **8** and **10**. The stereo centers in chiral alcohols were not affected at all by the de-emulsifiers tested (Scheme 1).

Product integrity remained unaffected even at product concentrations common in industrial processes. No effects were observed for typical maximum product concentrations of 20 g/L (**8**) and 90 g/L (**10**).

Since the reaction rate of enzymatic hydrolysis depends on the stereo configuration of a substrate,[24] instead of a chiral alcohol racemic $\beta$-keto ester, **3** was chosen as a model substrate for assaying hydrolytic stability. Saponification of **3** furnishes $\beta$-keto acid **11** which spontaneously decarboxylates to give cyclopentanone (**12**). Compounds **3** and **12** are easily and unambiguously distinguishable from each other by means of HLPC, where formation of **12** by competing thermal ester pyrolysis, as can happen under GC conditions, does not occur. In order to avoid thermal racemization, preferably highly volatile organic solvents such as *tert*-butyl methyl ether (TBME) were used, especially for the extraction of thermolabile products. The results of our experiments on product stability during microbial hydrolysis of bioemulsifiers are given in Table 3.

The highly efficient microbial de-emulsification of cell-free suspensions (short $t_p$) was not found to be accompanied by competing reactions, not even in trace amounts. In no case were stereo centers in chiral alcohols affected by the microorganisms tested, and ester functions proved hydrolytically stable under the conditions applied.

Thus far, the composition of the bioemulsifiers has remained unclear. Since biotransformation broths form variably composed mixtures of biosurfactants and since microbial de-emulsification allows unequalled fast phase separation, it hardly appears reasonable to invest further effort in the identification of the emulsifying agents.

From a large-scale application point of view, workup of whole-cell biotransformations has become more effective through microbial de-emulsification, as shorter $t_p$ allow continuous extraction procedures, for example on mixer−settler units.

However, it has to be noted that potential outcomes of microbial de-emulsification are dependent on the substrate/product investigated and on the reaction conditions applied. Therefore, product integrity needs to be assayed individually for any biotransformation.

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***Table 3.*** **Hydrolytic stability of the ester functionality ($t_i$ = 48 h[a], pH = 6.6, $T$ = 30 °C)**

| entry | microorganism | hydrolysis (%) |
|---|---|---|
| 1 | control[b] | 0 |
| | Aerobic | |
| 2 | *Bacillus subtilis* | <0.1 |
| 3 | *Escherichia coli* TG1[c] | <0.1 |
| 4 | *Pediococcus acidilactici* 519 | <0.1 |
| 5 | *Pseudomonas aureofaciens* ACN | <0.1 |
| 6 | *Pseudomonas fluorescens* BL 915 | <0.1 |
| 7 | *Rhodococcus erythropolis* DSM 743 | <0.1 |
| 8 | *Rhodococcus erythropolis* DSM 43297 | 0.3 |
| 9 | *Rhodococcus* sp. GK1 | <0.1 |
| 10 | *Lactobacillus kefiri* DSM 20587 | <0.1 |
| 11 | *Candida parapsilosis* DSM 70125 | <0.1 |
| 12 | *Candida boidinii* ATCC 26175 | <0.1 |
| 13 | *Saccharomyces cerevisiae* VW1A | <0.1 |
| | Micro-aerobic | |
| 14 | *Bacillus subtilis* | <0.1 |
| 15 | *Escherichia coli* TG1[c] | <0.1 |
| 16 | *Pediococcus acidilactici* 519 | <0.1 |
| 17 | *Pseudomonas aureofaciens* ACN | <0.1 |
| 18 | *Pseudomonas fluorescens* BL 915 | <0.1 |
| 19 | *Rhodococcus erythropolis* DSM 743 | <0.1 |
| 20 | *Rhodococcus erythropolis* DSM 43297 | 0.3 |
| 21 | *Rhodococcus* sp. GK 1 | <0.1 |
| 22 | *Lactobacillus kefiri* DSM 20587 | <0.1 |
| 23 | *Candida parapsilosis* DSM 70125 | <0.1 |
| 24 | *Candida boidinii* ATCC 26175 | <0.1 |
| 25 | *Saccharomyces cerevisiae* VW1A | <0.1 |

[a] $t_i$ - incubation time. [b] Without microorganism added. [c] $T$ = 37 °C (see Experimental Section).

**Combination of Microorganisms.** In addition, as a consequence of the heterogeneity of bioemulsifier composition, exploratory experiments were conducted in order to cleave emulsifying agents by a combination of microorganisms with the aim to reduce $t_p$ possibly even further. However, these experiments were unsuccessful, and in some cases $t_p$ even increased to >60 s. Obviously the tested microorganisms mutually digest their de-emulsifying proteins when combined.

**Effects of pH on Microbial De-emulsification.** The de-emulsifying effect of microorganisms is conceivable as extracellular enzymatic activity. Therefore, the pH of the aqueous medium may affect the effectiveness of enzymatic action.

There was no measurable effect of small pH variations on phase separation time ($t_p$). However, the microorganisms tolerated only a rather narrow pH range between 7.5 ≥ pH ≥ 6. Outside this pH range microbial de-emulsification did not work, and stable gels and slimes were formed. Below pH 4 even foams were observed.

**Effects of Biotransformation Substrate and Products on Microbial De-emulsification.** Where cytotoxic substrates are not fully converted or where product and byproducts display detrimental effects on cell vitality, microbial de-emulsification strategies may be affected. It is therefore strongly recommended to check for potential effects in the beginning of cultivating the microbial de-emulsifier by determination of cell-vitality or maximum tolerated dose for the substances in question. The easiest way to circumvent concentration-dependent impairment of the de-emulsifier is dilution of the reaction mixture to be extracted with water. The potentially disadvantageous handling of larger volumes is by far overridden by the superior extractability of microbially treated media.

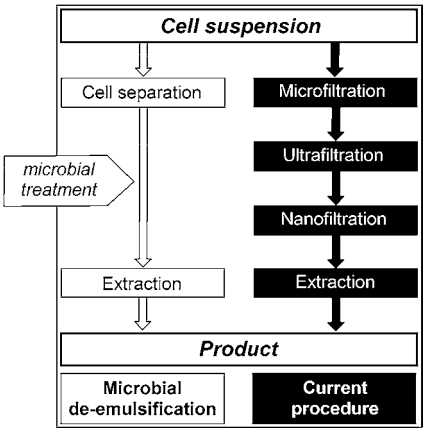


***Figure 3.*** **Comparison of current workup technology and the simplification accomplished by microbial de-emulsification.**

In case microbial methods are not applicable, enzymatic de-emulsification with hydrolases offer a powerful alternative, yet with potentially prolonged $t_p$.[14,15] The use of high solvent/water ratios during extraction involves large amounts of solvents which need to be handled, recovered, and disposed of while the risk of emulsion formation is still there.

**Alternative Cleavage of Bioemulsifiers with NaOH.** With respect to the chemical composition of bioemulsifiers, supplementary experiments with NaOH were conducted, since peptide and ester bonds are cleaved under alkaline conditions. Yet, $t_p$ was far beyond enzymatic variants. As this method would not apply for hydrolysis-sensitive products, this option was not further pursued.

## Conclusions

The results clearly demonstrate that the problems during extractive workup of whole-cell biotransformations can be overcome by microbial degradation of emulsifying agents. Furthermore, this novel approach offers a great advantage, since de-emulsification succeeds with both fungi and bacteria using the same set of enzymes. Also, competing enzymatic hydrolysis of the ester function has been negligible thus far. As can be seen from Figure 3, compared to current methodology the preparative effort is substantially reduced.

Microbial treatment of cell-free media is therefore a highly powerful workup technique for whole-cell biotransformations. Phase separations are complete in a short length time that is unequalled, and they allow continuous extraction techniques, e.g., in mixer−settler units. The process is cost effective and easily viable, for which reason it appears as the method of choice for the majority of applications.

## Experimental Section

**General.** All chemicals and organic solvents were purchased from Acros (Geel, Belgium), Aldrich (St. Louis, MO), and Wacker (Burghausen, Germany). Sucrose was obtained from a local store. Microorganisms were supplied by Julich

Chiral Solutions (Jülich, Germany) and the Collection Holdings. Medium ingredients were purchased from Merck (Darmstadt, Germany). Product identities were confirmed by $^1$H NMR analysis.

**Instruments.** $^1$H NMR spectra were recorded in $CDCl_3$ with a Bruker DRX-500 spectrometer at 500 MHz. Fermentations were performed in a 15-L Infors HT ISF200 fermenter. pH values were determined using a Mettler Toledo pH 320. Centrifugations were done using a Sorvall RC 5C Plus. All reactions were monitored by HPLC. HPLC analyses were done with a LiChrospher 100 RP-18 column on a Knauer Wellchrom system. GC analyses were conducted using a Perichrom GC ST200. The absolute configurations of ethyl (1*R*,2*S*)-2-hydroxycyclopentanoate (**3**) and ethyl (*S*)-3-hydroxybutanoate (**8**) were determined by the use of a Perkin-Elmer polarimeter 341 ($c$ = 1.0, $CHCl_3$) and by comparisons of the obtained results with reference data by refs 25 and 26 Optical measurements of phase separation were conducted on an Amersham Pharmacia Biotech Ultrospec 2100pro UV−vis spectrophotometer.

**Preparation of Cells for Biotransformation.** *Saccharomyces cerevisiae* L13 is the product of Societé industrielle de levure FALA, Strasbourg, France. Fresh cells were obtained directly from the local subsidiary in Kesselsdorf, Germany. To a fermenter containing tap water (10 L) were added *Saccharomyces cerevisiae* L13 (1250 g) and sucrose (1000 g). The aerated culture (2.0 L/L·min) was stirred for 30 min at 30 °C and 100 rpm, while pH was kept at 6.6.

*Lactobacillus kefiri* DSM 20587 was cultivated on MRS medium: casein peptone (10 g/L), sodium acetate trihydrate (5 g/L), meat extract (10 g/L), $K_2HPO_4$ (2 g/L), yeast extract (5 g/L), glucose (20 g/L), Tween 80 (1 g/L), diammonium hydrogen citrate (2 g/L), $MgSO_4$ (0.1 g/L), $MnSO_4 \cdot H_2O$ (0.05 g/L) at a starting pH of 6.6 in 10-L cultures. After 4 days of culture at 30 °C on a reciprocal shaker set to 150 rpm, cells were combined and stored at −20 °C. Cells (1000 g) were thawed and washed in 0.9% NaCl solution, and glucose (255 g) was added to a fermenter containing tap water (10 L). The aerated culture (2.0 L/L·min) was stirred for 30 min at 30 °C and 100 rpm, while pH was kept at 6.6

**Whole-Cell Biotransformations of Ethyl Acetoacetate with *Saccharomyces cerevisiae* and *Lactobacillus kefiri.*** After incubating for 30 min at 30 °C, the aerated culture (2.0 L/L·min) was fed ethyl acetoacetate (**1**) (100.0 mL, 103.0 g, 0.7915 mol) continuously for 20 h, while the pH was kept at 6.6. The reaction was monitored by GC analysis. After 24 h the biomass was removed by centrifugation at 3000*g* for 20 min (4 °C).

**Determination of Phase Separation Time ($t_p$).** Phase separation was determined in a 1-cm cuvette at $\lambda$ = 640 nm in a UV/vis spectrophotometer. Phase separation was complete when absorbance remained constant ($dA/dt = 0$). The $t_p$ values were obtained by triplicate determination in good conformity.

**Microbial Cleavage of Bioemulsifier in 1.0 L of Cell-Free Medium.** The de-emulsifying microorganism was grown at 30 °C in the exponential growth phase, e.g., on a reciprocal shaker set to 150 rpm, until optical density at 600 nm ($OD_{600}$) reached 0.8. *E. coli* TG1 was grown accordingly at 37 °C. Under these conditions no spores or capsules were formed. The de-emulsifier culture (1.6 L) was centrifuged at 3000*g*, and the pellet was added to freshly centrifuged (3000*g*) reaction medium (1.0 L) at 30 °C or 37 °C and pH 6.6. Frozen microorganisms (−80 °C) were also used for de-emulsification. The aerated suspension was incubated for 48 h at 100 rpm according to Tables 1 and 2. Micro-aerobic conditions were realized by closing the vessels by screw caps to inhibit diffusion of oxygen. *tert*-Butyl methyl ether (400 mL) was added, and after vigorous mixing for 60 s phase separation time ($t_p$) was determined photometrically. $t_p$'s > 10 min were not further determined. Each experiment was conducted three times.

**Microbial Cleavage of Bioemulsifier in 1.0 L of Technical Enzyme Preparations.** The procedure described above was applied accordingly for reaction medium (1.0 L) containing a technical enzyme preparation.

**Product Extraction after Microbial Treatment.** After microbial treatment of cell-free reaction medium (1.0 L) as described above, products were isolated by threefold extraction with *tert*-butyl methyl ether (400 mL). Volatile components were removed at $T$ = 50 °C by distillation under reduced pressure.

**Determination of Product Extractability.** Hydroxy esters **8**−**10** (20.0 g) were added to a culture (1.0 L) of *Saccharomyces cerevisiae* and *Lactobacillus kefiri*, respectively. The broth was worked up as described above. Measuring product extractability was done by determining the ratio of used and recovered material.

**Product Stability in the Presence of Microorganisms as Bioemulsifier Cleaving Agents.** In order to allow rational liquid chromatography analysis, the microorganisms (as indicated in Tables 1 and 2) and 0.5 mL of ethyl 2-oxocyclopentanoate (**3**) were added to 50 mL of 0.5 M potassium phosphate buffer, ph 6.6, at 30 or 37 °C, according to the method described above. After an incubation time ($t_i$) and aerobicity (as recorded in Tables 1 and 2) at 30 °C or 37 °C on a reciprocal shaker set to 100 rpm for 48 h, the ratio of cyclopentanone (**12**)/ethyl 2-oxocyclopentanoate (**3**) was determined by isocratic HPLC with water/$CH_3OH$ = 50:50 at $\lambda$ = 280 nm. $\beta$-keto ester **3** and consecutive hydrolysis product **12** were observed at retention times of 5.5 and 4.1 min, respectively. The percent conversions were determined using an integrator. Product identity was confirmed by $^1$H NMR.

**Effects of Microbial Lysis of Bioemulsifier on Reoxidation of Product and Product Stereopurity.** The tested microorganisms as indicated in Tables 1 and 2 and 0.5 mL of the chiral alcohols **8** and **10** were added to 50 mL of 0.5 M potassium phosphate buffer, pH 6.6, at 30 or 37 °C according to the method described above. After an incubation time ($t_i$) as indicated in Tables 1 and 2 at 30 °C or 37 °C on a reciprocal shaker set to 150 rpm for 48 h, product identity

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was confirmed by $^1$H NMR, and reoxidation of product and stereoisomer distribution were determined by GC. The chiral alcohol was converted into the respective trifluoroacetate by reaction with 1.2 mol trifluoroacetic acid anhydride in dry $CH_2Cl_2$ at 65 °C. After reaction was complete, volatile components were evaporated. For chiral GC analysis the trifluoroacetates of the respective $NaBH_4$-reduced ketones had been used to find the suitable conditions. GC analysis of the resulting trifluoroacetates was conducted with $N_2$ gas at 130 kPa; the temperatures of the injector and the detector were 240 and 250 °C, respectively. The relative amounts were determined using an integrator.

**Ethyl (*S*)-3-hydroxybutanoate (8):** J & W Scientific DB-5 column (30 m, 0.25 mm i.d.), 50 °C followed by a ramp-up to 85 °C for 2 min at a rate of 5 K/min. The product was observed at 5.0 min. Macherey & Nagel Lipodex E column (50 m, 0.25 mm i.d.), 80 °C followed by a ramp-up to 180 °C for 3 min at a rate of 10 K/min. The (*R*)-enantiomer was observed at 7.4 min, the (*S*)-enantiomer, at 7.6 min.

**Ethyl (1*R*,2*S*)-2-hydroxycyclopentanoate (10):** J & W Scientific DB-5 column (30 m, 0.25 mm i.d.), 50 to 115 °C at 5 K/min. Retention time of substrate **1** was 10.8 min. Products ***cis*-2** and ***trans*-2** were observed at respective retention times of 10.3 and 11.3 min. Macherey & Nagel Lipodex E column (50 m, 0.25 mm i.d.). The (1*S*,2*R*)-enantiomer was observed at a retention time of 17.3 min, the (1*R*,2*S*)-carbinol at 17.6 min, the (1*R*,2*R*)-diastereomer at 14.7 min, and the (1*S*,2*S*)-diastereomer at 15.3 min.

**Determination of Cell Vitality.** Cell vitality of yeasts was examined by the methylene blue method.[27] At each time point indicated, an aliquot of cells was taken from the reaction mixture and stained with 0.02% methylene blue. The numbers of stained and unstained cells were determined by microscopy. A minimum of 200 cells was counted for each measurement.

Cell vitality of bacteria was examined with 2,3,5-triphenyl tetrazolium chloride (TTC). An aliquot of cells was stained with 5 mM TTC for 5 min and centrifuged at 3000*g*.[28]

**Determination of Maximum Tolerance dose (MTD).** The maximum tolerated dose (MTD) was defined as the maximum dosage that did not result in cell death or a >5% reduced cell vitality compared to that in an untreated culture. Cell vitality was determined according to the methods described above.

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