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REVIEW

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Developments toward large-scale bacterial bioprocesses in the presence of bulk amounts of organic solvents

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Abstract Many pseudomonads and other bacteria can grow on aliphatic and aromatic hydrocarbons that occur in the environment. We are examining the potential of such organisms as biocatalysts for the oxidation of a variety of substituted aliphatic and aromatic compounds. To attain a high production rate of oxidation products via such biotransformations, we have focused on two-liquid phase culture systems. In these systems, cells are grown in liquid media consisting of an aqueous phase containing water-soluble growth substrates and droplets of a water-immiscible organic solvent containing bioconversion substrates and products. For industrial applications of such two-liquid phase processes, several questions remain. What are the maximum rates at which apolar compounds can be transferred from the apolar phase to cells growing in the aqueous phase, i.e., what are the maximum space-time yields attainable in two-liquid phase fermentations under practical conditions? What does an efficient downstream processing of two-liquid phase medium involve? What safety regimes should be considered in working with flammable organic solvents? Can elevated pressure be used to increase oxygen transfer? Based on answers to these questions, we have recently developed a high-pressure, explosion-proof bioreactor system with Bioengineering AG (Wald, Switzerland), which will be installed in our pilot plant and used to explore two-liquid phase bioconversions at a pilot scale.

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Introduction

Ongoing research activities are continuously revealing chemical reactions that are effectively catalyzed by microorganisms (Faber 1997). Of these bioconversions, the regio- and stereoselective oxidation of aliphatic and aromatic compounds represents an important class. Figure 1 shows several interesting biocatalytic systems studied in our laboratory that involve biotransformations of hydrophobic, water-immiscible organic compounds. Such biotransformations are all cofactor dependent (NADH, NADPH), and are often catalyzed by multicomponent enzyme systems, some of which are membrane proteins. Because of the complexity of these biocatalytic systems, whole-cell in vivo cultures are therefore usually favored over biotransformations using isolated enzymes.

Two-liquid phase bioprocesses in which bacteria are cultivated in media consisting of an aqueous solution and an organic, water-immiscible solvent present a valuable biotechnological tool for biotransformations of apolar compounds (Bosetti et al. 1992; de Smet et al. 1983; Furuhashi et al. 1986; Liu et al. 1996; Wubbolts et al. 1996). In such systems cells are grown in an aqueous medium containing water-soluble growth substrates, such as sugars and inorganic salts, while the hydrophobic oxidation substrate is dissolved in a second, apolar, organic solvent phase, typically amounting to 10%–50% of the total liquid volume. The cells form an active biocatalyst that transforms apolar substrate to products. Important is the fact that the product is not further converted or consumed by the cells. Hence the organic solvent, dispersed as fine droplets in the oil-in-water emulsion, often serves as an extractant for hydrophobic oxidation products (de Smet et al. 1983; Furuhashi et al. 1986; Kawakami and Nakahara 1994; Wubbolts et al. 1994a, 1996).

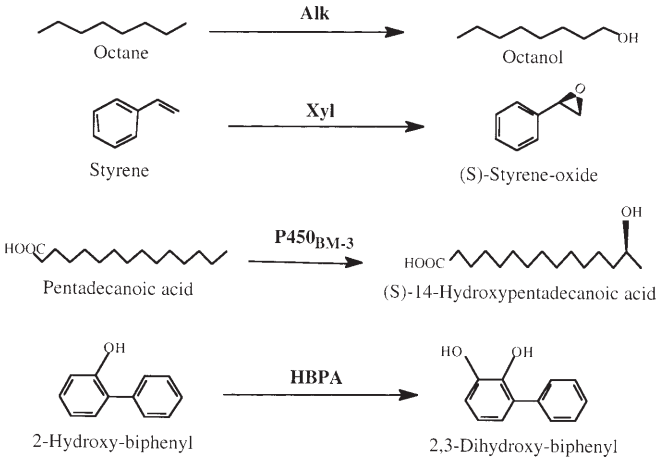


Fig. 1. Examples of regio- and stereoselective oxidation reactions of aliphatic and aromatic compounds performed by biocatalysts, from *top* to *battom*: terminal hydroxylations of medium chain length (mcl) alkanes catalyzed by the three-component alkane monooxygenase system (AlkB, AlkG, AlkT) (Bosetti et al. 1992; McKenna and Coon 1970; van Beilen et al. 1994b); epoxidations of styrene by the two-component xylene-monooxygenase system (XylM, XylA) (Harayama et al. 1989; Wubbolts et al. 1996; Wubbolts et al. 1994a); hydroxylation of long chain fatty acids in the ω-1, ω-2, and ω-3 positions by the single component cytochrome $P450_{BM-3}$ from *Bacillus megaterium* (Narhi et al. 1988); synthesis of substituted catechols from various phenol derivatives by the single-component hydroxy-biphenyl monooxygenase HBPA (Suske et al. 1997)

Thus far, the following significant achievements have been attained with such two-liquid phase bioprocesses. High cell densities, to $100\,g\,l^{-1}$ in fed-batch cultures (Hazenberg 1997) and to $20\,g\,l^{-1}$ in continuous cultures (Hazenberg 1997; Preusting et al. 1993) have been reached in bioreactors. Good enzyme activities, in the range of 10–$60\,U\,g_{CDW}^{-1}$, have been seen for these types of oxidation reactions at low cell densities (Favre Bulle and Witholt 1992; Preusting et al. 1991; Staijen 1996; van Beilen et al. 1994a; Wubbolts et al. 1994b). The first challenge is to maintain such activities as cell densities increase.

The volumetric productivities that have in fact been attained to date are in the range of 3–$10\,mmol\,l^{-1}\,h^{-1}$ (Favre Bulle et al. 1993; Hazenberg 1997; Wubbolts et al. 1996). We expect that ongoing developments in biocatalyst performance will permit specific productivities in the range of 10–$50\,U\,g_{CDW}^{-1}$ at cell densities of 20–$50\,g\,l^{-1}$, resulting in volumetric productivities in the range of 200–$1000\,U\,l^{-1}$. This level is equivalent to a space-time yield of 10–60 mmol of products formed per liter and hour. To utilize such activities, it is necessary to scale up fed-batch and continuous two-liquid phase bioprocesses. If this can be achieved, industrial processes with space-time yields of 10–$50\,tons\,m^{-3}\,yr^{-1}$ can be envisioned.

During the past few years we have considered whether productivities in this range in two-liquid phase bioprocesses are in fact attainable today and, if not, where the bottlenecks most likely lie (Kollmer 1997; Mathys 1997; Schmid 1997). We anticipated that the following could limit the large-scale development of two-liquid phase processes:

1. The maximum apolar substrate transfer rates from the organic phase to the cells could limit the volumetric bioconversion rates, thus negatively influencing the attainable space-time yield.
2. Downstream processing could present a major challenge because of the strong emulsification of organic solvents in the two-liquid phase culture medium.
3. The combination of aerobic operation and flammable solvents in two-liquid phase processes presents an explosion hazard. Unless means are found to operate two-liquid phase systems efficiently and safely, scale-up of such bioprocesses will not be feasible.

Regarding the first point, maximum apolar substrate transfer rates between 10 and at least $70\,mmol\,l^{-1}\,h^{-1}$ for the medium chain length alkanes heptane, octane, and decane have been determined (Schmid et al. 1998b). With respect to point 2, we have demonstrated recently the feasibility of efficiently isolating and purifying apolar products accumulated in the apolar phase of two-liquid phase *Pseudomonas oleovorans* cultures (Kollmer 1997; Mathys et al. 1998a,b). Point 3 we have examined in detail (Schmid 1997; Schmid et al., [in press c]), and we conclude it is possible to develop technical and operational systems to operate such bioprocesses safely under aerobic conditions in the presence of flammable compounds.

Mass transfer in two-liquid phase bioprocesses

In a multiphase system, mass and heat transfer processes provide the cells with nutrients and remove products and heat created by the whole-cell biocatalysts (Fig. 2). In two-liquid phase cultures, one of the phase barriers is the liquid–liquid interface between the organic solvent and the aqueous medium (Fig. 3a). Apolar substrates, dissolved in the organic phase, are transported from the solvent droplets to the cells (Fig. 3b) and apolar products are transferred from the cells to solvent droplets. An important question in this context is under which conditions will apolar substrate transfer from the organic phase to the cells limit productivities and how will maximal transfer rates relate to the biocatalytic potential of the cells. That is, if we succeed in designing a high-quality biocatalyst that remains very active at high cell densities, can we transfer substrate to the biocatalyst from the apolar phase across phase boundaries at a sufficiently high rate to take full advantage of the biocatalytic potential?

To answer this question, we determined octane mass transfer rates from the apolar phase to the cells in two-liquid phase *P. oleovorans* cultures, grown on octane as the sole carbon source (Schmid 1997; Schmid et al. [in press b]). In these cultures we monitored the rate at which carbon-containing metabolites accumulated in the cells, in the aqueous and organic medium and as CO_2 in the exhaust gas (Fig. 4). Thus, the rate at which metabolized carbon accumulated was a measure for the octane transfer rate from the solvent phase to the cells. We used this approach because it

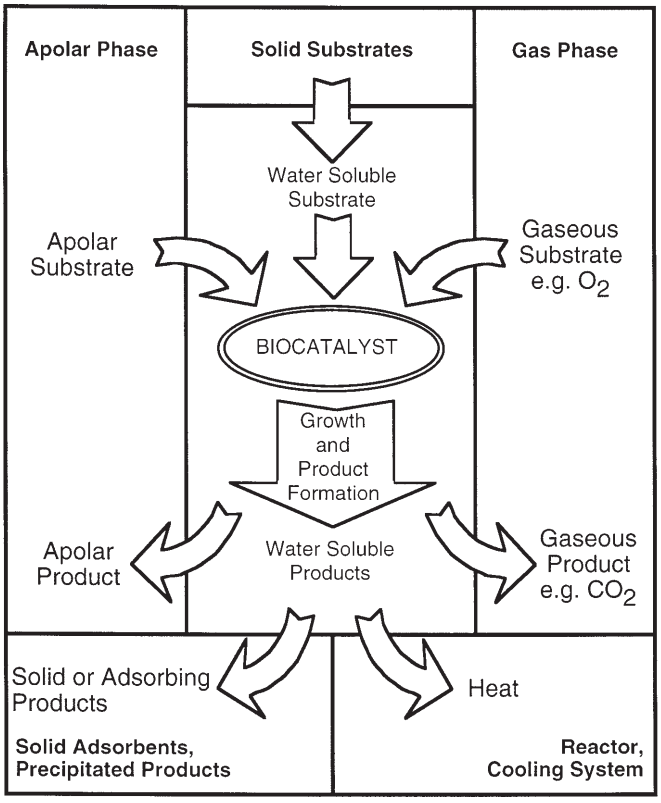
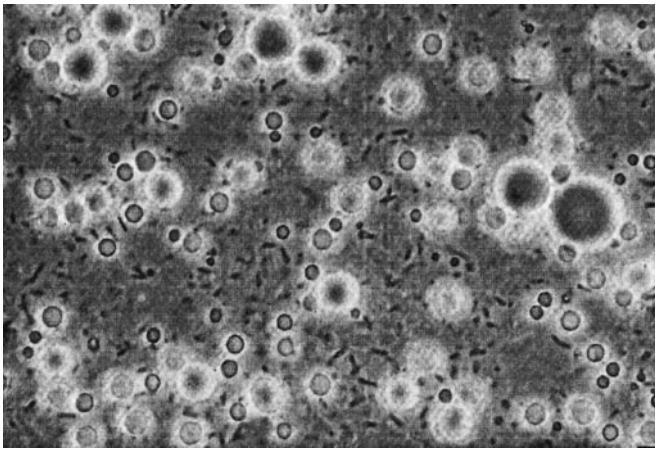


Fig. 2. Schematic diagram of substrate, product, and heat fluxes throughout a multiphase bioreaction medium. Gaseous, apolar, and solid substrates must cross phase boundaries to be taken up by cells. Likewise, products excreted by the cells, if they do not accumulate in the aqueous phase, are extracted into the organic solvent phase, stripped by the continuous flow of gas through the reactor, or adsorbed to a solid absorbent. Heat produced by the culture is transferred to the cooling system of the reactor. In a mass- or heat transfer-limited culture one of these fluxes, indicated by the *shaded arrows*, is rate limiting



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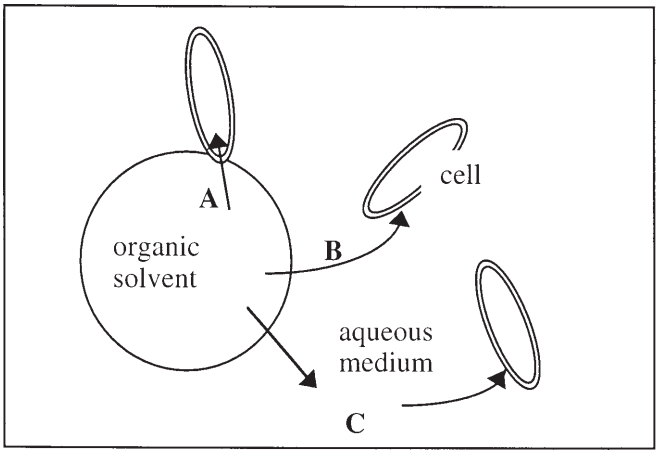


b

Fig. 3a,b. Interactions between cells and solvent droplets. **a** Photomicrograph of two-liquid phase medium. *Pseudomonas oleovorans* cells (*dark rods*) are suspended throughout the continuous aqueous phase. The organic solvent *n*-octane is dispersed as fine droplets, with an average diameter about 10 μm. The sample was taken from a two-liquid phase culture, stirred at 2500 rpm in a 3-l reactor, containing an organic phase volume fraction of 10% (v/v). **b** Schematic illustration of three possible mass transfer mechanisms of apolar substrates from the organic phase to the cells. *A*, direct droplet–cell interaction (Rosenberg and Rosenberg 1981); *B*, transfer mediated by organic carrier molecules (Goswami and Singh 1991); *C*, transfer via dissolution in the aqueous phase (Woodley et al. 1991)

is not possible to measure these transfer rates directly, and there are at present no models that permit reliable predictions. This method, although cumbersome, provided us with highly relevant information on the alkane transfer rates obtained under various operating conditions.

Table 1 shows that high transfer rates, within a range of 10–60 $mmol\,l^{-1}\,h^{-1}$, are attainable in systems stirred at high rates. Considerable rates are also reached at lower stirring speeds, when power inputs are lowered to values comparable to those attainable in industrial-scale reactors.

The results also showed that high mass transfer rates are attainable even when the apolar substrate is dissolved to low concentrations in nonmetabolizable carrier solvents, as is the case when octane is diluted to less than 3% in 1-hexadecene. This is an especially important finding, as many two-liquid phase processes operate at low substrate concentrations in the organic solvent to avoid inhibitory effects (Collins et al. 1995; de Smet et al. 1983; Kawakami and Nakahara 1994; Schmid et al. [in press b]; Tsai et al. 1996; Wubbolts et al. 1994a, 1996).

These results indicate that, so long as optimal biocatalyst activities can be maintained at elevated cell densities in two-liquid phase bioprocesses, high volumetric production rates will also actually be attainable, at least for some apolar compounds, because the apolar substrate mass transfer rates across phase boundaries will not limit the overall productivity of the process. This result is very welcome because it means that high biotransformation rates are feasible in two-liquid phase systems, where the cells grow on a water-soluble carbon source (e.g., glucose or glycerol) to maintain biocatalytic activity and an apolar substrate is oxidized to the desired product, but not consumed as a carbon or energy source. This situation applies especially to recombinant biocatalysts in which a host (generally *E. coli* strains in our laboratory) produces a specific monooxygenase, usually obtained from *Pseudomonas* strains or other natural isolates (see Fig. 1). The *E. coli* host serves only to produce and maintain the recombinant biocatalytic activity and to regen-

Table 1. Maximal alkane transfer rates in two-liquid phase fed-batch and continuous cultures of *Pseudomonas oleovorans*

Agitation	Organic phase	Organic phase volume fraction (%, v/v)	Maximal alkane transfer rate ($mmol\,l^{-1}\,h^{-1}$)
High power, $40\,kW\,m^{-3}$	Heptane, octane	20	≥60
	Decane	20	18
	Undecane, dodecane	20	<20[a]
	3% (v/v) octane in carrier solvent	12	≥30
Low power, $1–4\,kW\,m^{-3}$	Heptane–decane	20	14–≥45
	Undecane, dodecane	20	<15[a]

[a] Estimated based on data obtained from Lageveen (1986).
Source: Schmid et al. [in press b].

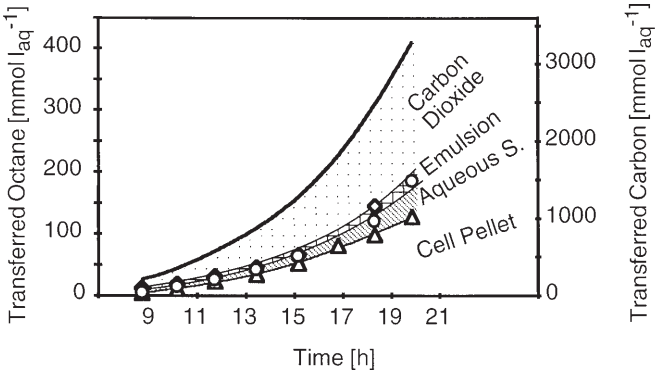


Fig. 4. Transfer of octane/carbon in two-liquid phase fed-batch cultivations of *P. oleovorans*, grown on *n*-octane as sole carbon source to a cell density of $30\,g\,l^{-1}$. Water-soluble substrates and oxygen were fed in excess. To determine the amount of octane that had been transferred from the organic liquid phase to the cells, the metabolized carbon accumulated in the different phases was measured and cumulated. ▩, Δ, octane/carbon in cells (cell pellet); ▨, octane/carbon in the aqueous supernatant; ○, cumulated octane/carbon in the cells and the aqueous supernatant; ⊞, octane/carbon in the emulsified organic phase; ◇, cumulated octane/carbon in the cells and in the organic and aqueous supernatant; ⁘, octane/carbon in the gaseous phase in the form of carbon dioxide; (*solid line* top curve), total cumulated octane/carbon in the cells, in the organic and aqueous supernatant, and in the gaseous phase. (From Schmid et al. [in press b], with permission)

erate the required cofactor, generally NADH or NADPH, while neither substrate nor product is consumed by the host strain. More work is needed to determine whether specific substrate–product combinations can in fact be transported across phase boundaries as effectively as octane and shorter *n*-alkanes.

Downstream processing of two-liquid phase media

During two-liquid phase cultivations, large amounts of surface-active material are set free by microorganisms such as *Pseudomonas oleovorans* (Witholt et al. 1990). These surfactants are mainly composed of lipopolysaccharides believed to be excreted by intact, growing cells and amphipolar membrane components set free by cell lysis (de Smet 1982; Schmid et al. [in press b]). At typical concentrations, $2–3\,g\,l^{-1}$, they lead to a strong emulsification of the two-liquid phase medium (Schmid et al. [in press a]). As a consequence, organic solvent droplets are stabilized and hardly coalesce, even after prolonged sedimentation or centrifugation (Mathys et al. [in press b]). Thermal treatment, heating (Mathys 1997) or cooling (Fig. 5), is an effective procedure to break the emulsion and to obtain a clear, continuous organic liquid, which can be further processed.

An alternative approach developed by Kollmer (Kollmer 1997) involves the continuous removal of the organic phase. A continuous-plate centrifuge is built into a bioreactor that continuously removes the emulsified organic phase, together with surfactant and small quantities of the aqueous phase (Fig. 6). Because this is done continuously from the start of a cultivation, no high concentrations of surfactant accumulate and the emulsion can easily be broken by mild mechanical treatment(e.g., by leading it through a coalescence filter). The organic effluent from the centrifuge then separates much more easily into two separate liquid phases, of which the organic phase can be recycled to the reactor or processed further for product isolation and purification.

We have further demonstrated in a model process the feasibility of purifying a bioconversion product, 1-octanol, from an organic solvent mixture containing a more volatile starting compound, *n*-octane. Distillation proved to be more effective than pervaporation, mainly because of insufficient selectivity and flux values obtained with pervaporation membranes commercially available today (Mathys et al. 1997). A two-step distillation process yielded a product purity exceeding 98% (v/v) (Fig. 7) (Mathys et al. 1998a).

A fully integrated bioconversion-downstream processing system for the specific oxidation of alkanes by recombinant *E. coli* containing the Alk-oxidation system has been designed and its performance modeled to determine the effect of different unit operations on product cost (Mathys 1997;

Mathys et al., in manuscript). This study showed that total costs for biochemically produced medium chain length alkanols would be of the order of US$10 kg^{-1}, which compares well to the present cost of highly purified alkanols. The largest contribution to the total production costs, 40%, is from the medium components required for cell growth (Fig. 8). Investment costs for equipment were calculated to be 20%–25% of the total production costs, when depreciated linearly within 12 years to 15% of the capital costs.

Explosion hazard in two-liquid phase bioprocesses

Recently we set out to build a pilot-scale, two-liquid phase, pressure reactor system in close collaboration with Bioengineering AG (Wald, Switzerland), with the aim of actually producing interesting biotransformation products on a kilogram scale. Our goal is to develop a system that enables two-liquid phase cultures to be operated efficiently, maximizing oxygen transfer by elevated pressure to optimize productivities of oxidation reactions. The system consists of a 16-l pressure reactor, operational up to 16 bar, and various feed tanks (Fig. 9).

One important aspect that had to be considered very seriously during the development of the pilot-scale reactor

Fig. 5. Effects of treating an octane-water emulsion by settling, autoclaving at 121°C/2 bar, or freezing at −20°C. The emulsion was obtained from a two-liquid phase *P. oleovorans* culture grown on *n*-octane. After the two-liquid phase culture medium was allowed to settle for a few hours, cells and the bulk of the aqueous phase separated to the bottom of the bottle. The emulsified organic phase, which accumulated at the top, was separated and treated as described. The emulsion contained the organic solvent and surfactants stabilizing the solvent droplets in an oil-in-water emulsion. The water content was in the range 10%–20% (v/v) (Mathys et al. 1998b)

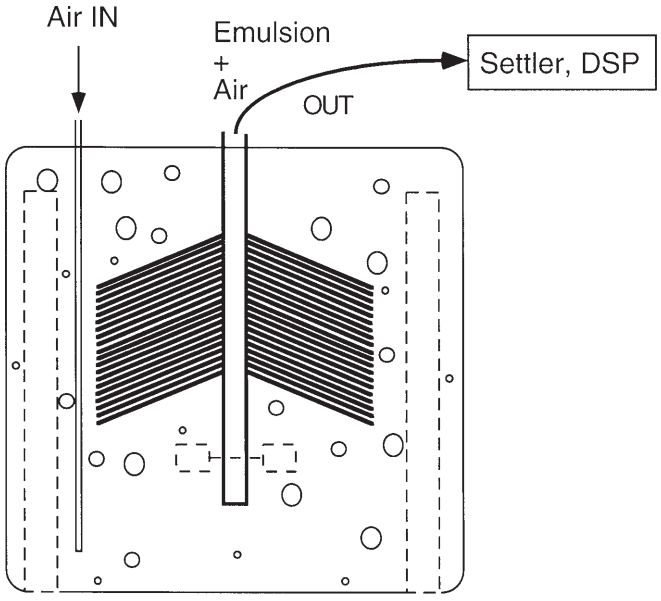
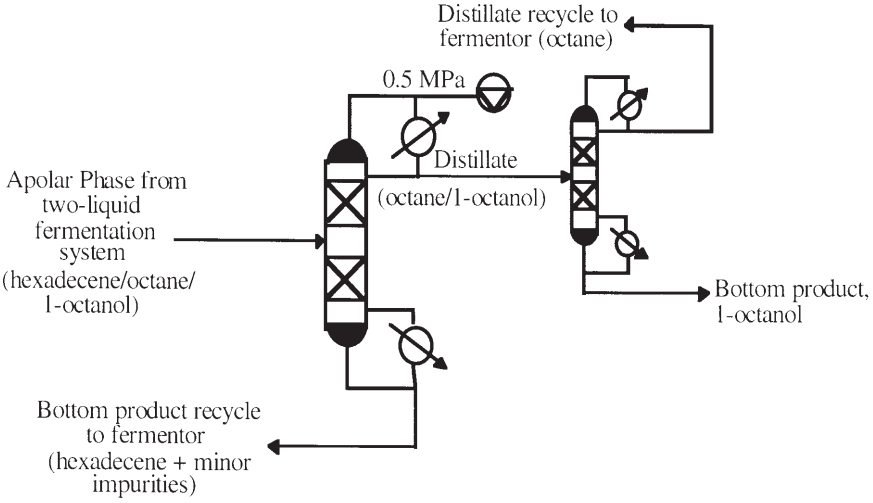


Fig. 6. Schematic diagram of a two-liquid phase bioreactor with an integrated plate centrifuge. A centrifugal force is exerted upon the two-liquid phase medium present between the plates of the centrifuge. The light gaseous and organic solvent phases thus accumulate in the center of the centrifuge and are continuously removed via the hollow axis from the reactor. The heavier aqueous phase and cells are continuously expelled radially out from the rotating plates. Additional impeller blades and baffles (indicated with *dashed lines*) can be added to improve mixing. *DSP*, down stream processing. (From Kollmer 1997, with permission)

Fig. 7. Flow diagram of a two-step distillation system for the continuous separation of 1-octanol from a solvent mixture of 1-hexadecene/octane/octanol. In the first distillation column the volatile octane (substrate of biotransformation) and octanol (product of biotransformation) are separated from the high-boiling 1-hexadecene (carrier solvent) and impurities. The more volatile substrate is distilled off in the second step, and octanol is recovered as the bottom product. Purities exceeding 98% 1-octanol were achieved. (From Mathys et al. 1998a)



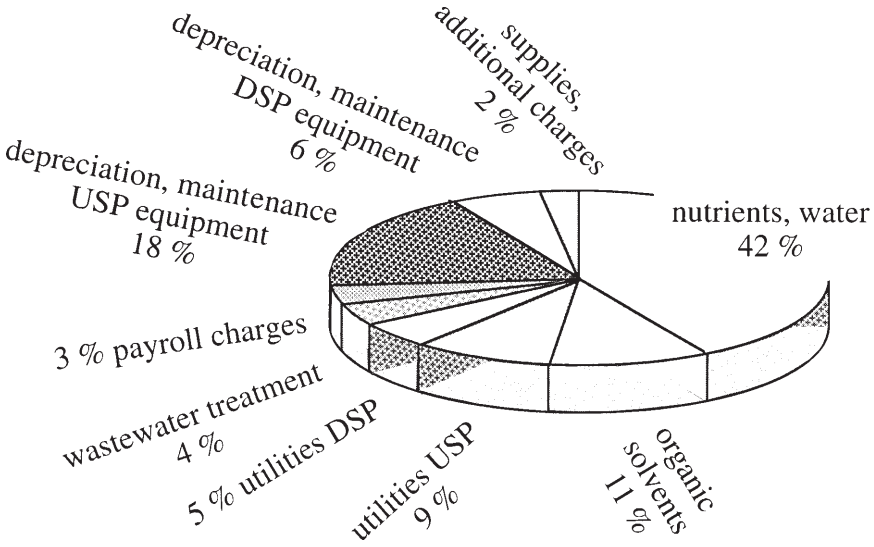


Fig. 8. Breakdown of costs for the production of 1-octanol in a two-liquid phase bioprocess. Costs were calculated based on a model fed-batch process for an annual production of 10 000 tons of 1-octanol by a recombinant whole-cell catalyst, using *n*-octane as starting material. *USP*, up stream processing. (From Mathys 1997; Mathys et al., in manuscript)

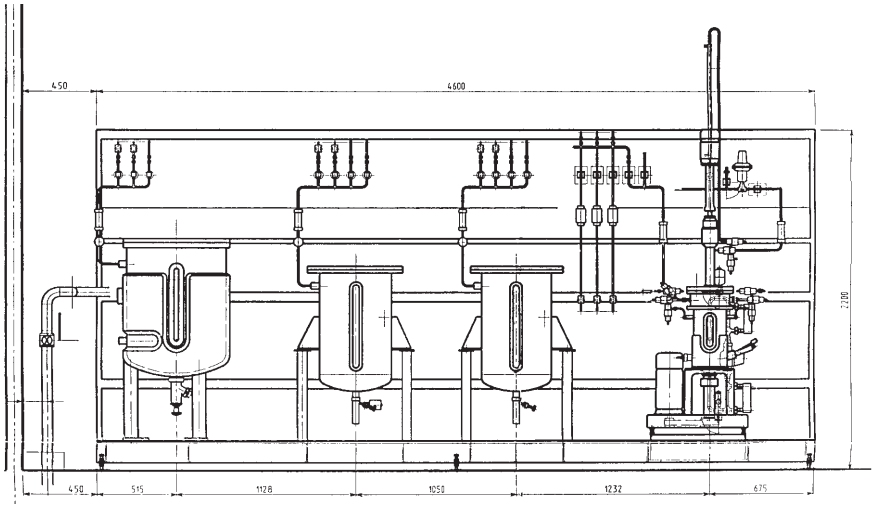


Fig. 9. Layout scheme of the pilot-scale pressure reactor built by Bioengineering AG, Wald, Switzerland. *From left to right*: 250-l harvest tank, two 100-l organic solvent tanks, and the 16-l pressure reactor, with a maximum operating pressure of 16 bar. Not visible: 50-l feed tank, two 30-l acid and base tanks, and a 5-l tank for antifoam. All vessels and connecting pipes are made of stainless steel 316 L. Medium feeds are provided via membrane-piston pumps (pompe cucchi, Italy), operational to a counterpressure of 20 bar. Shutoff valves (Burocco/Inoxvalvole, Italy) directly in line with the reactor also withstand pressures up to 20 bar. Distances indicated in the figure are in millimeters. (From Schmid 1997; Schmid et al., [in press c])

system concerned safety. In aerobic bioprocesses, air is sparged into the liquid phase of the stirred tank bioreactor. As a result, a mixture of gaseous oxygen, nitrogen, carbon dioxide, and vapors as well as liquids in the form of mist and foam accumulate in the reactor head space. In two-liquid phase processes, where volatile organic solvents are present as a second liquid phase, the organic vapor content can be significant and can lead to the formation of a dangerous explosive atmosphere within the gaseous head space of the reactor unless appropriate safety precautions are taken (Schmid 1997) (Fig. 10).

Technical precautions to maintain safe operating conditions have been evaluated in detail (Schmid 1997; Schmid et al., [in press c]). First and foremost, a reactor system operated with flammable solvents must be set up and operated within a designated and appropriately built explosion proof zone (Ex-zone), and standard precautions common to chemical plants must be followed. Additional safety measures are necessary because of the nature of aerobic bioprocesses, with the intentional coexistence of oxygen and flammable compounds within the reactor.

One possible way to cope with the potential explosion danger is an explosion-proof construction of the reactor. Because the pilot reactor is resistant to pressures up to 16 bar, it is capable of containing the maximum pressure generated by an explosion of air-solvent mixtures, when operated at atmospheric pressure, thus protecting personnel from accidental explosion risks.

Alternatively, special operating conditions can be used to avoid the formation of an explosive atmosphere within the reactor.

By operating above a minimal critical pressure and below a critical temperature, which both depend on the volatility of the flammable solvent, an explosive atmosphere is avoided (Kollmer and Rudolf von Rohr 1997).

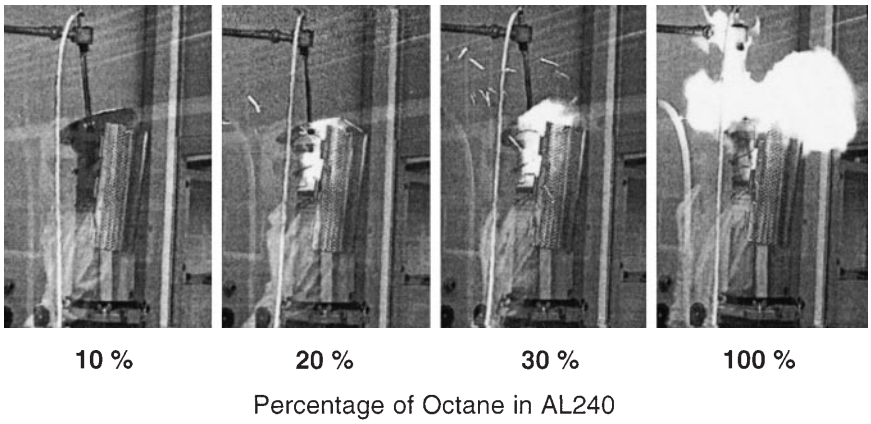


Fig. 10. Effects of igniting the reactor head space above the solvent/water two-liquid phase medium. The explosions, ignited with an electric spark of 4 J, were followed by video recording. The pictures represent moments when maximal flame expansion was observed, between 0.06 and 1 s after ignition. The reactor was operated with 90% water and 10% of the organic solvent consisting of the indicated amounts of flammable octane dissolved in the high-boiling hydrocarbon solvent AL240. The stirring speed was 2000 rpm and the aeration rate 1.7 vvm. (From Schmid 1997; Schmid et al., in manuscript)

Table 2. Safety regimes defined by operating limits given by pressure, temperature, and oxygen concentration in the exhaust gas to allow safe operation

Safety regime[a]	Pressure (bar)	Temperature (°C)	Oxygen concentration in exhaust gas (%, v/v)
Inert substrate[a]	No restrictions	No restrictions	No restrictions
Atmospheric pressure[b]	Max: 1.5	No restrictions	Max: 21
High pressure[c]	Min: $p_{crit.}$	Max: $T_{crit.}$	Max: 21
Oxygen[d]	No restrictions	No restrictions	Max: 6

[a] Inert substrate safety regime: no flammable compounds are used.
[b] Atmospheric pressure safety regime: the reactor is operated near atmospheric pressure and the explosion shock-resistant construction of the reactor acts as a constructional safety measure.
[c] High-pressure safety regime: the process is run above a minimal operating pressure and below a critical temperature, to avoid critical concentrations of the flammable solvent within the reactor head space and exhaust gas. Because of the reduced minimum ignition energy, oxygen concentrations greater than 21% should be used with caution.
[d] Oxygen safety regime: the oxygen concentration in the reactor head space and exhaust gas is maintained below an oxygen limit concentration of 6% (v/v), inertializing the gas phase in the head space and exhaust lines of the reactor.
Source: Schmid 1997; Schmid et al., [in press c].

By controlling the oxygen concentration in the inlet gas, the oxygen concentration in the reactor head space and exhaust gas can be maintained below a safe concentration of 6%–8%, thus avoiding an explosive atmosphere (ESCIS 1992). Limitations of the oxygen transfer rates, likely to occur because of the reduced oxygen concentrations, can be compensated by increasing the operating pressure within the pressure reactor.

While a major explosion occurs upon ignition when the gaseous head space is saturated with the vapor of a two-liquid phase mixture that contains pure octane as organic solvent, these effects are significantly lowered when octane is dissolved in a long-chain, nonvolatile hydrocarbon solvent (Schmid et al., [in press c]). Thus, using inert carrier solvents into which flammable organic solvents are dissolved presents a useful tool for maintaining safety (Fig. 10).

Based on these operational constraints, a total of four different operating windows were defined, which are being implemented in the automated process control system of the pressure reactor. By maintaining the conditions inside the reactor within these operating windows, termed safety regimes, safe operation can be ensured (Table 2). Based on our studies we conclude that potential explosion hazards in two-liquid phase bioprocesses can be handled so as to reliably avoid endangering personnel and equipment.

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