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Extractive Bioconversions with Emphasis on Solvent Production

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Introduction

In comparison with conventional chemical processes, biotechnological processes are characterized by being performed in aqueous solutions at low temperatures and by giving diluted product streams. If biotechnology is to compete with traditional technology in producing bulk chemicals, process intensification has to take place. This can be done by such means as increasing the catalyst density in the reactor, by continuously removing the product formed, by creating and maintaining optimal operational conditions for the catalysts, etc.

This review addresses the subject of product removal from the reactor. Such a step efficiently avoids product inhibition and the catalyst is forced to produce more. The term extractive bioconversion is used here in the broad sense, as technological membrane applications are also included. The examples discussed come mainly from the field of solvent production, but some examples from production of special biochemicals will also be given.

The review will deal with the reasons why product removal is a desirable process characteristic and, in conjunction with the presentation of some examples, will also discuss various methods of achieving these goals.

Product inhibition

This is a classic situation in biochemistry. In the reaction

$$A + B \rightarrow C + D$$

catalysed by the enzyme E, the products C or D, or both, inhibit the

Abbreviations: D_E, distribution coefficient for ethanol; D_P, distribution coefficient for product; D_w, distribution coefficient for water; IPMLD, intraperitoneal minimum lethal dose; PEG, poly(ethylene glycol); S, separation factor (D_P/D_w).

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enzymatic activity. A build-up of the concentration of C and/or D will reduce product formation until it ultimately ceases. Reactions following this pattern are those catalysed by, for example, isomerases and hydrolases.

A classic example is the hydrolysis of benzylpenicillin to form 6-aminopenicillanic acid and phenyl acetate. The proton formed changes the pH to such an extent that the reaction is completely inhibited (Lagerlöv *et al.*, 1976).

The reaction catalysed by glucose isomerase is another good example where it is important to try to operate at as favourable an equilibrium constant as possible (Baker, Bowes and Somers, 1980).

PRODUCT INHIBITION DUE TO TOXIC EFFECTS ON THE METABOLIC PATHWAY

Production of solvents normally involves the formation of substances causing general inhibition phenomena. When continuous processes with extractive removal of the product are being developed, the inhibitory effects of by-products may begin to increase in importance.

In an earlier study on extractive fermentation in aqueous two-phase systems, Kühn (1980) showed that the formation of glycerol may cause problems in ethanol fermentations. This is because the volatile ethanol is removed by intermittent distillation whereas the glycerol formed accumulates in the remaining broth. A step to remove the glycerol must be introduced if a continuous process is to be set up. This is only one example of a manœuvre that has to be taken into consideration when dealing with new process technology.

The effects of the solvents produced by organisms are not fully elucidated. Non-specific influences on membrane permeability have been discussed and implicated as a major cause of the observed product inhibition. Bazua and Wilke (1977) have demonstrated a non-competitive inhibition of ethanol on the glucose-to-ethanol pathway.

Organic acids such as acetic acid and butyric acid have been shown to influence performance markedly during the fermentation process. Thus, in the case of *Saccharomyces cerevisiae*, acetic acid was found to interfere with the membrane transport of phosphate (Maiorella, Blanch and Wilke, 1983). Production of acetone and butanol by *Clostridium acetobutylicum* is another example of a product-inhibited fermentative process. Of the products formed during this heterofermentative process, acetate and butyrate are by far the most potent inhibitors (Costa and Moreira, 1983), even if the solvents cause changes to the membrane fluidity and thereby modify the mechanisms for transport of substrate into the cell (Moreira, Ulmer and Linden, 1981).

Morphological changes in *Saccharomyces cerevisiae* have been shown to appear in the presence of 1-propanol and 2-methyl-1-butanol (Maiorella, Blanch and Wilke, 1983). The inhibited yeast is pseudomycelial (long and rod-shaped) as if the cells, when budding, have lost their ability to pinch off the new cell as an individual entity. This morphological change resembles that reported in immobilized yeast cells (Jirky, Turkova and Krumphanzl, 1980).

Feedback inhibition in the classic sense means that the end-product of a

sequence of catalytic steps has the ability to control the activity of one key enzyme at the beginning of the metabolic pathway leading to the production of the substance itself. Kominek (1975a,b) demonstrated the possibility of continuously removing cycloheximide produced in a fermentation with *Streptomyces griseus*, thereby substantially improving productivity.

MORE GENERAL TOXIC EFFECTS

A well-known example of the general influence on cells during cultivation is demonstrated by *Clostridium tetani* in which an extracellular enzyme is produced by the cells. The enzyme attacks the organism itself and at high concentrations causes cell lysis. Puziss and Hedén (1965) indicated that extractive bioconversion could be used when growing *C. tetani*. In an aqueous two-phase system the cells partitioned to the bottom phase while the toxin was more evenly distributed, as judged from the partition constant, between the small cell-rich phase and the larger extraction phase. In this case a 100-fold increase in productivity was reported.

The phenomenon of suicidal extracellular proteins seems to appear in other *Clostridium* species. Recently it was reported that complications appeared during continuous culture of *C. acetobutylicum* because of the liberation of inhibitory enzymes, so-called autolysins, when the solvent content exceeded a certain level (Goma *et al.*, 1984; Soucaille *et al.*, 1984). Moreover, in this case there is a marked demand for an efficient process technology if continuous highly productive processes are to be set up.

PRODUCTION OF 'SURFACTIVE' MATERIAL

An area of growing importance in biotechnology is the microbial production of surface-active components (Frautz, Lang and Wagner, 1984; Wagner *et al.*, 1984). These substances generally affect the cells and must be removed continuously during cultivation. When dealing with continuous processes, even producers of very low levels of surface-active by-products may ultimately create problems if the surfactant is not removed.

Steps to protect a labile product from the fermentation environment

The environment in a fermenter is characterized by high shear forces, the presence of proteases and, in many cases, an oxidative capacity by the air distributed through the broth. Such an environment may be harmful to many of the products of microbial origin. Extracellular peptides and proteins may be attacked by proteolytic enzymes. In dense cell populations this may be a severe problem because a steady state is quite rapidly established between formed and degraded products. To be able to capitalize on high cell densities a new process technology must be adopted.

Shear forces are harmful to many proteins and conformational changes may occur; high oxygen tension may be expected to have similar effects. In these cases also it may be advantageous to insert an extractive step in the process.

INTEGRATION OF BIOCHEMICAL SEPARATION AND PURIFICATION STEPS IN FERMENTATION PROCESSES

Together with experience of extractive fermentations with processes characterized by product inhibition, an awareness has grown of the need to integrate the fermentative stage with those of downstream processing. The point is to optimize the whole process instead of optimizing individual steps in production, as was earlier the case. A closer integration in terms of combining the fermentation with the first step in the purification scheme has started. The incentive for this to develop has been the need to protect the product from a tough environment and the need to achieve an initial enrichment and partial purification of the product.

Besides all these positive effects, some unpredictable factors can be anticipated. The build-up of concentrations of non-extractable or non-volatile by-products or non-fermentative substrate components will slowly change the character of the medium so that quite different production conditions may be present after operations over extended periods of time, for example there may be increases in glycerol, ions, non-degradable materials, etc. The physiological role of such changes has been discussed and in some but not all cases may be advantageous (Mattiasson and Hahn-Hägerdal, 1982; Maiorella, Blanch and Wilke, 1983). To compensate for this build-up of unwanted products, a bleed procedure must be introduced into the process scheme. This can be arranged in many different ways, but remains outside the scope of this chapter.

In the following sections certain other technologies used when creating extractive bioconversions are described. The choice of which method should be used in each case must be individual; hence the examples described here may fulfil a demand in a specific application. The general criteria for a separation step, when creating an integrated fermentation–separation process, are:

Selectivity;
Low or no toxicity;
Low operational costs;
Asepticity;
High capacity.

Bioconversions in aqueous–organic solvent phase systems

There are several advantages in extracting a fermentation broth or an enzymatic reaction mixture with an organic solvent. As mentioned previously, it is possible to overcome some inhibitory effects of desired end-products from fermentations or other bioconversions by transferring these products to an organic solvent; in some cases this has resulted in high productivities in the processes studied. Products dissolved in the organic solvent can easily be upgraded with conventional downstream processing techniques, such as distillation or ultrafiltration, especially if the product has high specificity to

the solvent. Another possible way to concentrate charged organic molecules is a second extraction from the solvent into a strong base or acid.

The recycling of process water in the extractive process has potential advantages in reducing both waste-treatment costs and the input of fresh water (Murphy, Blanch and Wilke, 1982). Another benefit includes the recycling of medium components such as nutrients and carbon sources. Water recycling has been successfully applied to ethanol fermentation using batch processes (Wall *et al.*, 1981).

In the realization of an extractive bioconversion, certain criteria must be met for the desired extractant (Lilly, 1982; Wang, 1983). These are listed in *Table 1*. When using the organic solvent to dissolve hydrophobic reactants, solubility must be high (Lilly, 1982).

Table 1. Critera for selection of the correct extractant

1. Immiscible with the aqueous solution.
2. High partition coefficient for the product.
3. Non-toxic to the biocatalyst.
4. Product can easily be separated from the extractant.
5. Cheap and available in large quantities.
6. Can be sterilized.
7. Inability to form stable emulsions with the fermentation broth.
8. Non-flammable.
9. Non-toxic to personnel.

Solvent extraction has been suggested as an effective method of maintaining a low product concentration in fermentation processes. As mentioned above, a high distribution coefficient for the product, D_p, is needed, otherwise the required solvent flows will be too large to handle and a practical process will be uneconomical. D_p is defined as the ratio of the molarity of the product in the solvent to that in the aqueous phase at equilibrium, i.e. $D_p = C_{p,s}/C_{p,w}$ where $C_{p,s}$ and $C_{p,w}$ are molarity of product in the organic and aqueous phases, respectively.

The separation factor, S, which is the ratio of D_p to D_w (the distribution coefficient for water, $C_{w,s}/C_{w,w}$ where $C_{w,s}$ and $C_{w,w}$ are molarity of water in the organic and aqueous phases, respectively), should be as high as possible to achieve a high separation of the product from water. Production of azeotropic ethanol from a 1M ethanol fermentation broth requires a separation factor greater than 350 (Murphy, Blanch and Wilke, 1982). Properties of different organic solvents as extractants for ethanol are presented in *Table 2* (Murphy, Blanch and Wilke, 1982). This corroborates an observation by Roddy (1981), that the relative capacities for extraction of ethanol from water mixtures were roughly: hydrocarbon < ether < ketone < amine < ester < alcohol.

Table 2. Properties of extraction solvents for ethanol at 25°C (EtOH = 1–2M) (after Murphy, Blanch and Wilke, 1982)

	Distribution coefficient (D_E)*	Separation factor (S)†
Alkanes	0·005–0·008	up to 300
Aromatics	0·02–0·1	90–120
Esters and ketones	0·1–0·3	10–20
Alkyl phosphates	up to 0·7	1–20
Chlorinated hydrocarbons	0·1–0·15	75–170
Higher alcohols	0·2–0·8	10–40

* $D_E = C_{E,S}/C_{E,W}$
† $S = D_E/D_W$ (*see* text)

Matsumura and Märkl (1984a) have investigated the ability of 25 organic solvents to extract ethanol. The solvents were selected from several classes of organic compounds including alcohols, esters and amines. 2-Ethyl-1-butanol gave the highest distribution coefficient and selectivity ratio ($D_E = 0{\cdot}83$; $S = 103{\cdot}8$); *sec*-octanol ($D_E = 0{\cdot}60$; $S = 75$) and tri-*n*-butyl phosphate ($D_E = 0{\cdot}79$; $S = 29{\cdot}3$) also gave fairly good values.

To improve the extraction efficiency further, addition of inextractable material to the water phase will increase the extractability of ethanol. This can be explained by the reduced water activity which decreases the solubility of ethanol in the aqueous phase. Thus Tedder *et al.* (1984) added glucose to the broth before extraction and then recycled the extraction raffinate to the fermenter to utilize the sugar. Similar observations were made by Murphy, Blanch and Wilke (1982) who noted an increase in the partition constant of 1·5 when 1 M KCl was added to the water phase. Similar effects are also observed in extractive bioconversions in aqueous two-phase systems (Mattiasson, 1983c).

The fact that many organic solvents are toxic to micro-organisms or denature enzymes limits the number of solvents that can be used in extractive bioconversions. Unfortunately, organic solvents with a high capacity and high separation factors are often the most toxic (Murphy, Blanch and Wilke, 1982; Roffler, Blanch and Wilke, 1984). This was also found by Matsumura and Märkl (1984a). The effect of 10 solvents with a fairly high D_E on the growth of typical ethanol-producing micro-organisms is shown in *Table 3*.

Playne and Smith (1983) examined the toxicity, to a commercial mixed culture of facultative anaerobic acid-producing bacteria, of 30 organic chemicals which could be used to extract volatile fatty acids. Thirteen chemicals were non-toxic and included the paraffins (C_6–C_{12}), phthalates, organophosphorus compounds, Freon 113, Aliquat 336 (tricaprylyl methyl ammonium chloride), di-isoamylether and trioctylamine. Other amine extractants were

partially toxic. Alcohols (C_5–C_{12}), ketones (C_5–C_8), benzene derivatives, isoamylacetate and di-isopropyl ether were toxic.

Datta (1981) found that no toxicity was observed to anaerobic bacteria at saturation levels of toluene, diesel and isoamylacetate in water, and that triisooctylphosphine oxide or Alamine 336 (similar to trioctylamine) dissolved in these solvents did not affect toxicity.

For *Lactobacillus delbrueckii*, a lactic-acid-producing bacterium, solvent toxicity increased in the order: alkane < cumene < ketone < tertiary amine < secondary amine < quaternary amine (Roffler, Blanch and Wilke, 1984). The extractive capacity and toxicity is shown in *Table 4*.

A new technique to overcome the problem with extractives has been presented by Matsumura and Märkl (1984b). Extraction of ethanol was performed through a permeable membrane. The membrane used was a hollow-fibre cuprous ammonium rayon which was found to be impermeable to 2-ethyl-1-butanol, *sec*-octanol and tri-*n*-butylphosphate. The yeast cells were co-immobilized with Porapac Q (Waters Associates Inc.), which acted as a barrier to any toxic solvent molecules that passed through the membrane. Matsumura and Märkl called this technique perstraction. In a continuous ethanol fermentation, using tri-*n*-butylphosphate as the extractant, coupled with the perstractive process, high-glucose syrups (containing 34–47% glucose) at 1·9 and 2·6 M were successfully fermented without ethanol inhbition.

Some examples of extractive bioconversions using organic solvents as extractants are given below.

In an early study, Finn (1966) investigated the possibility of reducing product inhibition when producing prodigiosin with a strain of *Serratia*. The extractant chosen was kerosene (a paraffin). No improvement in production could be demonstrated.

Schultz and Gerhart (1969) and Kominek (1975b) studied extraction of cycloheximide (an antifungal antibiotic) from a fermentation medium. First, a dialysis membrane was used to separate the cells from the broth; then the dialysate was extracted using methylene chloride. Residual organic solvent was removed by bubbling sterile air through the extracted dialysate before mixing back to the fermenter. The product yield could be doubled by means of this on-line extraction method.

Minier and Goma (1981, 1982) described a new technique using a plug-flow reactor and liquid–liquid extraction to achieve continuous extractive fermentation of ethanol. Porous pieces of brick were used to adsorb yeast cells. The fermentation broth was pulsed into a stream of dodecanol to increase the interfacial area between the broth and the extractant. With this technique it was possible to complete the fermentation of 409 g/ℓ glucose and to improve the productivity by a factor of four compared with a conventional control. The dodecanol feed rate was 1 ℓ/h compared with the medium feed rate of 0·057 ℓ/h. This shows that the required solvent flow would be too great to handle in a full-scale process unless further improvements were introduced.

Production of liquid alkane fuels from biomass has been proposed (Levy, Sanderson and Wise, 1981). The process includes three steps: mixed culture anaerobic fermentation, liquid–liquid extraction and electrolytic oxidation.

Table 3. Effects of solvents on growth of ethanol-producing micro-organisms. Solvent concentration, 0·1% (v/v) (after Matsumara and Märkl, 1984a)

Solvent	Ethanol distribution coefficient	Micro-organism				
		Schizo-saccharomyces pombe IFO 0344	*Saccharomyces cerevisiae* IFO 0309	*Candida brassicae* IFO 1664	*Saccharomyces uvarum* ATCC 26602	*Zymomonas mobilis* IFO 13766
Methyl crotonate	0·54	++	++	++	++	++
tri-*n*-Butyl phosphate	0·79	—	—	+	—	+
2-Ethyl-1-butanol	0·83	+	+	+	+	+
n-Octanol	0·59	—	—	—	—	—
sec-Octanol	0·60	—	—	—	—	—
2-Ethyl-1-hexanol	0·52	—	—	—	—	—
3-Phenyl-1-propanol	0·77	+	+	+	—	—
2-Ethyl-1,3-hexanediol	0·85	++	++	++	++	++
iso-Eugenol	0·52	—	—	+	—	—
Polypropylene glycol P-1200	0·58	++	+	+	+	+

++ indicates 80–100%
+ indicates 20–79%
— indicates 0–19%
} of the growth observed in solvent-free control.

Table 4. Lactic acid extraction and toxicity to *Lactobacillus delbrueckii* of several extraction solvents (after Roffler, Blanch and Wilke, 1984)

Solvent	Lactic acid distribution coefficient*	Solvent concentration in fermentation broth	Maximum specific growth rate (h^{-1})	Glucose conversion (%)
Control (no solvent)	—	—	0·58	100
n-Heptadecane	0·01	Saturation	0·55	100
Kerosene saturated with TOPO	0·6–0·8	10% of saturation	0·54	100
		Saturation	0·38	100
Tributyl phosphate	0·9	10% of saturation	0·65	100
		Saturation	Toxic	0
30% (w/w) TOPO in cumene	1·0–1·4	10% of saturation	0·62	100
		Saturation	Toxic	0
Aliquat 336 (quaternary ammonium chloride salt)	1·0–4·5†	10% of saturation	Toxic	0

* Ratio of lactic acid (g/ℓ) in solvent phase to lactic acid (g/ℓ) in aqueous phase
† 25% (w/w) Aliquat 336 in cyclo-octane at pH 5·5.

Butyric acid and longer organic acids are selectively extracted using kerosenes. In the second extraction the organic acids are transferred from the kerosene into an aqueous solution of sodium hydroxide. The base solution showed a tenfold increase in the concentration of the product compared with the concentration of higher acids in the fermenter liquid.

Bioconversions of substances with low solubility in water have been exploited previously only to a limited extent. Examples of such poorly soluble substances are steroids, fats and hydrocarbons. The introduction of organic solvents in extractive bioconversions may circumvent these problems. *Table 5* (Carrea, 1984) gives examples of bioconversions in biphasic systems including steroid transformations, hydrocarbon oxidation, methylester hydrolysis, iodine production, glucose conversion and tryptophan production. The area of bio-organic synthesis is at present being actively studied by biotechnologists.

As was indicated in the introduction, different reasons may lie behind the desire to set up an extractive fermentation. The examples discussed so far have dealt with the need to improve yields. In a recent report, Becker *et al.* (1984) have described the use of a lipophilic phase in plant-cell cultures in order to trap lipophilic substances produced and excreted by the cells. A fluid triglyceride was used in the organic phase in some experiments, and a solid phase (a Li-chroprep (Merck) which is a modified silica) was used in some others. Both systems operated well. The solid phase did not have as good partition constants as that of the triglyceride. The system using a solid sorbent, however, was much easier to handle and the subsequent separation of the product from the sorbent was easier to carry out than when triglyceride extraction was used. The advantage of these systems over the traditional ones was the improved productivity plus the increased stability of the product. In some cases it was possible, using this new technique, to recover products from cultures of cell lines that, under conventional conditions, were impossible to produce.

Table 6 summarizes the possible advantages and disadvantages of running extractive bioconversions with an organic solvent.

Extractive bioconversions in aqueous two-phase systems

In biotechnology, extraction with organic solvents is not generally applicable because of the toxicity to cells and the denaturing effects on enzymes. By-products and feed components may also cause problems by inhibiting the reaction. The use of extractive bioconversions exploiting aqueous two-phase systems offers alternatives that circumvent most of these constraints (Hahn-Hägerdal *et al.*, 1983; Mattiasson, 1983c). When aqueous solutions of two different polymers (such as polyethylene glycol and dextran) are mixed, the mixture becomes turbid and when it is left for a while, phase separation occurs. As the concentrations of each polymer are usually less than 10%, the phase system is characterized by containing 85–95% water. Aqueous two-phase systems therefore provide mild conditions for cells and proteins. The interfacial tension is very low compared with that of organic-solvent–

Table 5. Bioconversions in biphasic systems (after Carrea, 1984)

Catalyst	Bioconversion	Organic solvent	Reference
Fungal laccase	Formation of oestrogen oligomeres	Ethyl acetate	Lugaro *et al.*, 1973.
Hydroxysteroid dehydrogenase (free or immobilized)	Oxidation of hydroxyl groups (3β; 17β; 3; 12) of steroids	Ethyl acetate or butyl acetate	Cremonesi *et al.*, 1973, 1974; Ter Meulen and Annokkee, 1983; Carrea *et al.*, 1979, 1984.
20β-hydroxysteroid dehydrogenase (free or immobilized)	Reduction of 20-keto steroids	Ethyl acetate, butyl acetate or hexanoloctane	Cremonesi *et al.*, 1975; Ter Meulen and Annokkee, 1983; Carrea *et al.*, 1979; Hilhorst, Laane and Veeger, 1983; Dunnill and Lilly, 1979.
Nocardia (free or immobilized)	Oxidation of cholesterol	Carbon tetrachloride or toluene	Ter Meulen and Annokkee, 1983; Buckland, Dunnill and Lilly, 1975; Atrat, Hüller and Hörhold, 1980; Dunnill and Lilly, 1979.
Nocardia rhodocrous (free or immobilized)	1-Dehydrogenation oxidation of 3β- or 17β-hydroxysteroids	Benzene–heptane	Yamané *et al.*, 1979; Fukui *et al.*, 1980.
Pseudomonas oleovorans	Epoxidation of hydrocarbons	Cyclohexane or hexadecane	Schwartz and McCoy, 1977; De Smet *et al.*, 1983.
Rhodotorula minuta (immobilized)	Resolution of DL-menthol	Heptane	Omata *et al.*, 1981.
Peroxidases	Oxidation of iodide to iodine	Carbon tetrachloride, chloroform or xylene	Neidleman and Geigert, 1981.
Escherichia coli	Synthesis of tryptophan from indole and serine	Toluene, benzene or cyclohexane	Bang *et al.*, 1983.
Saccharomyces cerevisiae	Conversion of glucose to ethanol	Dodecanol	Minier and Goma, 1982.

Table 6. Advantages and disadvantages of running extractive bioconversions with an organic solvent

Advantages:	Decrease of product inhibition
	High catalytic density
	Continuous operation
	High substrate concentration in feed
	Reduction of waste treatment costs
	Reduction of fresh water input
	Recycling of nutrients
	Ease of product separation
	High solubility of reactants
Disadvantages:	Organic solvents may often be toxic
	Catalyst denaturation at liquid-liquid interphase
	By-product inhibition
	Feed component inhibition

water-phase systems, normally less than 0·1 dyne/cm (1 μN/cm) (Albertsson, 1971). This means that phase systems require only gentle mixing to form a finely dispersed emulsion with a large surface area and short diffusion distances from one phase to the other, i.e. conditions very favourable for mass transfer. For the two polymers used, a phase diagram can be drawn from experimental data (*Figure 1*). Point S represents a homogeneous solution and

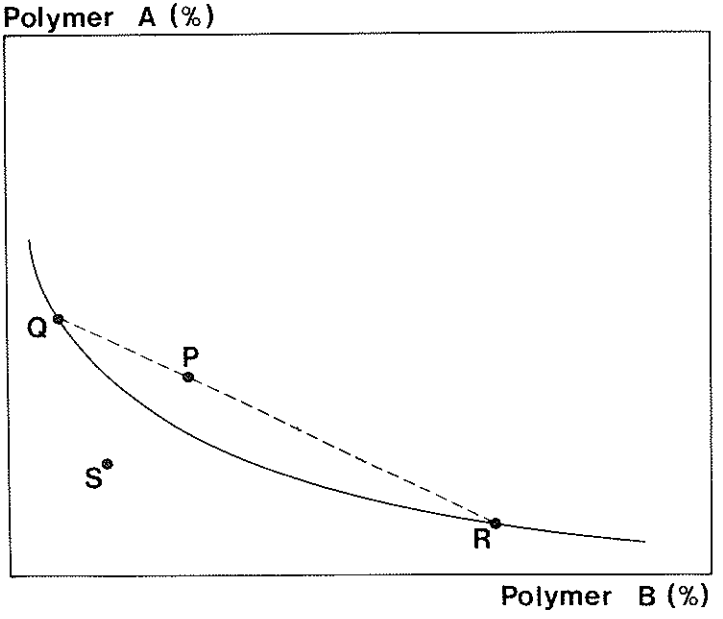

Figure 1. Mixtures of two water-soluble polymers A and B, represented by points above the binodial (curved line) (such as point P) give two liquid phases, whereas mixtures below the binodial (such as point S) result in a homogeneous solution. The line QR is called the tie-line and the points Q and R represent the compositions of the phases in a two-phase system with the total composition P.

point P denotes a two-phase system. Many polymers have been tested (Albertsson, 1971). Even if we deal with polymer solutions of varying hydrophobicity, all solutions are very close to water when hydrophobicities are compared with those of organic solvents. This fact is illustrated by the so-called hydrophobic ladder in *Figure 2*. A more general picture of the partitioning of products, enzymes and cells and particles is given in *Table 7*.

In one of the first studies of bioconversion in aqueous two-phase systems, Puziss and Héden (1965) investigated production of toxin from *Clostridium tetani*. The phase system consisted of 2% dextran (MW = 480 000) and 12% polyethylene glycol (EG) (MW = 4000). The ratio between upper and lower volumes was 15:1. The toxin produced had a partition coefficient near

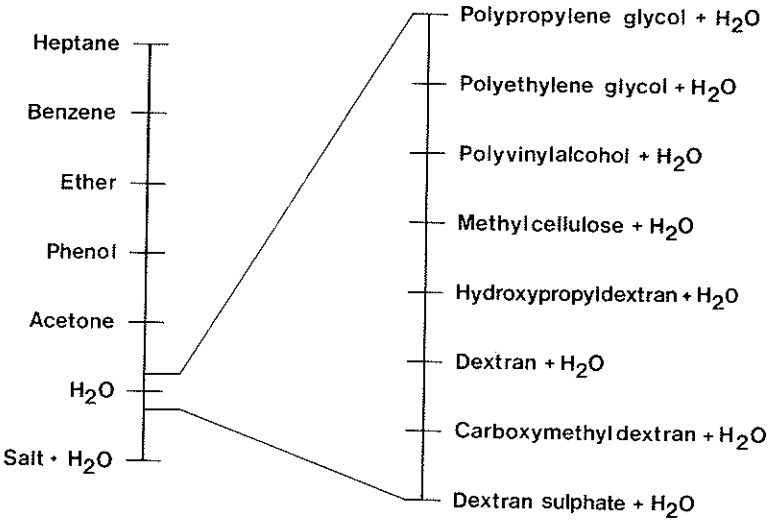

Figure 2. Hydrophobic ladder. To the left are some commonly used organic solvents listed in order of increasing hydrophobicity. Aqueous solutions of the polymers to the right are mutually immiscible and thus form phase systems. Because all the solutions consist mainly of water, all fall within a narrow part of the spectrum to the left (after Albertsson, 1971).

Table 7. Partition coefficient (K_{part}) in aqueous two-phase systems (after Mattiasson and Larsson, 1985)

	K_{part}
Small molecules, substrates, products	0·5–2
Proteins	0·01–100
Particles (cells etc.)	Extreme partitioning
Soluble separator molecules	0·001–1000 Normally 0·01–100
Separator particles	Extreme partitioning

unity and the cells were mainly partitioned to the lower phase. As a consequence of the volume ratios, the upper phase contained 15 times more toxin than the lower phase. Production increased from 10^7 mouse intraperitoneal minimum lethal doses (IPMLD)/ml to 10^9 IPMLD/ml. This corresponds to a 100-fold increase in productivity.

The fermentative production of acetone-butanol is, as mentioned earlier, a very good example of fermentation inhibited by product formation. Mattiasson *et al.* (1982) used a phase system consisting of 6% dextran and 25% PEG and found a slight decrease in product inhibition. The productivity of the extractive fermentation was the same as that for a conventional batch process. It was also found that the composition of the phase system is very important for the partitioning of the product (*Table 8*).

Andersson, Mattiasson and Hahn-Hägerdal (1984) studied the conversion of benzylpenicillin to 6-aminopenicillanic acid using penicillin acylase in an aqueous two-phase system. The system consisted of 8·9% PEG 20000 and 7·6% potassium phosphate. The enzyme was partitioned to the bottom phase

Table 8. Effects of variations in the phase composition on the partition behaviour of ethanol, acetone and butanol (after Mattiasson, 1983c)

% (w/v)			$K_{part} = C_T/C_B$‡		
Dextran	PEG*	$V_T:V_B$†	Ethanol	Acetone	Butanol
3	15	8:1	1·2	1·3	1·6
3	17	9:1	1·3	1·5	1·9
3	20	12:1	1·2	1·5	1·9
4	15	7:1	1·2	1·3	1·6
6	10	3:1	1·5	1·0	1·3
6	15	4:1	1·3	1·5	1·8
6	25	6:1	1·9	1·9	2·0
Dextran	PVA*				
2	8	8:1	1·0	1·2	1·2
2·25	8	4:1	1·1	1·1	1·3
2·5	8	3:1	1·1	1·3	1·3
Dextran	Pluronic*				
4	14	5:1	1·4	1·4	1·5
6	14	4:1	1·3	1·4	1·4
Dextran	Ucon*				
4	14	4:1	1·3	1·1	1·4
4	15	5:1	1·1	1·3	1·5
5	12	3:1	1·2	1·3	1·3

* PEG = polyethylene glycol, PVA = polyvinyl alcohol. Pluronic and Ucon = copolymers of PEG and polypropylene glycol.
† V_T and V_B = volume of top and bottom phases.
‡ C_T and C_B = concentration of each product in top and bottom phases.

($K_{part} < 0{\cdot}01$). No improvement in productivity was observed, compared with that in an immobilized system.

Acetic acid has been produced by *Escherichia coli* in a phase system containing 6% PEG 8000 and 7·5% dextran (Mattiasson and Hahn-Hägerdal, 1983); *E. coli* produced about 50% more acetic acid than in a batch fermentation process.

Extractive bioconversions have also been used in ethanol fermentation using glucose (Kühn, 1980), cellulose (Hahn-Hägerdal, Mattiasson and Albertsson, 1981) and starch (Larsson and Mattiasson, 1984b). In *Table 8* it can be seen that it is possible to achieve partition constants for ethanol of 1·0–1·9 by carefully selecting the phase system.

The cellulose conversion showed a slight increase in productivity in the dextran–PEG phase system. In the case of glucose fermented to ethanol, repetitive batch fermentations were performed. The top phase was separated off and ethanol was liberated by distillation. The top phase was then returned to the phase system, and more glucose was added (*see Figure 3*). After this cycle had been repeated a few times, the fermentative capacity was almost totally inhibited by glycerol and other non-volatile products that were concentrated during the prolonged use of the fermentation system. The problem was solved by dialysing the broth and by adding more yeast cells.

A continuous process solving the problem of by-product inhibition has been developed (Larsson and Mattiasson, 1984b). The process is based on the integrated use of aqueous two-phase systems and membrane technology (*Figure 4*). The phase system used consisted of 5% PEG 20 000 and 3% crude

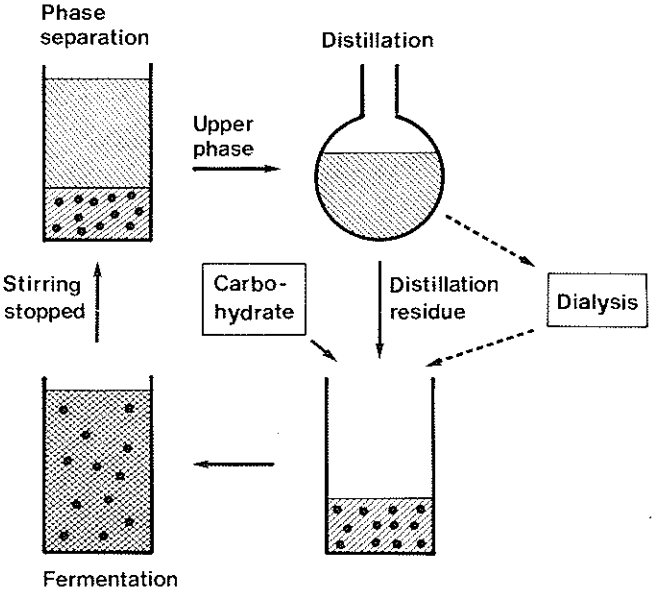

Figure 3. The fermentation–distillation–separation cycle (after Kühn, 1980).

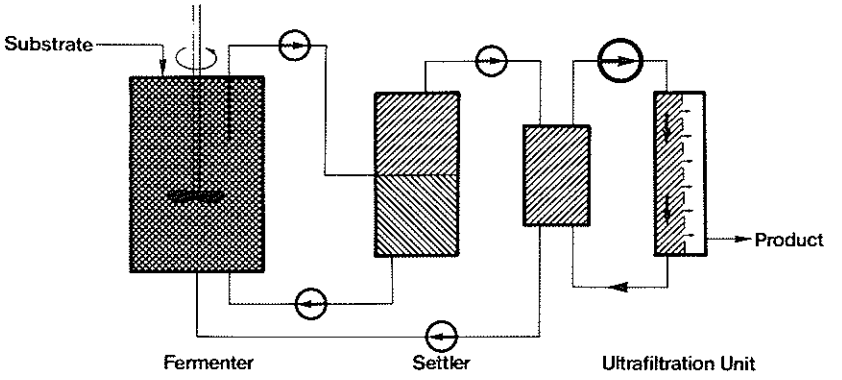

Figure 4. Experimental flow sheet for continuous production of ethanol using a aqueous two-phase system integrated with ultra-filtration.

dextran. Native starch was converted to glucose by amylases and simultaneously fermented to ethanol by yeast cells. Ethanol was extracted to the upper phase, which was continuously treated by cross-flow ultrafiltration. The upper-phase polymer (PEG 20000) was retained in the system and recirculated to the reactor. A clean ethanol–water filtrate without phase components was obtained. If the process were optimized by using an optimal phase system (*see Table 8*), it would be possible to concentrate the ethanol by a factor of 1·9 in the extraction step and by a factor of 1·4 in the membrane step. This would mean that an overall concentration by a factor of 2·6 would be attainable.

The advantages of extractive bioconversions in aqueous two-phase systems are the same as those of organic solvent extraction (*see Table 6*) but they are, in addition, biocompatible and in some instances they also stabilize enzymes in the solution. The disadvantages of by-product and feed-component inhibition are reduced to a minimum if ultrafiltration is used to recover phase components.

Extractive production using critical fluid carbon dioxide

The use of supercritical and near-critical fluids in extraction processes has been under development for two decades. (Supercritical fluids exist at pressures above the critical point—for CO_2, 7373 kPa (73·8 atm)—and at moderate temperatures. The critical density is about 40% of that of the normal liquid (de Filippi and Moses, 1982).) So far, there appear to be possible applications within the food industry (Kurzhals, 1982). Their biotechnological potential is still not clear. Supercritical carbon dioxide has gas-like transport and diffusivity characteristics but has solvent characteristics similar to those of an organic solvent (Kohn and Savage, 1979). The distribution coefficient for ethanol in water at saturation pressure (*ca.* 70 atm $\approx$ 7070 kPa) is 0·25 (expressed as mole ethanol fraction in the CO_2 phase

divided by the mole ethanol fraction in the aqueous phase), and the value for *sec*-butanol is about 2 (de Filippi and Moses, 1982).

There are few examples of supercritical extraction in fermentation processes. De Filippi and Moses (1982) extracted ethanol from corn fermentation broth using a pilot-plant unit. The conditions in the extractor were 27·2°C and 81·6 atm (8241·6 kPa) and the conditions in the still were 17·2°C and 51 atm (5151 kPa). Ethanol concentration in the feed was 9·7%. The solvent:feed ratios in two experiments were 10 and 12, respectively, and the feed rates were 0·14 ℓ/min and 0·27 ℓ/min. Ethanol concentration in the raffinate after extraction was approximately 4·5% and the maximum ethanol concentration after the still was 87%.

On-line adsorption

By using adsorbents such as activated carbon, zeolites and polymeric resins, it is possible to reduce product inhibition in a fermentation by continuous adsorption of the formed products. The adsorbent can be added directly to the fermentation broth, or can be applied in a way that avoids direct contact with the cells. When saturation of the adsorbent is complete it can be treated separately to liberate the adsorbed products.

Milestone and Bibby (1981) used silicalite, a zeolite analogue, to adsorb alcohols (C_2–C_5) from aqueous solutions. The alcohols were adsorbed from diluted solutions and were highly concentrated after a subsequent desorption step (*see Figure 5*). It was possible to concentrate ethanol from a 2% solution to 35% in one step. Adsorption of butanol from a 0·5% solution followed by drying the silicalite at 40°C and then heating it to 150°C resulted in a condensate containing 98% butanol.

In a preliminary investigation Maddox (1982) adsorbed butanol from a fermentation broth without any prior removal of cells. The silicalite used could adsorb butanol up to 8·5% of its own weight. Butanol/acetone fermentations using an adsorbent kept outside the fermenter have been investigated by Larsson, Holst and Mattiasson (1984). Butanol and acetone were continuously produced from an anaerobic fermentation using *Clostridium acetobutylicum*. The cells were separated from the broth by membrane filtration using a hollow-fibre unit. The cells were recycled to the fermenter and the solvent-rich permeate was passed over a column packed with adsorbent before being recycled to the fermenter. The experimental set-up is shown in *Figure 6*. The solid sorbent used was a commercial nitrated divinylbenzene–styrene copolymer. The capacity of the sorbent was found to be 82 mg butanol/g sorbent and 51 mg acetone/g sorbent when tested against an equilibrium concentration of 5 g/ℓ. A total amount of 400 g glucose was added to the 1·5 ℓ fermenter. Solvent adsorbed to the sorbent was liberated by treating the sorbent under reduced pressure. A total of 67 g solvent was liberated when heating 930 g polymer. During this fermentation it was possible to maintain the low level of products, thereby reducing inhibition.

Other adsorbents such as mordenite (a zeolite) have been tested for butanol adsorption (Larsson and Mattiasson, 1984a).

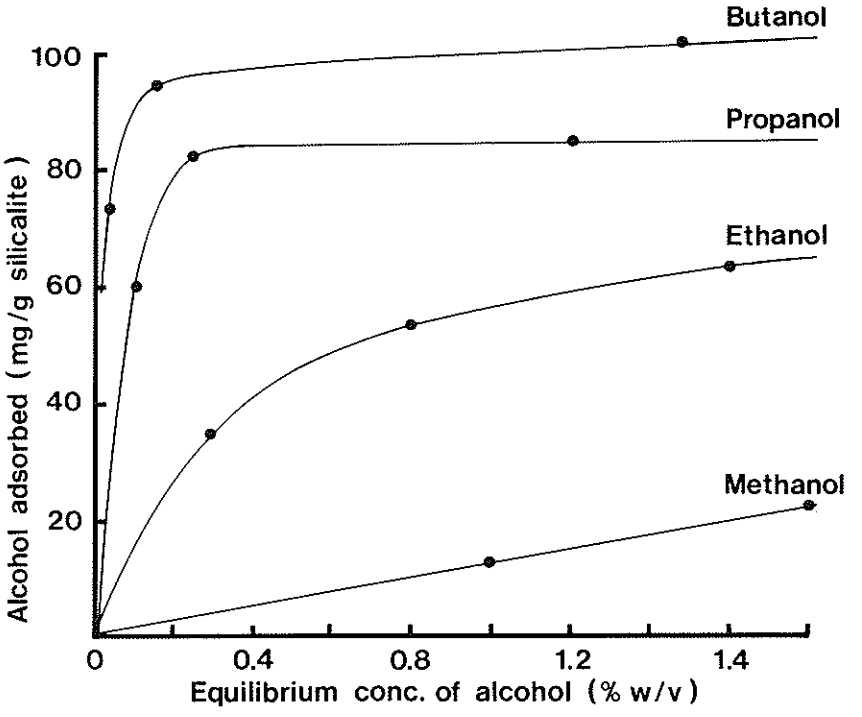

Figure 5. Equilibrium adsorption isotherms on silicalite for straight-chain alcohols from aqueous solution (at 20°C) (after Milestone and Billy, 1981).

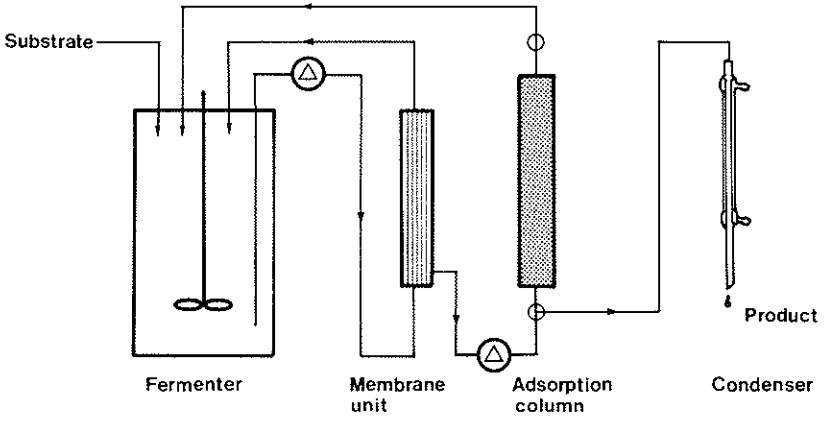

Figure 6. Flow-sheet for a semi-continuous adsorption process.

Ethanol adsorption on activated carbon has been reported in several studies. Direct addition of activated carbon to an ethanol fermentation has been attempted (Wang, Robinson and Lee, 1981). The ethanol concentration decreased but without the concomitant increase in productivity: the latter fact was ascribed to low yeast viability. In a similar experiment with the activated carbon outside the fermenter, the yeast cells were separated by centrifugation before the broth was brought into contact with the active carbon (Lee and Wang, 1982). After adsorption, the broth was returned to the fermentation and more glucose was added. The productivity using this technique was 25 g/ℓ/h.

Walsh *et al.* (1983) proposed an ethanol separation and purification system using stripping with CO_2 and adsorption of the ethanol from the gas stream with activated carbon. In a desorption step more than 95% ethanol could be recovered.

In a study investigating batch kinetics of glucose utilization by *Saccharomyces cerevisiae*, the use of three different hydrophobic adsorbents showed no improvement of productivity (Lencki, Robinson and Moo-Young, 1983). Using silicalite, XAD-4 or XAD-7, the ethanol concentration decreased in the broth but the cell growth was inhibited because of nutrient and cell adsorption to the resin.

Wang and co-workers obtained increased productivities when using a polymeric resin (XAD-4) in a fermentation with *Streptomyces griseus* to produce cycloheximide (Wang, Kominek and Jost, 1980; Wang, Connors and Robinson, 1982; Wang, 1983). If an amount of resin equivalent to 6% of the reactor volume was added directly to the broth, the productivity increased by a factor of two compared with a conventional fermentation. When a polymeric resin was kept behind an ultrafiltration membrane, productivity increased by a factor of 2·25.

The direct contact between the adsorbent and the fermentation broth creates certain problems. Adsorption of nutrients and/or cells has been observed in several cases. Furthermore, attachment of cells or cell fragments to the sorbent may interfere with subsequent desorption of the product. Adding cells to the adsorbent may also reduce the adsorptive capacity in repetitive steps.

The way to improve performance may be to develop better adsorbents or to avoid direct contact between the adsorbent and cells/cell fragments. Examples of this latter approach are membrane filtration (Larsson and Mattiasson, 1984a), centrifugation and cell recycling (Lee and Wang, 1982) and entrapping the sorbent in a more inert gel (H. Y. Wang, personal communication).

Table 9 lists some of the systems studied using adsorbents. This technique may grow in importance in biotechnology, especially when affinity adsorbents are introduced and the fields of application are broadened to other types of cell-catalysed reactions.

Table 9. Fermentation systems using solid sorbents for *in situ* product recovery

Micro-organism	Product	Sorbent	Productivity	Reference
Clostridium acetobutylicum	Butanol/acetone	Bonopore*	2·2†	Larsson, Holst and Mattiasson, 1984
Saccharomyces cerevisiae	Ethanol	Activated carbon	25 g/h	Lee and Wang, 1982
Streptomyces griseus	Cycloheximide	Amberlite XAD-4	2·25†	Wang, 1983
Monascus sp.	Red pigment	Amberlite XAD-7	1·5†‡	Evans and Wang, 1984
Pseudomonas aeruginosa	Salicylic acid	Amberlite IRA-400	5·5†	Tone, Kitai and Ozaki, 1968
Corynebacterium renale	Salicylic acid	Amberlite IRA-400	2†	Tangan and Ghose, 1981

* A nitrated divinylbenzene-styrene copolymer.
† Increased productivity compared with conventional batch fermentation.
‡ Extracellular production.

Vacuum fermentations

By conducting a bioconversion under reduced presure it is possible to distil off volatile products without heat denaturation of cells and/or enzymes. This results in low product concentrations in the broth and a corresponding reduction in product inhibition. Vacuum fermentations can be performed by two different techniques: either by keeping the fermentation under vacuum and letting the product boil off, or by maintaining the fermenter at normal pressure and circulating the broth into a vacuum chamber for distillation.

A problem with reducing the pressure of the entire fermentation broth is the low solubility of oxygen under these conditions. Alcoholic fermentation by *Saccharomyces cerevisiae* requires trace amounts of oxygen (Akbar, Richard and Moss, 1974; Cysewski and Wilke, 1977). Ramalingham and Finn (1977) added ergosterol to the growth medium to eliminate the oxygen requirement of the yeast. A pressure of 32 mmHg (≈ 4·26 kPa) was maintained in the fermenter, and the cells were recycled by adding the centrifuged overflow back to the fermenter. A 50% glucose feed was continuously fermented. The residence time was 19 h and the glucose utilized for ethanol production was 45·5%. From these figures, the ethanol productivity can be calculated as 12 g/ℓ/h.

Cysewski and Wilke (1977) were able to obtain ethanol production rates of 40 g/ℓ/h in continuous fermentations with *S. cerevisiae*. When cell recycle was used to increase the cell density in the reactor, a production rate of 82 g/ℓ/h was obtained. The accumulation of toxic non-volatile by-products was reduced by bleeding some of the medium from the fermenter. The ethanol concentration in the broth was held lower than 15 g/ℓ and the substrate level was 5–10 g/ℓ. Pure oxygen was added to retain cell viability. Feed containing

33·4% glucose was completely fermented and the ethanol concentration in the condenser was 160–200 g/ℓ.

The large amounts of carbon dioxide produced in ethanol fermentations make it expensive to keep the entire fermentation under vacuum conditions. By keeping the fermenter at atmospheric or slight over-pressure and recirculating the broth to a vacuum chamber where distillation can take place, it is possible to overcome these difficulties and at the same time solve the problem of low oxygen solubility.

This technique has been used in two studies for ethanol production, the first using *Zymomonas mobilis* (Lee *et al.*, 1981), where a productivity of 8·5 g ethanol/ℓ/h was achieved, and the second using *Candida acidothermophilum* (Ghose, Roychoudhury and Ghosh, 1984). In the latter study, cellulose was utilized as substrate, the broth being supplemented with cellulase and β-glucosidase. The enzymes produced dextrose which was simultaneously fermented to ethanol by the yeast. The process was semi-continuous and the ethanol level never exceeded 24 g/ℓ in the fermentation broth, thereby reducing ethanol inhibition of yeast cells and cellulolytic enzymes. The feed (pretreated rice straw) was added periodically and after 10 distillation cycles the medium in the fermenter was replaced by fresh medium to avoid by-product inhibition. The ethanol concentration in the condenser was 121 g/ℓ and the productivity 4·5 g/ℓ/h: 97·5% of the cellulose was utilized.

The major disadvantages of vacuum fermentation are by-product and feed-component inhibition. These problems can be solved by bleeding some of the broth but this loss of broth causes a decrease in overall ethanol productivity (Maiorella, Blanch and Wilke, 1983).

Membrane technology

Membrane separation processes have found wide applications in biotechnological laboratories and it can be foreseen that they will soon be applied to commercial production (Michaels, 1980), so that the bioprocess can operate at higher levels of productivity. There are several biotechnological fields in which membranes can be used:

1. To assist in the separation of solids from solutes, e.g. the separation of cells from a fermentation broth;
2. In ultrafiltration for the separation of small molecules from large ones;
3. In the confinement of biocatalysts to a membrane-surrounded volume;
4. To protect sensors controlling biotechnological processes;
5. The use of stereoselective membranes in certain applications has been discussed and some reports are also available.

The special value of membranes in the field of extractive bioconversion concerns (1) the separation of cells from the fermentation broth; (2) the entrapment of biocatalysts in spaces bounded by membranes; (3) the separation of large molecules from small ones.

SOLID–LIQUID SEPARATION

In many fermentation processes a separation step in which cells have been removed from the fermentation broth has involved filtration or membrane separation. In these applications, microfiltration as well as ultrafiltration membranes have been used. In the field of extractive bioconversions the removed cells are recycled to the fermenter whereas the broth is treated for purification of the product. By recycling the cell mass several advantages are achieved, including the very low requirement for new cell mass, and the ability of the system to operate under very high cell densities and thus to maintain high productivities. Because of the recovery of the cells in a membrane separation step, such a system may be operated at very high dilution rates without the risks of wash-out of the fermenter. Some published examples of ethanol fermentation using membrane technology are given in *Table 10*. From all these examples it is clear that the volumetric productivities are far superior to those of conventional batch systems and are at least equivalent to those reported for immobilized-cell systems.

A question that has to be addressed is whether exposure to the shear forces used is harmful to the cells. Two separate investigations on micro-organisms (*Zymomonas mobilis* and *Streptococcus lactis*) have failed to demonstrate a decrease in viability or growth rate or even leakage of intracellular proteins from the cells to the medium (Beyler, Rogers and Fiechter, 1984; O. Holst, L. Hansson, A.-C. Berg and B. Mattiasson, unpublished work). Cells susceptible to shear forces may not be cultivated in a membrane recycling reactor but merely in reactors, as discussed below.

CELLS CONFINED IN MEMBRANE REACTORS

There are essentially two ways of using a membrane unit in combination with fermentation. In the case of hollow-fibre units this means that either the cells are freely floating with the perfusing medium in the fibre lumen and passing out with the effluent, or they are captured behind the membrane on the shell side of the unit (the space between the fibres and the cover in a hollow-fibre unit). In this latter case a quite different situation is reached. If substrate is fed with the main stream in the lumen of the fibres, the substrate molecules must pass the membrane before reaching the cells. The cells, on the other hand, will grow in a protected environment close to the substrate pool and so colonize the outer surface of the membrane. This is especially so in cases of anisotropic membranes. In such systems it has been shown that the cells tend to grow in extremely dense populations and form more or less compact cell masses (Inloes *et al.*, 1983). *Table 11* lists some of the applications where this approach has been used.

All these applications of membrane technology have raised the question: how useful are the membranes? Is it economically possible to use such technology in large-scale processes?

Experience so far is rather limited with regard to long-term stability with microbial cells which grow relatively rapidly. More experience has been published on work with mammalian cells (Adamson *et al.*, 1983; Hopkinson,

Table 10. Ethanol fermentations using membrane technology for cell recycling

Micro-organism	Initial cell concn (g/ℓ)	Feed	Sugar concn (g/ℓ)	Ethanol concn (g/ℓ)	Productivity (g/ℓ/h)	Reference
Saccharomyces cerevisiae	14	Glucose	100	49	30	Mehaia and Cheryan, 1984a
	60	Glucose	100	49	100	
	100	Glucose	100	49	100	
Zymomonas mobilis	35	Glucose	100	45	120	Rogers, Lee and Tribe, 1980
	50	Glucose	140	70	120–200	
		Starch	100	48	60	Lee, Pagar and Rogers, 1983
Kluyveromyces fragilis	41	Lactose	50	24	26	Cheryan and Mehaia, 1983
	90	Lactose	150	71	75	
	90	Lactose	150(60% conversion)		240	

Table 11. Fermentations using cells captured on the shell side in hollow-fibre units

Micro-organism	Initial cell concn	Feed	Substrate concn (g/ℓ)	Product concn (g/ℓ)	Productivity (g/ℓ/h)	Reference
S. cerevisiae	10^7 cells/ml	Glucose	89	12 (ethanol)	26	Inloes *et al.*, 1983
Kluveromyces fragilis	82 g/ℓ	Lactose	150	48 (ethanol)	91	Mehaia and Cheryan, 1984b
Pseudomonas denitrificans		Nitrate		(nitrogen)		Mattiasson *et al.*, 1981

1983; Seaver *et al.*, 1984) and more recently with plant cells (Jose, Pedersen and Chin, 1983).

From the experiments using anisotropic membranes with the cells growing on the outer surface of the membrane, a common problem has been reported by several investigators: after a period of culture, cells tend to pass through the membrane and leak out with the effluent (Gan, 1963; Mattiasson *et al.*, 1981; Inloes *et al.*, 1983). This was first interpreted as resulting from imperfections in the membranes, but recent interpretations seem to agree that the pressure built up by the growing, extremely dense, cell population is high enough to rupture the thin membrane skin (it took about 2 days for both *Saccharomyces cerevisiae* (Inloes *et al.*, 1983) and *Pseudomonas denitrificans* (Mattiasson *et al.*, 1981) to penetrate). If this is so, then anisotropic membranes cannot be used with fast-growing cells unless special precautions are taken. A successful solution to this problem has not yet been reported. The closest has been a recommendation to use isotropic membranes with smooth surfaces so that the cells will not grow in such dense colonies. In this way the risks of membrane rupture are markedly decreased but, concomitantly, there is a marked impairment of the mass transport (Inloes *et al.*, 1983).

Membrane fouling can cause considerable problems. The build-up of a secondary layer can reduce flux through the membrane in dead end filtrations to such an extent that the membrane may only be used for a few minutes. However, the introduction of tangential flow filtration has permitted much better flow properties, although fouling is still a major problem.

SEPARATION OF SOLUBLE MOLECULES FROM EACH OTHER

The basic concept in membrane separation is that size difference will enable some molecules to pass through a membrane while others will be retained. The material of the membrane itself will of course impart certain characteristics such as charges and hydrophobicities, etc. to the membrane. It has been extremely difficult to obtain membranes of consistently high quality with regard to the nominal molecular weight cut-off in the filtration process. The separation processes used are ultrafiltration and reverse osmosis, and their main applications in biotechnology are in concentration of protein solutions. In the field of bioconversions there are some examples of membrane reactors for enzyme conversions (Drioli *et al.*, 1975; Bückmann *et al.*, 1981; Wichman *et al.*, 1981; Glomon *et al.*, 1982). Separation of product streams is very rare (*see also* pages 146–152).

A special version of membrane technology is termed pervaporation. Here a gas flow or vacuum is applied on one side of the membrane and the unit is brought into contact with a solution containing a substance to be isolated by extraction through the membrane. This technology may be considered as a combination of vacuum fermentation and membrane filtration; it may prove interesting to evaluate in combination with, for example, biotechnological solvent production.

Pervaporation based on a gas flow on the other side of the membrane has been used in analytical applications. The membrane gas-sensor devices developed for analysis of volatile compounds are based on this concept (Yano, Kobayashi and Shimizu, 1978; Mandenius and Mattiasson, 1983; Shioya, Takamatsu and Dairaku, 1983).

The pervaporation technique was tested recently on butanol and isopropanol fermentations using *Clostridium beyerinckii* (Groot, Van den Oever and Kossen, 1984; Groot *et al.*, 1984). The pervaporation membranes used consisted of silicon tubing and the transport gas was oxygen-free nitrogen. The experimental set-up was as shown in *Figure 7*. In a batch experiment it was demonstrated that product inhibition started later during the fermentation when pervaporation was used for product removal and, in a continuous process, a higher conversion of the substrate was observed when continuous removal of the product took place.

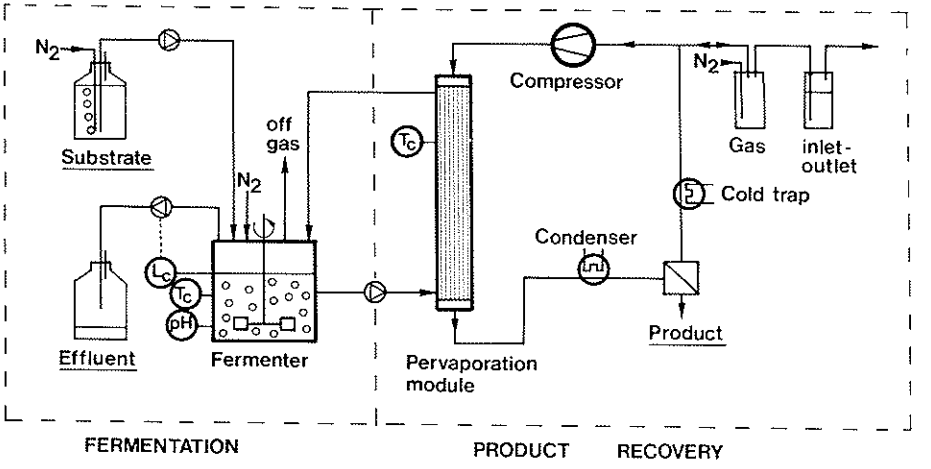

Figure 7. Experimental set-up of a fermentation using pervaporation for product recovery (after Groot *et al.*, 1984). T_c = temperature control; L_c = level control; off gas = gas leaving the fermenter.

Other combinations of extractive techniques are also possible. Recently Matsumura and Märkl (1984b) presented perstraction which is a combination of membrane technology and extraction with organic solvents (*see* pages 140–146).

Separation by gravity

A simple and inexpensive way of separation is to let the solid material settle and then to process the supernatant in one way and the sediment in another. A limiting factor, however, has been the low efficiency under normal gravity; the process is too slow. Several methods of improving the separation stage have been studied:

1. Use of larger particles, i.e. flocculation of the solid material;
2. Separation at higher G values, i.e. centrifugation;
3. Separation by exploiting the Boycott effect (Boycott, 1920).

Points (1) and (2) are in practical use today, either separately or in combination.

FLOCCULATION

A classic method of removing cells from a liquid is to add flocculating material and then separate the precipitate; this is an established method in waste-water treatment plants. If cells are to be used repetitively in a continuous process, addition of chemical flocculants may introduce problems in the subsequent processing. If this step is to be used then flocculation must be easily controlled. In many ways this system resembles immobilized-cell systems.

Use of spontaneously flocculating yeasts and bacterial strains has been found to be attractive because flocculating chemicals are not needed. A settler is attached at the outflow of the fermenter, where the flocculated cells are collected as sediment and are then recycled to the fermenter while the supernatant is passed on for further processing. The efficiency of this sedimentation step is important because high cell losses may be detrimental to the process, especially at high dilution rates.

Flocculation is governed by the surface structure of the cells. A better understanding of cell physiology and cell culture conditions has led to a better understanding of the flocculation mechanisms (Kefford, Kjelleberg and Marshall, 1982).

Flocculating organisms have been exploited in ethanol fermentation (Ramirez and Boudarel, 1983; Scott, 1983).

CENTRIFUGATION

Increased G numbers will improve separation and so reduce the need for flocculation. The drawback with continuous centrifuges is their price and their maintenance costs. However, in some applications, use of centrifuges has so improved the performance of the process that the outlay was fully justified. A good example of a process involving cell recycling through centrifugation is the Biostill process for ethanol fermentation, developed by Alfa-Laval and based on their own separators. Here, full-scale plants have been developed for continuous fermentation using only one fermenter and cell recycling by separation in a centrifugal separator. From the flow scheme in *Figure 8* it can be seen that a continuous stream from the fermenter is separated into a yeast-rich stream, which is fed back to the fermenter, and a beer stream which continuously passes through a distillation step and through a heat exchanger, for temperature regulation, before return to the fermenter. Several advantages are claimed for this process layout, although for small-scale processes this procedure may be too capital intensive. There are other similar process layouts for continuous ethanol production (Guidoboni, 1984).

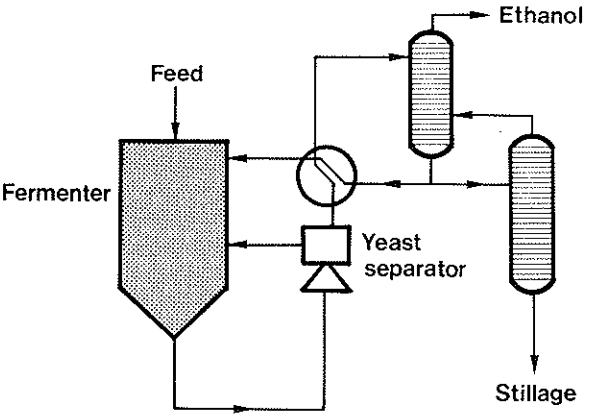

Figure 8. Flow-sheet of the Biostill process (after Alfa Laval, 1981).

STATIC SETTLERS

Walsh and Bungay (1979) have put forward a static separator that is very cheap and also seems to have the potential of efficiently removing yeast cells from a flow stream. Inserted inclined planes facilitate much faster sedimentation and therefore also a more efficient separation. This Boycott effect may be exploitable in other biotechnological situations and deserves further attention.

Immobilized cells

During the last 15–20 years much work has been devoted to the development of immobilization technology, to immobilization and coupling procedures, to the development of suitable supports and, to a lesser extent, to process technology using immobilized cells and/or enzymes. The need for efficient methods of producing solvent has focused interest on certain key questions that have to be addressed when dealing with immobilized cells. In some recent reviews much of the work has been covered (Durand and Navarro, 1978; Klein and Wagner, 1978; Messing, 1981; Mattiasson, 1983a,b). The intention here is merely to illustrate when, and under what conditions, immobilized-cell preparations have something to offer in the context of extractive bioconversions, and in certain cases to discuss the limiting factors of the solid-state technology.

The characteristics of immobilized enzyme or cell preparations are listed in *Table 12*. Dead as well as viable cells have been used in bioconversion processes. The definition of a viable cell needs clarification; some criteria for defining viability of immobilized cells have been noted (Mattiasson, 1983b).

The use of high cell densities under relatively strict control permits conversion of a greater proportion of the substrate to products and less to generation of new cell masses (Esener *et al.*, 1981; Mattiasson and Hahn-

Table 12. Characteristics of immobilized biocatalysts

High catalytic density permitting high reaction rates
Higher specific product yield
Can be used in continuous processes
High dilution rates without the problems of wash-out
Better and easier control
Reduced demands on costly fermenters
Easier product upgrading

Hägerdal, 1982; Mattiasson, Larsson and Hahn-Hägerdal, 1984). This is an observation that has been reported from several laboratories and has been fitted into general ideas about how the microenvironmental conditions influence the cell metabolism of immobilized cells.

A characteristic of immobilized cells is that they may be used in continuous processes by, for example, packing them in a column and letting substrate pass over them. In this way, there is a continuous removal of product and a concomitant supply of fresh substrate. In practice this may be regarded as equivalent to an extractive process, but with the difference that, when operating along the column, there is a linear decrease in substrate concentration and a simultaneous build-up of product, so that towards the end of the reactor marked product inhibition may occur. Furthermore, within each particle of immobilized biocatalyst, diffusion limitations appear. This also may cause enrichment of products, again hampering the reaction. These problems have been recognized and several alternative solutions have been proposed to circumvent, or at least to reduce them: the size of the particle in which the biocatalyst is immobilized may be varied—the smaller the size, the less the diffusion restrictions; reduction of the density of the catalysts leads to similar effects, but prevents the use of high catalyst concentrations. For immobilized cells, fixation at a surface has been suggested as a competitive method, in that diffusion restrictions are then held to a minimum and often very cheap immobilization methods may be adopted. Furthermore, when operating at low substrate concentrations it has proved advantageous to use surface-bound catalysts because the organic material in the solution will accumulate at the surface.

Still another problem when dealing with gas-forming processes is the generation of gas within the immobilized particles (beads). In many cases this may lead to rupture of the beads. This is particularly important and well documented in the case of ethanol fermentation.

Much work has been carried out in order to set up ethanol fermentation based on immobilized-cell technology. Several process layouts have been tested and, as seen from *Table 13*, some can be highly productive; nevertheless, for economic operation, high production alone may not suffice. It is the combination of high productivity, high concentration in the final product stream and total utilization of the substrate supplied that is difficult to achieve. A high ethanol yield per unit substrate utilized is governed partly by

Table 13. Immobilized *Saccharomyces cerevisiae* for ethanol production

Immobilization technique	Substrate	Productivity (g/ℓ/h)	Dilution rate (h^{-1})	Yield* (%)	Reference
Adsorption					
Ion exchange resin	12% glucose	62	1·44	70	Daugalis *et al.*, 1981
Entrapment					
Polyacrylamide	19·6% glucose	34	0·56	60	Shiotani and Yamané, 1981
Alginate	19·6% glucose	46	0·57	80	Shiotani and Yamané, 1981
Alginate	5% cellobiose†	2.2	0·1	80	Hahn-Hägerdal, 1984
Alginate	5% glucose	18·3	0·73	100	Hahn-Hägerdal and Mattiasson, 1982
Alginate	10% glucose	3·6	0·1	70	Kierstan and Bucke, 1977
Alginate	17·5% cane molasses	14–17·5	0·2–0·25	80	Linko and Linko, 1981
Alginate	10% xylose‡	32	0·75	84	Chiang *et al.*, 1982
Carrageenan	10% glucose	43	1	84	Wada, Kato and Chibata, 1979
Carrageenan	10% glucose	50	1	100	Wada, Kato and Chibata, 1980
Gelatin	3% glucose	7·4	0·48	100	Sitton and Gaddy, 1980

* Percentage of theoretical yield.
† Yeast cells coimmobilized with β-glucosidase.
‡ Using a separate isomerase column for converting D-xylose to D-xylulose.

decreasing cell growth and partly by maintaining a very high cell density so that all the substrate is converted. It is, of course, also desirable to keep any side reactions to a low level. Two organisms in particular have been studied: *Saccharomyces cerevisiae* and *Zymomonas mobilis*, which utilize different metabolic pathways and therefore will differ in their metabolic behaviour. The bacterium utilizes the Entner–Douderoff pathway (in which 6-phosphogluconate is converted anaerobically to pyruvate and triose phosphate via 2-oxo-3-deoxy-6-phosphogluconate) whereas the yeast uses the conventional glycolytic pathway. As the Entner–Douderoff pathway yields only one molecule of ATP when converting glucose to pyruvate, *Z. mobilis* must convert more sugar than the yeast, to obtain essential metabolic energy. Similar effects can be achieved by applying metabolic inhibitors. Thus, a higher productivity and yield of ethanol can be obtained from the yeast in the presence of azide (Hahn-Hägerdal and Mattiasson, 1982). Similar effects are observed when cells are stressed, e.g. by lowering the water activity (Hahn-Hägerdal, Larsson and Mattiasson, 1982), as happens when cells are immobilized in a polymer.

A characteristic of processes based on immobilized viable cells is that very often there is a leakage of cells into the product stream. This is often harmless, but needs to be considered when discussing such processes.

Permeabilization

A biotechnological process involves several steps of mass transfer: (1) transfer from the bulk to the environment of the catalyst; (2) transfer within the pores of the catalyst; (3) if cells are participating, transport over the cell membrane, of the substrate as well as of products; (4) transport problems within the cell.

Much process development work has been devoted to decreasing the external mass-transfer limitations, but very little has been done so far to improve transport in and out of the cell. A group of permeases (also called translocases) have been identified and their operation mechanisms have been described, but if more efficient mass transfer is needed, other means are necessary. Such a method is permeabilization, which involves the treatment of cells in such a way that the membrane becomes less selective, thereby making it possible to move substances more efficiently across the cell membrane. Such a possibility is especially interesting if the potential catalytic capacity within the cell is higher than that which the transfer of substrate according to normal routes can supply, or when the product accumulates within the cell and is only slowly transported away, as in the well-documented case of ethanol fermentation where the ethanol accumulates within the cell, thereby amplifying product inhibition.

Permeabilization may be accomplished in different ways. The general principle is to treat the membrane with chemicals so that it loses its selectivity, but still functions as a membrane in the sense that it keeps enzymes and coenzymes within the cell (Felix, 1982). Only a few organic chemicals have been found to be useful for permeabilization and then only under rigorous

control of concentration, time and temperature (Matsumura and Märkl, 1984a). Permeabilization is thus a very subtle, finely tuned process: a very gentle method is simply to change the membrane fluidity by, for example, the introduction of unsaturated fatty acids (Panchal and Stewart, 1981; *see also* Moulin, Boze and Galzy, 1984). In an experiment with *Saccharomyces uvarum* it was clearly shown that the addition of linoleic acid to the medium had a marked effect on both glucose uptake and ethanol productivity. The effect of the detergent Tween 80 at low concentrations (0·1–0·2% w/v) was studied in ethanol fermentations using *Saccharomyces cerevisiae* (Panchal and Stewart, 1981). If Tween was added after a fermentation was over, a substantial amount of ethanol was liberated from the cells. This observation is also in line with those claiming that ethanol concentration in the cells is markedly increased compared with that in the surrounding medium.

In the area of secondary metabolism, there have been several investigations into the possibility of using permeabilized cells as enzyme packages where substrate and coenzyme may more or less freely diffuse in and out. This has also been applied to immobilized cells and especially to immobilized plant cells (Felix, Brodelius and Mosbach, 1981). It was found that only a very limited number of solvents could be used. That secondary metabolites could be extracted, even if they were stored within the vacuoles of the cell, was perhaps not surprising. It was more unexpected, however, that the cells after treatment with organic chemicals were able to start synthesizing more products which could be liberated in a subsequent permeabilization step. Careful permeabilization in this case obviously permits the cell itself to close the imperfections in the membrane and leaves us with a cell fully capable of synthetic work.

The borderline between permeabilization and extraction with organic solvents is not at all clear. As described above, it was possible to increase the amount of ethanol liberated from a cell preparation by adding Tween 80. If the definition is that permeabilization involves treatment which does not affect the viability of the cell (this not being the case for extraction using organic solvent), then very few true permeabilization experiments have been carried out. If the definition of permeabilization is widened to include dead cells, then many examples have been documented.

Concluding remarks

In this paper we have discussed the different techniques used in extractive bioconversions. Most of the work carried out so far has focused on solvent production. This does not necessarily mean that the greatest potential of the techniques discussed is within this area, but merely that this field of research concerning process development is the most active at present.

Very few examples are available as yet within the field of plant- and animal-cell cultures and microbial production of macromolecules, but as these techniques become more developed there will also be a need for new process technologies and then some of the techniques discussed above will be more widely applied.

The trend to integrate bioconversion and downstream processing will certainly continue and this will also play a part in making extractive bioconversions more widely applicable within biotechnology in the future.

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