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The Acetone-Butanol-Ethanol Fermentation: Recent Progress inTechnology

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Introduction

The acetone–butanol–ethanol (ABE) fermentation process, using *Clostridium acetobutylicum* or *C. beijerinckii*, epitomizes the problems of all fermentation processes *vis-à-vis* chemical synthesis:

1. Low reactor productivities;
2. Presentation of a dilute aqueous solution for product recovery.

In particular, the process is subject to severe product inhibition, at concentrations no greater than 20 $g\ l^{-1}$, and this, in turn, restricts the concentration of sugar that can be fermented.

The purpose of this chapter is to review some recent developments in fermentation technology as applied to the ABE process. The aim of such technologies is to increase reactor productivity and/or remove product inhibition. In this way, the economics of the overall process can be improved.

Detailed accounts of the microbiology and biochemistry of the ABE process (outlined in *Figure 1*) have been presented in several recent reviews (e.g. Linden, Moreira and Lenz, 1985; Ennis, Gutierrez and Maddox, 1986; Jones and Woods, 1986; Awang, Jones and Ingledew, 1988). In this article, techniques for improving productivity in batch fermentation will be considered first, followed by the use of continuous culture techniques and some novel fermentation technologies, such as the application of immobilized cells. Finally, some integrated fermentation/product recovery technologies will be described, because the key to future commercial development of the process may depend on minimizing product inhibition while reducing the costs of product recovery.

Abbreviations: ABE, acetone–butanol–ethanol; CFM, capillary crossflow microfiltration; CSTR, continuous stirred tank reactor.

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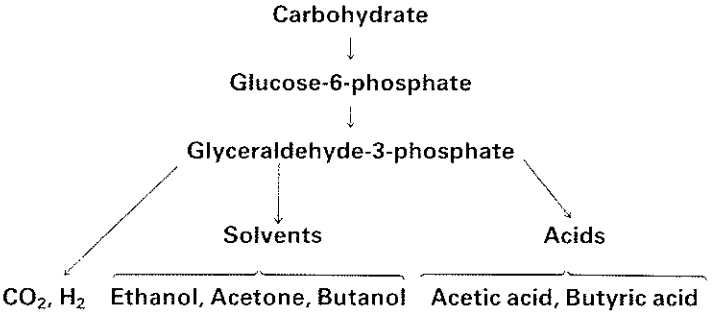

Figure 1. Major metabolites produced from carbohydrate on anaerobic fermentation by *Clostridium acetobutylicum* (or *C. beijerinkii*).

Batch fermentation

pH AND INITIAL SUGAR CONCENTRATION

It has been known for many years that the culture pH value has a strong influence on the course of the ABE fermentation. The general concensus has been that fermentations performed at relatively high pH values produced acids rather than solvents, whereas in fermentations performed at relatively low pH values, the reverse was true (e.g. Monot, Engasser and Petitdemange, 1983). However, there have been several reports of solvents (i.e. total butanol + ethanol + acetone) being produced at pH values approaching neutrality, and it is now clear that there is a strong interaction between the pH value and the initial sugar concentration, and also the strain of bacterium used. Thus, higher initial sugar concentrations encourage solvent production, even at pH values approaching neutrality (Monot *et al.*, 1982; George and Chen, 1983; Monot, Engasser and Petitdemange, 1983; Holt, Stephens and Morris, 1984; Ennis and Maddox, 1987). Marchal, Blanchet and Vandecasteele (1985), working with a substrate derived from the Jerusalem artichoke (*Helianthus tuberosus*), re-emphasized the marked influence of pH value, and showed that while a low pH value favoured solvent production over acid production, it also led to an increase in the total fermentation time. Their solution to the problem was to control the pH profile during the process. Two such profiles were described. In one, the pH was held at pH 6·0–6·5 during the growth phase, and was then allowed to decrease by self-acidification. In the second, the pH was similarly held during the growth phase, and was then allowed to drop by self-acidification. On reaching pH 5·3, it was re-adjusted to pH 6·5, followed by another natural decrease. By this means, substantial improvements in solvent production were achieved. A similar approach has been described by Ennis and Maddox (1987), working with a substrate of whey permeate. They reconfirmed that at relatively low sugar (lactose) concentrations (45 g l^{-1}), low pH favoured solventogenesis, but growth and sugar utilization were poor. Conversely, at higher pH values, although growth and sugar utilization were much improved, solvent production was poor. The pH profile that was developed to optimize the fermentation involved holding the pH near neutrality during the growth phase, and then allowing it to fall naturally to the optimum value for the solvent

production rate (pH 5·1–5·5), at which point it was maintained by addition of alkali (ammonia).

The conclusion that can be drawn from these studies is that to achieve a high solvent productivity, it is necessary to control the pH profile during the process, rather than simply to maintain a constant pH value. The optimum profile for a given substrate and bacterial strain is probably case-specific, and should be evaluated for each situation.

AGITATION AND HEAD-SPACE PRESSURE

Spivey (1978), in his article describing the commercial process then operating at National Chemical Products, South Africa, stated that during the fermentation the head-space pressure was allowed to rise to 35 KPa above ambient pressure (due to the carbon dioxide and hydrogen produced by bacterial metabolism). Further, the fermentation vessels were not fitted with mechanical agitators. Presumably, sufficient mixing occurred due to gas evolution so that agitation was not required. Maddox, Gapes and Larsen (1981) performed experiments in a stainless steel fermentation vessel where the head-space pressure could be controlled. Pressures of up to 105 KPa were generated naturally, and held at the required value using a relief valve. It was shown that maintenance of a positive head-space pressure resulted in a marked improvement in solvent productivity, the value increasing from 0·05 g/l.h to 0·2 g/l.h when the process was conducted at 105 KPa above ambient pressure rather than at atmospheric pressure. Furthermore, it was demonstrated that sparging or sweeping the culture with hydrogen gas at atmospheric pressure had no effect on the solvent productivity, and it was suggested that this is because the partial pressure was not sufficiently high to achieve the necessary concentration of hydrogen in solution. It was postulated, based on remarks by Spivey (1978), that the hydrogen gas produced early in the fermentation is subsequently reused by the bacterium to provide reducing power for butanol production.

In contrast to the above, Griffith, Compere and Googin (1983) reported that at a head-space pressure of 1400 KPa, produced by pressurizing with hydrogen gas, there was a slight decrease, rather than an increase, in butanol production. Unfortunately, no details were provided for production rates or yields.

In support of the beneficial effects of an increased head-space pressure, Doremus, Linden and Moreira (1985) compared fermentations performed at 105 KPa with those performed under non-pressurized conditions. They demonstrated a 60% improvement in butanol productivity in the pressurized fermentations, and they postulated that this was due to supersaturation of the broth with hydrogen. Lobos, Lamed and Su (1982) had previously reported that high levels of hydrogen supersaturation can occur in unstirred cultures of various anaerobic bacteria, including *C. acetobutylicum*. This supersaturation favours the formation of reduced products, such as butanol and ethanol, at the expense of oxidized products, such as acetate and butyrate. Depending on the organism, mole ratios of reduced : oxidized products can increase as much as tenfold under conditions of supersaturation, and this can also be achieved by application to the culture of a positive hydrogen head-space pressure (Lobos,

Lamed and Su, 1982). Yerushalmi, Volesky and Szczesny (1985) have also investigated the effect of increased hydrogen partial pressure on solvent production. Using hydrogen gas, they achieved head-space pressures of up to 1479 KPa, and observed increased yields of 18% and 13% for butanol and ethanol, respectively, compared to non-pressurized fermentations. The corresponding acetone yield was decreased by 14%. Although no data were provided for product concentrations or productivities, this shift in product ratio from acetone towards butanol is of practical interest.

The effect of carbon dioxide pressure on solventogenesis has been studied by Klei, Sundstrom and Miller (1984). However, their methodology was such that the fermenter head-space contained hydrogen (from bacterial metabolism) in addition to carbon dioxide, so their results should be interpreted with some caution. Nevertheless, their findings show that maximum solvent concentrations were achieved at a head-space pressure of 175 KPa. At a pressure of 280 KPa ethanol production was eliminated. Doremus, Linden and Moreira (1985) have stated that the concentration of dissolved carbon dioxide has no effect on solvent production.

More recently, Brosseau, Yan and Lo (1986), using *C. saccharoperbutylacetonicum*, have confirmed that maintenance of a positive head-space pressure favours solvent production, but no details were provided of the pressures attained.

When investigating the effects of hydrogen supersaturation in cultures of clostridia, Lobos, Lamed and Su (1982) commented that supersaturation was dramatically reduced upon agitation of the broth. The effect of agitation on the ABE fermentation has been studied subsequently by other investigators. Welsh and Veliky (1984) compared the effect of non-agitation with agitation at 100 r.p.m. (1·2 litre working volume fermenter), and observed that agitation of the culture had a detrimental effect on the solvent concentration, yield and production rate. In addition, there was a decrease in the ratio of butanol to acetone in the broth. Doremus, Linden and Moreira (1985) reported similar results when investigating the effect of agitation over the range 25–300 r.p.m. (3 litre working volume fermenter). Thus, solvent productivity was inversely proportional to the agitation rate. No real effect was observed on the final solvent concentration or ratio of butanol to acetone, but this may reflect the fact that no experiments were performed with a zero agitation rate. As expected, the effect of agitation was much less in pressurized than in non-pressurized fermentations.

In contrast to the above, Yerushalmi and Volesky (1985), working in the agitation range 190–560 r.p.m. (10 litre working volume fermenter), observed no real effect of agitation on the solvent production rate, concentration or yield. However, this may again reflect the lack of a non-agitated control experiment. Nevertheless, a slight increase in the specific solvent production rate was observed as the impeller speed increased from 190 to 340 r.p.m. At 560 r.p.m., growth of the culture was inhibited, presumably due to shear effects.

Overall, it is now apparent that operation of the batch fermentation process under conditions of non-agitation and a positive head-space pressure is conducive to solvent production. Technically, these requirements are simple to

achieve. The effect appears to be caused by the high partial pressures of hydrogen which are attained, resulting in hydrogen supersaturation of the culture.

CARBON MONOXIDE AND VIOLOGENS

Closely related to the effects of agitation and head-space pressure on the ABE process are the effects of carbon monoxide and methyl and benzyl viologens. Carbon monoxide is an inhibitor of the enzyme hydrogenase, and thus its action increases the pool of reduced nucleotides in the cell, which in turn leads to increased production of products which require reduced nucleotides for their formation, i.e. butanol and ethanol (Datta and Zeikus, 1985). Methyl and benzyl viologens also alter electron flow in favour of the alcohols, but the exact mechanism is unknown (Rao and Mutharasan, 1986).

Kim *et al.* (1984) and Meyer, McLaughlin and Papoutsakis (1985), working in batch culture, have investigated the effect of gassing with carbon monoxide, in mixtures with nitrogen. They observed an inhibitory effect on cell growth, hydrogen production and acid production, while there was a stimulatory effect on ethanol and butanol production. This work was extended by Datta and Zeikus (1985), who demonstrated that metabolic modulation by carbon monoxide was particularly effective when acetate or butyrate, at 5 g l^{-1}, were added to the fermentation as electron sinks. Using levels of carbon monoxide in the range 10–20 KPa partial pressure, it was observed that solventogenesis commenced earlier and higher butanol concentrations could be achieved. The latter was related to an increased ratio of butanol to acetone. Indeed, in some circumstances, acetone production could be completely eliminated. As mentioned earlier, this manipulation of end-product ratios is not without some commercial significance. Meyer, Roos and Papoutsakis (1986) subsequently extended these observations to glucose-limited continuous cultures. Normally, solvents are not produced under conditions of glucose limitation, but when the culture was sparged with carbon monoxide, ethanol and butanol (but not acetone) were produced at very high specific production rates.

The use of carbon monoxide in ABE fermentation processes may prove to be a commercial proposition, provided that a cheap supply of the gas is available. The major benefit appears to be increased production of ethanol and butanol at the expense of acetone.

The application of methyl and benzyl viologens to solvent production gives similar effects to those of carbon monoxide, but its practical utility may be limited. Rao and Mutharasan (1986), working in batch culture at pH 5·0, showed that addition of methyl viologen (0·1 g l^{-1}) led to increased production of ethanol at the expense of acetone, while the butanol concentration remained unchanged. At pH 6·8, where there was no solvent production in control experiments, addition of methyl viologen stimulated ethanol production. These observations have since been extended to continuous culture experiments (Rao and Mutharasan, 1987, 1988). Under glucose-limited conditions at pH values above pH 6·3, where solvent production is not normally observed, addition of methyl or benzyl viologen led to a decreased

production of acetate and butyrate, and an increased production of ethanol and butanol. Acetone was not observed. Similarly, the use of benzyl viologen in a non-nutrient-limited continuous fermentation led to an increased ratio of butanol to acetone. Kim and Kim (1988) have recently reported similar results, and, in addition, have successfully used electrochemical energy as a source of reducing equivalent to achieve the same effect.

NUTRIENT LIMITATION

It is often difficult to interpret the literature with regard to the effect of a nutrient limitation on solvent production in batch fermentation, since although initial nutrient concentrations are usually described, the nutritional status of the culture during and after the process is often not. For commercial media, it has been stated that all nutrients are normally present in excess (Jones and Woods, 1986), but it is not clear whether this is the optimum situation. Technically, it is simple to add supplementary nutrients to commercial media if required, and also to remove certain nutrients, although this may be costly.

Nutrient limitation in batch culture may be defined as a situation where cellular growth is restricted (terminated) due to the exhaustion of an essential nutrient. In the ABE fermentation, glucose limitation is well known to be detrimental to solvent production, and will not be considered further (Monot, Engasser and Petitdemange, 1983; Long, Jones and Woods, 1984).

Nitrogen limitation has been studied by Monot and Engasser (1983) who reported that strong solvent production occurred after exhaustion of nitrogen from the medium. In contrast, Long, Jones and Woods (1984) concluded that nitrogen-limited cultures did not produce solvents. Their results showed that there must be a minimum nitrogen concentration remaining after the growth phase for solventogenesis to occur. Roos, McLaughlin and Papoutsakis (1985) performed experiments where the ratio of nitrogen to glucose was varied, to investigate the effect on solventogenesis. Although it is not clear if nitrogen was ever a limiting nutrient, the data suggest that an excess of nitrogen is detrimental to solvent production, and that as the ratio of nitrogen to glucose decreases, the rate of solvent production increases. Thus, solvent production may be enhanced by a lowered availability of nitrogen.

With regard to phosphate-limited batch cultures, Bahl, Andersch and Gottschalk (1982) have described how solvent production occurred after exhaustion of phosphate from the medium. In conditions of excess phosphate, the fermentation produced acids rather than solvents, and thus there may be a case for removal of this anion from commercial media. Recently, Bryant and Blaschek (1988) have described the effect of buffering on solvent production, but it is possible to interpret some of their data as an effect of the phosphate concentration. Although the nutritional status of the culture at the end of the fermentation was not described, the results appear to confirm that solvent production can occur under phosphate-limited conditions.

Kanchanatawee and Maddox (unpublished data) have studied the effects of nitrogen and phosphate limitation, and conditions where all nutrients are in excess, on solvent production. The results confirm that solvent production can

occur under conditions of nitrogen or phosphate limitation, but that the optimum conditions are where both the nitrogen and phosphate concentrations are just sufficient for growth, but are not growth-limiting. If either nitrogen or phosphate is present in too great an excess, there is a decrease in solvent production. Hence, there is an interaction between the nitrogen and phosphate nutrient concentrations, which may help to explain some of the apparent contradictions in the literature, particularly for nitrogen. Similar results have been observed by Yerushalmi and Volesky (1987) with regard to the nitrogen concentration, i.e. the optimum condition for solvent production occurs when the supply is just in excess of that required for biomass growth.

Iron limitation in batch culture may be a useful technique for manipulation of the ratio of butanol to acetone. Junelles *et al.* (1988) performed experiments whereby the iron content of the medium was reduced from 10 mg l^{-1} to 0·2 mg l^{-1} (as $FeSO_4.7H_2O$), with the result that the butanol to acetone ratio increased from 3·7 : 1 to 11·8 : 1. It was suggested that iron limitation, in addition to inhibiting the enzyme hydrogenase, directly affected the carbon and electron flow. Previous to this, Bahl *et al.* (1986) reported on experiments carried out in chemostat culture, in a low-phosphate medium. Under iron limitation, the ratio of butanol to acetone was increased from 2 : 1 to 8 : 1. Hence, on the grounds that butanol is a more desirable product than acetone, there would appear to be a case for close examination of the iron content of commercial media.

Fed-batch fermentation

The technique of fed-batch culture is widely used in the fermentation industry. In some cases, e.g. the penicillin production process, the glucose consumption rate of the culture, and hence the growth rate, is controlled via the rate of glucose supply. This is used to direct the metabolism of the organism. In other cases, e.g. in the glutamic acid production process using fed-batch culture, a controlled supply of sugar is used as a means of overcoming substrate inhibition and of obtaining a high concentration of product in the broth, thus reducing product recovery costs. For the ABE fermentation, fed-batch culture is of limited value unless coupled with continuous product removal. This is due to the problem of product inhibition which restricts the solvent concentration in the broth to less than 20 g l^{-1}. From the rather limited literature that is available on the application of fed-batch culture to the ABE fermentation, it appears that high feed rates of sugar favour solvent production, whereas low rates favour acid production (Fond *et al.*, 1984, 1986).

Continuous fermentation using free cells

The criteria for a commercially successful continuous fermentation process may be listed as:

1. High reactor productivity;
2. High product concentration in the broth;
3. Long-term operational stability.

It has long been recognized that continuous fermentation is a very useful means for attaining high reactor productivities. This is achieved by maintaining the culture for long periods of time at the value of its maximum instantaneous production rate (as observed in batch culture). In addition, continuous culture avoids the non-productive downtime which is a feature of batch fermentation. Ennis, Gutierrez and Maddox (1986) have summarized some of the culture conditions and productivities which have been observed in ABE continuous culture. Values of up to 2·5 g/l.h (for total solvents) have been recorded, but are more usually in the range 0·5–1·0 g/l.h. Unfortunately, high productivities are often achieved at the expense of high solvent concentrations and sugar utilization, and this remains a problem. Furthermore, it is becoming increasingly clear that it is difficult to maintain continuous ABE fermentation (using free cells) in a long-term stable steady-state. This is a major drawback to commercial application, and thus it is a problem that must be solved.

OPERATIONAL STABILITY

Instability of continuous culture of solvent production using *C. acetobutylicum* has been recognized for more than 50 years. Wheeler and Goodale (1932), in a patent application, described how attainment of stability required a cascade of fermenters in series, because of the complicated life cycle of the organism. Since then, the problem has been remarked upon by many authors (Dyr, Protiva and Praus, 1958; Finn and Nowrey, 1958; Yarovenko, 1962; Hospodka, 1966; Gottschal and Morris, 1981; Jobses and Roels, 1983; Stephens *et al.*, 1985; Clarke and Hansford, 1986; Fick and Engasser, 1986). There appear to be two distinct types of instability (Barbeau, Marchal and Vandecasteele, 1988). The first is represented by metabolic oscillations, and appears to be caused by 'product inhibition' ('acid shift'). Thus, long-term stability of single-stage continuous fermentations has only been obtained at relatively low butanol concentrations, conditions that do not favour economical product recovery. The second type of instability is represented by a long-term metabolic drift towards acid, rather than solvent, production. This is sometimes known as 'degeneration' (or 'acid drift') (Finn and Nowrey, 1958; Barbeau, Marchal and Vandecasteele, 1988).

In single-stage continuous culture, solvent production is favoured at low dilution (growth) rates, while acid production occurs at high dilution (growth) rates (Bahl, Andersch and Gottschalk, 1982; Monot and Engasser, 1983). Hence, most workers have operated continuous culture at low dilution rates, conditions where 'product inhibition' ('acid shift') occurs. Barbeau, Marchal and Vandecasteele (1988) have documented the metabolic oscillations that occur in a product-limited (nutrient excess) continuous fermentation in a single-stage reactor at low dilution rate (D) ($0{\cdot}035\ h^{-1}$). Thus, oscillations were observed in the concentrations of biomass, solvents and acids, and all metabolic activity ceased when the solvent concentration exceeded 12 g l^{-1}. Similarly, Fick, Pierrot and Engasser (1985) have identified butanol as the

main factor responsible for the limited viability of *C. acetobutylicum* in a single-stage, product-limited continuous culture, and long-term stability depends on holding its concentration below 9 g l^{-1}. Clearly, such a low concentration does not aid the economics of product recovery. Clarke, Hansford and Jones (1988) have recently described the same phenomenon, and have provided an explanation for the oscillations, involving two physiological types of cells in the culture, i.e. acidogenic and solventogenic types. At any given time, the higher growth rate of acidogenic cells leads to their selective retention in the culture, giving an increase in biomass and acid concentrations. As the acid concentration increases, so cells are triggered into the solventogenic type, causing increased solvent and decreased acid concentrations. However, the lower growth rate of the solventogenic cells results in their preferential washout from the culture, accompanied by a decrease in solvent concentration, and this triggers a new cycle of cell growth and acidogenesis.

These metabolic oscillations prevent the attainment of a true steady-state, and are undesirable in a commercial process because of the loss of carbon into unwanted acidic products.

The cause of 'degeneration' ('acid drift') is not yet clear. However, it has been reported to be promoted by high pH values, high dilution rates and by the absence of nutritional limitation (Barbeau, Marchal and Vandecasteele, 1988). These authors suggested that a key element to prevention may be nutritional limitation by, for example, nitrogen, possibly in a two-stage culture.

Solutions to the problems of 'product inhibition' and 'degeneration' have been sought by many workers. As mentioned above, Wheeler and Goodale (1932) recognized the problem of instability, and avoided it by using several fermenters in series. They stressed the importance of maintaining a high growth rate in the first fermenter ($D>0{\cdot}1\ h^{-1}$). Later, Dyr, Protiva and Praus (1958) described the use of three-stage and four-stage systems, but they still encountered the problem of 'degeneration'. They suggested that this was due to insufficient differentiation of the growth and solventogenic phases. Eventually, a five-stage system was developed whereby a high growth rate was attained in the first vessel ($D = 0{\cdot}3\ h^{-1}$), acidogenesis in the first and second, and solventogenesis in the third, fourth and fifth vessels. Hospodka (1966) and Yarovenko (1962) subsequently described an 11-stage continuous process, but they admitted to problems of 'degeneration'.

Bahl, Andersch and Gottschalk (1982) further developed the concept of a multistage system, using a two-stage phosphate-limited chemostat. In the first stage, the dilution rate was $0{\cdot}125\ h^{-1}$, leading to good cell growth, while in the second stage the dilution rate was $0{\cdot}04\ h^{-1}$ and solventogenesis, but not growth, occurred. They demonstrated that this system was extremely stable. Stephens *et al.* (1985) confirmed the instability of one-stage continuous cultures under a variety of conditions, and suggested that a two-stage process, using a turbidostat as the first stage, might be a solution to the problem. Barbeau *et al.* (1986) have also suggested a two-stage process, as have Godin and Engasser (1988). Hence, there now seems little doubt that a two- or multistage process, whereby the different physiological phases of the organism are separated from

each other, is a potentially useful solution to the problem of instability in continuous culture using free cells. The key appears to lie with obtaining unrestricted cell growth in the first vessel, followed by solventogenesis, but not growth, in the latter vessels. Solventogenesis may be obtained via a nutrient limitation.

Another solution to the stability problem has been suggested by Fick and Engasser (1986) who considered that the metabolic oscillations may be due to flocculation of cells, as well as to butanol toxicity. By incorporating KCl (0·5 g l^{-1}) into the feed medium, they prevented flocculation and achieved stable steady-stages in a one-stage fermenter. Soni, Soucaille and Goma (1987) have linked instability of continuous cultures with autolysin production (which is stimulated by butanol), and have suggested the use of divalent cations as a means of preventing autolysin production, and hence 'degeneration'. Finally, Rao and Mutharasan (1988) have noted the use of benzyl viologen in preventing 'degeneration', but practical application of this technique may be limited.

NUTRIENT LIMITATION

There have been many studies into the use of chemostats for solvent production. In addition to being a means of increasing fermenter productivity over batch culture, chemostat culture is an excellent tool for the elucidation of biochemical mechanisms, and for studying the effects of various environmental parameters on microbial physiology. Unfortunately, some reports in the literature are difficult to interpret as it is not always clear whether a nutrient-limited condition has, in fact, been attained. Some claims of chemostat culture provide no evidence for a nutrient limitation, and examination of feed-medium composition casts considerable doubt on such claims. Furthermore, it is now realized that the effect of nutrient limitation on solvent production must be considered in interaction with other important environmental parameters, such as culture pH, dilution rate, etc. Nevertheless, it is now clear that solventogenesis in continuous culture can occur under several different nutrient-limited conditions, including phosphate limitation (Bahl and Gottschalk, 1984; Soni, Soucaille and Goma, 1986), nitrogen limitation (Andersch, Bahl and Gottschalk, 1982; Monot and Engasser, 1983; Stephens *et al.*, 1985), magnesium limitation (Stephens *et al.*, 1985; McNeil and Kristiansen, 1987) and sulphate limitation (Bahl and Gottschalk, 1984). Recently, Clarke and Hansford (1986) studied solvent production under a variety of nutrient-limited conditions (including nitrogen and phosphate), and concluded that although solventogenesis occurs under these conditions, such limitation is not essential for solvent production. However, the possible role of nutrient limitation in preventing culture 'degeneration' has been mentioned above, and this may represent an advantage over the non-nutrient-limited condition.

During their studies into chemostat and turbidostat culture, Gottschal and Morris (1982) and Stephens *et al.* (1985) made the observation that solventogenesis is favoured by high cell densities whereas acidogenesis is

favoured by low cell densities. Interestingly, Hospodka (1966) had also stressed the importance of maintaining a high cell denstiy ($>7 \times 10^9$ cells ml^{-1}) during his description of a multistage pilot plant process. Unfortunately, there has been no further study into this phenomenon, despite its potential commercial application with regard to the novel fermentation technologies described below.

In conclusion, free-cell continuous culture allows superior productivities (up to 2·5 g/l.h) to be achieved when compared with batch culture. Such systems are notoriously unstable, although multistage systems may provide a solution to the problem. Unfortunately, the solvent concentrations that are achieved in continuous culture are considerably lower than in batch culture, aggravating the problem of economic product recovery.

Novel fermentation technologies

CONTINUOUS CULTURE WITH CELL RECYCLE

Continuous culture (using free cells) with cell recycle is a technique by which the cells in the effluent stream are collected and recycled to the fermenter. The result of this is that higher biomass concentrations are achieved in the fermenter, and this should lead to higher reactor productivities. Furthermore, the higher biomass concentration within the fermenter may allow the process to operate at higher product concentrations than in conventional systems, with the result that the higher productivities achieved are not entirely at the expense of product concentration. Potential disadvantages of cell recycle include the need for additional capital equipment, and the process may be complex and difficult to operate. *Table 1* summarizes the studies using this technology.

Table 1. Cell recycle techniques applied to the ABE fermentation

Recycle technique	Productivity g/l.h	Comments	Reference
Centrifugation	<1·0	Cells were heat-shocked during recycle	Vijjeswarapu, Chen and Foutch (1985)
Capillary CFM	5·4	'Degeneration' occurred	Afschar *et al.* (1985)
	3·0	Two-stage, stable system	Afschar *et al.* (1985)
Mineral tubular membrane	6·0	Metabolic oscillations and 'degeneration' were observed	Ferras, Minier and Goma (1986)
Plate and frame filter	6·5	Phosphate-limited, stable system	Schlote and Gottschalk (1986)
Hollow-fibre CFM	6·5	Stressed the importance of bleed dilution rate	Pierrot, Fick and Engasser (1986)
Tubular CFM	2·9	Proposed hypothesis to explain metabolic oscillations	Ennis and Maddox (1989)

An early report of a continuous culture with cell recycle applied to the ABE fermentation was that of Vijjeswarapu, Chen and Foutch (1985). Cells, and presumably spores, in the effluent stream were heat-shocked by passage through a glass tube held at 80°C, and were then harvested by centrifugation before being returned to the fermenter. Although product concentrations and productivities were low, the use of recycle increased the value of these parameters up to fourfold, compared with a conventional continuous

fermentation. No comments were made regarding the long-term operational stability of the system.

The application of microporous membranes to cell recycle in the ABE fermentation has been described by several authors. Here, the fermenter effluent is pumped to a membrane through which liquid, but not cells, can pass. The cells are then returned to the fermenter. Afschar *et al.* (1985) used a capillary crossflow microfiltration (CFM) device for the recycle, and applied turbidostat control to the fermenter biomass concentration. At a biomass concentration of 8 g l^{-1}, and a dilution rate of 0·64 h^{-1}, a solvent productivity of 5·4 g/l.h was achieved. The authors did not mention any metabolic oscillations, but stated that after 'prolonged' periods of cultivation at high product concentrations, 'degeneration' occurred, i.e. a similar phenomenon to that observed in non-recycle continuous systems. The problem was solved using a two-stage system, with cell recycling and turbidostatic biomass control in both stages. Productivities in the range 2–3 g/l.h were achieved, at solvent concentrations of 12–15 g l^{-1}. Biomass concentrations were 2–3 g l^{-1} and 10–12 g l^{-1} in stages 1 and 2, respectively. This system apparently provided long-term operational stability. Ferras, Minier and Goma (1986) have described the use of a mineral tubular membrane device for the cell recycle, and achieved a biomass concentration of 125 g l^{-1} (no cell-bleed device was used). At this high cell concentration, however, plugging of the membrane occurred. Considerable metabolic oscillation was observed, and after 22 days of operation 'degeneration' was evident. Nevertheless, transient productivity values of up to 6 g/l.h were observed. Schlote and Gottschalk (1986) have described a plate and frame ultrafiltration system for cell recycle in a phosphate-limited continuous culture. Productivities of 6·5 g/l.h and 2·2 g/l.h were recorded at solvent concentrations of 16·25 g l^{-1} and 22 g l^{-1}, respectively. No mention was made of instability problems, perhaps reflecting the use of a nutrient-limited culture.

The application of a hollow-fibre ultrafiltration system has been described by Pierrot, Fick and Engasser (1986). These authors stressed the importance of two parameters, the total dilution rate and the bleed dilution rate. The former determines the throughput of the system, and the latter defines the biomass growth rate. Productivities of 4·5–6·5 g/l.h were achieved at solvent concentrations of 13–16 g l^{-1} and a biomass concentration of 20 g l^{-1}. The authors made no reference to operational stability of the system. More recently, Pierrot *et al.* (1987) have described a two-stage continuous reactor with cell recycle in the second stage. High productivities were claimed at solvent concentrations in excess of 17 g l^{-1}, but no experimental details were provided.

In contrast to most workers, who have used laboratory media for their experiments, Ennis and Maddox (1989) have described the application of a cell recycle system (one-stage fermenter) to a technical substrate, i.e. whey permeate. Initial experiments showed that a tubular CFM unit was superior to plate and frame or hollow-fibre systems, but problems were encountered with filter blockage, necessitating the development of a complex backwashing system. Stable steady-states were difficult to achieve, and culture 'degenera-

tion' was invariably observed. The reasons for the metabolic oscillations were discussed, and similar conclusions were drawn to those of Clarke, Hansford and Jones (1988), i.e. the culture consists of several different morphological/physiological types whose relative proportions fluctuate. Ennis and Maddox (1989) commented that for the substrate used the cell recycle system was inferior, in terms of productivity, stability and ease of operation, to fermentation systems using cells immobilized by either entrapment or adsorption.

In conclusion, it appears that cell recycle systems are useful for achieving increased reactor productivities, although their application to technical substrates remains relatively unknown. Stable steady-states can be achieved and, in contrast to non-recycle systems, high product concentrations are not sacrificed for high productivity. Technically, however, cell recycle is a complex system, and pilot plant studies are required before a full technological assessment and comparison with other types of reactors can be made.

IMMOBILIZED CELLS

Cell immobilization is defined as a technique that confines cells within a reactor system, permitting their easy reuse. Compared to free cell suspensions, greatly increased cell densities can be achieved, resulting in increased fermenter productivities. Further, in continuous culture systems, high dilution rates can be used without fear of washout. Phenomenonologically, a continuous fermentation system using immobilized cells is similar to that using recycle of freely suspended cells. However, immobilized cells have the advantage that they can be used in a variety of reactor types, e.g. packed beds, fluidized beds, that are not readily applicable to free cells. Overall, it appears that continuous fermentation systems using immobilized cells do not suffer from the same stability problems as do free cells. However, a disadvantage of immobilized cells is the additional cost and operation of immobilization.

Alginate-immobilized cells

The majority of reports describing this entrapment technique originate from two laboratories, one in Sweden and one in The Netherlands. The first report was from the former, when Haggstrom (1979) demonstrated the successful immobilization of both spores and cells, and then used the immobilized cells (after germination in the case of spores) in a non-growing mode for solvent production from glucose. Productivities were reported to be superior to those using free cells, and it was stated that immobilization of spores was preferable to that of cells (Haggstrom and Molin, 1980). Subsequent reports from this group described the use of glutaraldehyde to improve the mechanical strength of the alginate beads, and use of the beads in a packed-column reactor with liquid recirculation (Haggstrom, 1981; Haggstrom and Enfors, 1982). The reactor productivities obtained were up to 2·8 g/l.h, but during operation these rapidly decreased to approximately 1 g/l.h. This decrease was attributed to a lack of nutrients, since the feed medium contained only glucose plus salts.

Nevertheless, the continuous fermentation was operated for 42 days, after which time the beads remained mechanically intact. Subsequently, a technique was described for maintaining a constant productivity in non-growing immobilized cells, based on the intermittent addition of nutrients to the reactor (Forberg, Enfors and Haggstrom, 1983). It was suggested that the cells must be maintained in an active, but non-growing, condition for maximum solvent productivity and yield to be achieved. However, the usefulness of this technique to technical substrates is unknown.

In contrast to the Swedish group, workers in The Netherlands did not use glutaraldehyde to mechanically strengthen the alginate beads, and the feed media used contained all necessary nutrients for cell growth. Krouwel, van der Laan and Kossen (1980) immobilized spores in alginate beads, and used them, after germination, in a packed-bed reactor for continuous solvent production. The productivity (1 g/l.h) was approximately fourfold greater than that of traditional batch fermentation using free cells. Although the reactor was operated for nine days, it did suffer from problems of stagnant zones and poor gas release. As with the Swedish group, the Dutch workers preferred to immobilize spores rather than cells, and they have described a start-up procedure involving 'sterilization' and 'activation' of the alginate beads using an ethanol/water mixture (Krouwel, van der Laan and Kossen, 1981).

Because of the difficulties encountered with the packed-bed reactor, a continuous stirred tank reactor (CSTR) was investigated (Krouwel, Groot and Kossen, 1983). The advantages of this reactor type include easy gas release, no concentration gradients, and, because the mixing state is known, the system at steady-state can be modelled relatively easily. Using the CSTR, productivities of up to 4 g/l.h were achieved, and the system was stable and simple to operate. During a further study into a packed-bed reactor, Krouwel *et al.* (1983) reiterated its disadvantages of poor spore germination during start-up (because of pH gradients) and presence of stagnant zones; and the system is not true plug flow. They concluded that this reactor type was not suitable for continuous solvent production using alginate-immobilized cells.

A comprehensive assessment of various types of reactors for alginate-immobilized cells has been performed by Schoutens *et al.* (1986a). Because the ABE process suffers from product inhibition, a plug flow (e.g. packed-bed) reactor should be favoured. However, as mentioned above, this system gives

Table 2. Comparison of reactor types for alginate-immobilized cells (Schoutens *et al.*, 1986a)

Type	Advantages	Disadvantages
CSTR	Allows good spore germination Easy gas release	Solids hold-up only 30% High bead attrition at high stirrer rates Settling of beads at low stirrer rates
Packed bed	Solids hold-up 50–60%	Gas release is difficult Plug flow is unfavourable for biomass development
Fluidized bed	Solids hold-up 40–45% Easy gas release Attrition rates lower than CSTR	Without recycle, plug flow characteristics hinder start-up Recycle increases mixing
Gas-lift loop reactor	Easy gas release Well-mixed Low bead attrition rates	Solids hold-up 20–40%

poor spore germination and poor gas release. Nevertheless, a process kinetic study, in addition to demonstrating that the process is controlled by product inhibition, revealed that a high bead concentration in the reactor favoured increased product concentration and productivity (Schoutens, van Beelen and Luyben, 1986). *Table 2* summarizes the advantages and disadvantages of four reactor types, as described by Schoutens *et al.* (1986a). Subsequently, a more detailed study was made of the fluidized-bed reactor and the gas-lift loop reactor (Schoutens *et al.*, 1986b; Schoutens *et al.*, 1986c). For both reactors, bead attrition rates were low and stable operation was attained. However, on the basis of its overall performance, which was due to its higher reactor loading, the fluidized-bed reactor was considered to be the superior type.

Other reports describing the use of alginate-immobilized cells include that of Ennis, Maddox and Schoutens (1986) who used a CSTR and a fluidized-bed reactor with a substrate of whey permeate. The results confirmed that the process is controlled by the inhibitory butanol concentration, and that the reactor productivities varied as a function of the dilution rate and the bead fraction in the reactor. Stable operation was achieved for 17 days. Taya, Yagi and Kobayashi (1986) described the use of a CSTR, while Largier *et al.* (1985) used a sporulation-deficient mutant in a fluidized-bed reactor. The latter allowed a high productivity (3 g/l.h) at a high solvent concentration (15 g l^{-1}), but the system was operated for only a short period of time.

In addition to alginate, the use of carrageenan-immobilized cells has been studied for continuous solvent production. A stable system with a butanol productivity of 5·2 g/l.h has been described, but no details were provided (Anon., 1981). Frick and Schugerl (1986) compared the use of alginate, κ-carrageenan and chitosan for cell immobilization. The alginate and chitosan beads were dried to improve their stability, while the carrageenan beads were treated with diaminohexane and glutaraldehyde. The fermenter system was a two-stage stirred tank cascade. The alginate-immobilized cells proved to be the most productive (1·9 g/l.h at a solvent concentration of 15 g l^{-1}), and remained stable after 30 days of operation.

Overall, alginate immobilization is a useful technique for improving the productivity of the ABE fermentation, while retaining reasonably high product concentrations. In contrast to free cell systems, there are no reports of instability during operation. In addition, the technique is technically simple to operate, does not require expensive capital equipment, and appears suitable for technical substrates. However, the cost of cell immobilization will need to be considered before its commercial potential is known.

Immobilization by adsorption

In contrast to immobilization in alginate, which is an entrapment technique, immobilization by adsorption of cells onto a solid surface should avoid any problems of nutrient/product diffusion in the bulk liquid to/from the cell. However, accumulation of multicell layers may negate this advantage. Technically, immobilization of cells by adsorption is a cheap and mild procedure, and should be readily amenable to scale-up.

The adsorption of *C. acetobutylicum* onto beechwood shavings has been described as giving reactor productivities comparable to those obtained with alginate immobilization (Forberg and Haggstrom, 1985). The glass reactor was packed with beechwood shavings arranged as parallel sheets on a supporting base of wire netting, under which was a magnetic stirrer. The reactor was started-up using a spore suspension, and was then operated continuously using a glucose/salts feed medium with intermittent dosing of a nutrient solution. In this way, the cells were maintained in an active, but non-growing state. The system was operated under stable conditions for several weeks, with a productivity of 1·5 g/l.h. The excellent cell adsorption that occurred was attributed to an extracellular polysaccharide material produced by the bacterial cells.

Welsh, Williams and Veliky (1987) adsorped cells onto coke and achieved a stable steady-state with a productivity of 1·2 g/l.h at a solvent concentration of 12 g l^{-1}. Bonechar, as used in sugar refining, has been used as a solid support by Qureshi and Maddox (1987). Here, the bonechar was placed in a glass column, and was inoculated by pumping in a growing culture. The system was then operated as a packed-bed reactor. During start-up, biomass accumulation was favoured by operating at dilution rates high enough to prevent growth-inhibitory concentrations of solvents. Once biomass was accumulated, the dilution rate was decreased so that excessive biomass growth was prevented by the inhibitory solvent concentration. Although the system was operated successfully for 62 days, it finally suffered from problems of blockage, due to excess biomass growth, and gas hold-up. Recently, a mathematical model has been presented to describe this packed-bed reactor (Qureshi, Paterson and Maddox, 1988). The model postulates the presence of different morphological/physiological cells types within the reactor, similar to those described by Clarke, Hansford and Jones (1988) for a free cell continuous fermentation. The point is made that further increases in reactor productivities, at sufficiently high solvent concentrations, will depend on achieving a greater understanding of the factors affecting the life cycle of the organism.

Because of the problems encountered with the packed-bed reactor, investigations were made into various configurations of this reactor type, and into a fluidized-bed reactor. With the packed bed, productivities of up to 6 g/l.h were achieved at solvent concentrations of 6–7 g l^{-1}, but blockage and gas hold-up always presented problems. The fluidized-bed reactor, however, proved to be extremely stable, and was operated for 50 days at productivities approaching 5 g/l.h. Interestingly, based on the bonechar loading, the fluidized-bed reactor was six times more productive than the packed bed, probably due to greater amounts of biomass being present (Qureshi and Maddox, 1988).

Maddox (1988) has compared the use of bonechar-immobilized cells, alginate-immobilized cells and a cell recycle technique, using crossflow microfiltration, for solvent production from a potential commercial substrate, whey permeate. The highest reactor productivities were achieved using bonechar-immobilized cells, and the system was stable and very simple to operate. Alginate-immobilized cells also provided a simple and stable process,

but reactor productivities were lower. The cell recycle technique was technically difficult to operate, and stable conditions could not be attained. However, it was stressed that the cell recycle technique could be more useful for other substrates, not suffering from the handicap of precipitation, and hence membrane-fouling, problems that are always encountered when using whey permeate as a substrate.

Integrated fermentation/product recovery techniques

As mentioned earlier, one of the major problems of the ABE fermentation process is that it suffers severely from product inhibition at total solvent concentrations of less than 20 g l^{-1}. This leads to a dilute aqueous solution being presented to product recovery, with concomitant adverse effects on the economics of the process. In addition, product inhibition restricts the application of fed-batch culture to the fermentation process, and so advantage cannot be taken of this technique to improve reactor productivities. One solution to these problems is to develop an integrated fermentation/product recovery process whereby toxic solvents are removed from the culture at a rate similar to that at which they are being formed. In this way, it should be possible to ferment more concentrated sugar solutions (giving reductions in capital costs), obtain higher fermenter productivities, and, depending on the technique used, reduce the costs of product recovery. Possible integrated processes have been described by Ennis, Gutierrez and Maddox (1986), and some applications are given below.

ADSORPTION

The desired characteristics of an adsorbent for use in the ABE process include high adsorbent capacity for ethanol, acetone and butanol, but not sugar, acetic acid, butyric acid or any nutrients; favourable adsorption kinetics; and efficient adsorption over varying solvent concentrations (Ennis, Gutierrez and Maddox, 1986). Adsorbents which have been investigated are summarized in *Table 3*.

Silicalite (a zeolite analogue) is a molecular sieve which can preferentially adsorb alcohol from alcohol–water mixtures. Equilibrium adsorption isotherms for ethanol and butanol have been presented (Milestone and Bibby, 1981). The latter was concentrated from 0·5% (w/v) to 98% (w/v), with subsequent thermal desorption at 150°C and reuse of the silicalite. Maddox (1982) subsequently used silicalite at the end of a batch fermentation process, and demonstrated its effectiveness at adsorbing butanol. More recently, silicalite has been used between stages of a two-stage continuous fermentation process (Ennis, Qureshi and Maddox, 1987). The fermentation was performed in two packed-bed reactors in series, using bonechar-immobilized cells. In the control experiment, without between-stages product removal, little fermentation took place in stage 2, despite the presence of sufficient sugar, indicating that product inhibition was occurring. However, when the stage 1 effluent was treated with silicalite prior to stage 2, further fermentation occurred. The data

Table 3. Adsorbents that have been investigated for the ABE fermentation

Adsorbent	Comments	Reference
Zeolite and silicalite	Adsorption capacity 80–90 mg butanol/g resin May adsorb some nutrients	Milestone and Bibby (1981); Maddox (1982); Larsson and Mattiasson (1984); Ennis, Qureshi and Maddox (1987)
XAD series	Adsorption capacity inferior or similar to silicalite May adsorb some nutrients	Groot and Luyben (1986); Das, Soni and Ghose (1987); Ennis, Qureshi and Maddox (1987); Nielsen *et al.* (1988)
Bonopore	Adsorption capacity similar to silicalite Does not adsorb nutrients	Larsson and Mattiasson (1984); Nielsen *et al.* (1988)
Charcoal and activated carbon		Groot and Luyben (1986); Das, Soni and Ghose (1987)
IRC-50		Das, Soni and Ghose (1987)

showed good adsorption of solvents onto the silicalite, but not of sugar. However, there was evidence that some other nutrients were removed from the broth. Similar results were observed for the polymeric resin XAD-16. A synthetic zeolite has also been investigated by Larsson and Mattiasson (1984). In their experiments, the fermenting culture was pumped through a membrane unit, from which the cells were returned to the fermenter while the broth was passed through a zeolite column before recycling to the fermenter. The adsorption capacity of the zeolite was given as 5%, but few experimental details were provided. Similar experiments were performed with a crosslinked divinylbenzene-styrene copolymer, whose adsorption capacity was given as 7%.

The XAD series of resins (Rohm and Hass, Philadelphia) have been investigated by several authors. Groot and Luyben (1986) added the resins to a batch fermentation medium prior to inoculation, and determined the amount of butanol produced. The adsorbents became fouled with cells, but this did not seem to affect their adsorption capacities (50–80 mg g^{-1} resin) in subsequent fermentations. XAD-8 was the most successful of the series studied. Other members of the series have been investigated by Nielsen *et al.* (1988) and Das, Soni and Ghose (1987). The overall conclusion seems to be that while they have reasonable adsorption capacities for butanol, some essential nutrients are also removed from the broth. While this would not be a problem if the resins were to be used only at the end of a batch fermentation as an intermediate step prior to product recovery by distillation, it may preclude their use in integrated fermentation/product recovery processes, unless additional nutrients are added to the culture.

Bonopore (a divinylbenzene-styrene copolymer; Nobelkemi AB, Sweden) has been investigated by Nielsen *et al.* (1988), who showed that it does not remove nutrients from the fermenting broth. Since its adsorption capacity for butanol is similar to that of the XAD series, it appears to be a more suitable

adsorbent than either this series or silicalite. Although Bonopore does adsorb butyric acid, this can be minimized by raising the value of the culture pH. Bonopore was tested in repeated batch cultures, where it was shown to improve cell growth, sugar utilization and solvent production. The bacterial cells were removed from the culture prior to the adsorption stage, and subsequently added back.

GAS STRIPPING

The use of gas stripping to remove inhibitory solvents during the ABE fermentation, followed by condensation for product recovery, has been demonstrated by Ennis *et al.* (1986). In a batch fermentation, using nitrogen as the stripping gas at a flow rate of 2 vvm, significant increases were recorded in sugar utilization rate and solvent productivity. Further experiments have been performed using gas stripping as a between-stages product removal technique in a two-stage continuous fermentation process (Ennis, Qureshi and Maddox, 1987). The effluent from stage 1, which contained sugar but in which fermentation had ceased due to product inhibition, was gas stripped prior to being passed to stage 2, where further fermentation occurred. In comparison with solid adsorbents and liquid extractants, gas stripping removes only volatile solvents from the cultures, and the technique has been suggested to be superior to the use of the XAD series or silicalite (Ennis, Qureshi and Maddox, 1987). However, no information is available regarding the costs associated with solvent recovery from the vapour phase following gas stripping.

REVERSE OSMOSIS

There are few reports on the application of reverse osmosis to product removal during solvent production, so it is difficult to assess its potential. Garcia, Ianotti and Fischer (1986) have used polyamide membranes in an integrated continuous fermentation process. Ultrafiltration of the fermenter effluent was used for cell recycle, and the ultrafiltrate was passed to reverse osmosis for solvents recovery. The permeate was recycled to the fermenter. Hollow-fibre ultrafiltration was considered to be a suitable pre-treatment for reverse osmosis, although fouling and plugging were problems. The flux of fermentation liquor through the reverse osmosis membrane was only one-third of that of a model solution. Butanol rejection values of 98% were possible, at recoveries of 20–45%. The authors concluded that the technique can overcome the problems of dilute solvent solutions and low fermenter productivities.

LIQUID–LIQUID EXTRACTION (EXTRACTIVE FERMENTATION)

In extractive fermentation, a solvent is contacted with the broth during fermentation; inhibitory products dissolve into the solvent, and product inhibition is reduced. Products dissolved in the solvent phase can be recovered by distillation or back-extraction into another solvent (Roffler, Blanch and Wilke, 1987a). The basic requirements of the technique have been well described by Mattiasson and Larsson (1985) and Ennis, Gutierrez and Maddox

(1986). The following criteria need to be considered in the selection of a solvent for extractive ABE fermentation:

1. Immiscibility with the fermentation broth;
2. High partition coefficient for the fermentation products;
3. High selectivity for the fermentation products, i.e. no removal of nutrients or intermediates;
4. Non-toxicity to the producing organism;
5. Products can be separated easily from the solvent, and the solvent can be recovered easily;
6. The solvent is cheap and available in large quantities;
7. The solvent can be sterilized, is non-flammable, and has relatively low viscosity;
8. The solvent does not form stable emulsions with the fermentation broth.

A list of solvents that have been investigated is given in *Table 4*.

An early report on the application of extractive fermentation to the ABE process was that of Wang *et al.* (1979). These authors used corn oil in repeated batch fermentation, and demonstrated an improvement in butanol production. However, there was a rapid loss of culture activity after a second replacement of the corn oil. Subsequently, several groups have surveyed a range of solvents for their use as butanol extractants, but not all of these solvents have been

Table 4. Solvents that have been investigated for extractive ABE fermentation

Solvent	Fermentation technique	Reference
Alcohols	Extractive batch	Roffler, Blanch and Wilke (1987a) Dadgar and Foutch (1985) Griffith, Compere and Googin (1983)
Decanol	Cell recycle to avoid toxicity	Eckert and Schugerl (1987)
Decanol/oleyl alcohol	Extractive batch	Evans and Wang (1988)
Hexanol	Dialysis	Traxler *et al.* (1985)
Oleyl alcohol	Extractive batch and fed batch	Roffler, Blanch and Wilke (1987a) Roffler, Blanch and Wilke (1987b) Roffler, Blanch and Wilke (1988) Taya, Ishii and Kobayashi (1985) Ishii, Taya and Kobayashi (1985) Honda *et al.* (1987) Shimizu and Matsubara (1987)
Alkanes	Extractive batch	Roffler, Blanch and Wilke (1987a) Dadgar and Foutch (1985)
Aromatic hydrocarbons		Dadgar and Foutch (1985)
Corn oil	Extractive batch	Wang *et al.* (1979)
Esters	Extractive batch	Roffler, Blanch and Wilke (1987a) Dadgar and Foutch (1985) Griffith, Compere and Googin (1983)
Dibutylphthalate	Extractive batch	Wayman and Parekh (1987) Parekh, Parekh and Wayman (1988)
Vegetable oil fatty acid esters		Griffith, Compere and Googin (1984)
Halogenated hydrocarbons	Extractive batch	Levy (1984) Dadgar and Foutch (1985)
Ketones		Dadgar and Foutch (1985)
Polyoxyalkylene ethers	Extractive batch	Griffith, Compere and Googin (1983)

tested during an actual fermentation process. Griffith, Compere and Googin (1983) measured the partition coefficients of a range of alcohols, esters and polyoxyalkylene ethers, and tested several in culture. Although alcohols and esters generally displayed superior partition coefficients, they were more toxic to *C. acetobutylicum* than were the polyoxyalkylene ethers (partition coefficient 1·5–3). Hence, the latter were considered to be more useful. Subsequently, some vegetable oil fatty acid esters were also investigated (Griffith, Compere and Googin, 1984). These esters are useful because of their partition coefficient (up to 3), low toxicity and low viscosity, but no fermentation data have been presented regarding their use. Other reports which include surveys of a range of potential extractants include those by Dadgar and Foutch (1985) and Roffler, Blanch and Wilke (1987a). The results may be summarized:

1. Alkanes are poor extractants, but are non-toxic and separate rapidly from the fermentation broth. Substitution with halogen groups improves the partition coefficient, e.g. Levy (1984) used Freon F11, but few experimental data were presented.
2. Esters are good extractants, are relatively non-toxic, but their phase separation behaviour may be a disadvantage. Nevertheless, dibutylphthalate has been studied in some detail (*see* below).
3. Alcohols are good extractants, but some suffer from toxicity to *C. acetobutylicum*.

From the available information, it appears that there is no one solvent that meets all of the selection criteria, e.g. good extractants tend to be toxic, but some have been considered in further detail.

Oleyl alcohol has been the subject of several recent reports on extractive ABE fermentation. Taya, Ishii and Kobayashi (1985) described its use in a fed-batch extractive fermentation, and demonstrated increased sugar consumption and butanol production. However, they commented on the relatively low partition coefficient of oleyl alcohol for acetone. Although acetone concentrations are unlikely to become inhibitory during the fermentation process, this point must be of some concern with respect to total product recovery. Possibly, some authors have paid insufficient attention to extraction of acetone and ethanol. The choice of oleyl alcohol as a suitable butanol extractant has been confirmed by other reports (Ishii, Taya and Kobayashi, 1985; Honda *et al.*, 1987; Shimizu and Matsubara, 1987).

In a series of papers, Roffler and co-workers have provided further information on the use of oleyl alcohol (Roffler, Blanch and Wilke, 1987a, 1987b, 1988; Roffler, Wilke and Blanch, 1988). In an extractive batch fermentation, glucose utilization was increased from 80 g l^{-1} to 100 g l^{-1}, while the butanol productivity was 60% higher than in a traditional batch fermentation. In fed-batch extractive fermentation, a feed glucose solution of 339 g l^{-1}, was fermented at an overall butanol productivity of 1·3 g/l.h. Thus, the extraction technique allows advantage to be taken of fed-batch culture. The fermentation was commenced in batch culture, and when solventogenesis began oleyl alcohol was added to the fermenter and the glucose/nutrient feed

was initiated. Because a nutrient solution, rather than simply glucose, was continuously fed, cell growth continued and biomass concentrations of up to 14 g l^{-1} were attained (Roffler, Blanch and Wilke, 1987b). The authors commented that when separate aqueous and organic phases are maintained in the fermenter during extractive fermentation, the rate of butanol transfer from the aqueous to organic phase may be much slower than the rate of butanol production. Although the interfacial area could be increased by agitation, this could make phase separation difficult. Hence, it would be preferable to contact solvent and fermentation broth in a separate extraction vessel. This concept of a fed-batch continuous extraction was subsequently applied by recycling whole broth, containing cells, to a Karr reciprocating plate extraction column in which butanol was extracted by oleyl alcohol flowing countercurrently through the column (Roffler, Blanch and Wilke, 1988). In this way, a feed solution containing glucose at 300 g l^{-1} was fermented at an overall butanol productivity of 1 g/l.h.

As mentioned above, some potentially useful butanol extractants suffer the disadvantage of being toxic to *C. acetobutylicum*. However, some workers have been able to minimize this disadvantage, and thus use solvents with superior partition coefficients. Traxler *et al.* (1985) used dialysis fermentation in combination with extraction using hexanol. In this way, the toxic hexanol was kept apart from the biomass, and significant increases in butanol productivity were obtained. A similar approach has been adopted to prevent contact of toxic *n*-decanol with the bacterial cells (Eckert and Schugerl, 1987). Here, a continuous culture, cell-recycle fermenter was utilized. The effluent stream from the fermenter was pumped to a crossflow microfiltration unit, from where the cells were returned to the fermenter while the permeate was extracted with decanol in a four-stage mixer–settler cascade. The extracted broth was returned to the fermenter. A productivity of 3·08 g/l.h was achieved, but the reactor-extraction system was rather complex. Interestingly, the decanol that was used was saturated with butyric acid, to prevent extraction from the broth of this metabolic intermediate.

Another approach with *n*-decanol has been to use it in admixture with oleyl alcohol (Evans and Wang, 1988). Thus decanol, an extractant with a high partition coefficient (6·2), but high toxicity, was mixed with oleyl alcohol, an extractant with a relatively low partition coefficient (3·2) but low toxicity. Up to 40% (v/v) decanol in oleyl alcohol proved to be non-toxic to *C. acetobutylicum*, provided that the fermenting culture was maintained at pH 4·5. Using a mixture of 20% (v/v) decanol in oleyl alcohol, butanol production was increased by 72%.

An example of an ester being used as an extractant is that of dibutylphthalate (Wayman and Parekh, 1987; Parekh, Parekh, and Wayman, 1988). Although this solvent has only a low partition coefficient for butanol (1·39), it was chosen on the basis of its low water solubility and lack of toxicity. In addition, it is commercially available, sterilizable and does not form stable emulsions. Using a cell-recycle technique to obtain a high biomass concentration, Wayman and Parekh (1987) performed repeated batch extractive fermentations. Butanol

plus acetone concentrations of up to 32 g l^{-1} (based on aqueous volume) were achieved.

In summary, it appears that there are two approaches to the application of extractive fermentation to the ABE process. The first uses extractants with relatively poor partition coefficients but which are non-toxic, while the second uses extractants with higher partition coefficients but, because of their toxicity, additional equipment and/or technology may be required to avoid contact between cells and extractant. Given the low value of butanol as an industrial chemical, and hence the need to reduce capital costs during production, it may be that the first approach is the more realistic for commercial application.

AQUEOUS TWO-PHASE EXTRACTION

One of the problems of extraction using organic solvents is that the extractant is often toxic to the bacterial cells. Aqueous two-phase extraction presents a possible solution to this problem. The principles of the technique, and its application to the ABE process, have been described (Mattiasson, 1983; Larsson and Mattiasson, 1984; Mattiasson and Larsson, 1985). The phase system consisted of a glucose nutrient medium containing 6% dextran, and 25% polythylene glycol (molecular weight, 20 000), in a ratio of 1 : 6. Hence, the bacterial cells were concentrated in the bottom nutrient phase, and the fermentation products were free to equilibrate between the phases. Compared with a control fermentation, the two-phase system gave a similar productivity (0·25 g/l.h). The commercial potential of this technique is difficult to assess, but it appears that the exact composition of the phase system is very important (Mattiasson and Larsson, 1985).

PERVAPORATION

Pervaporation is a membrane process in which liquids diffuse through a solid membrane, and are then evaporated and removed by a gas stream, or by applying a vacuum. When the components of the liquid show different diffusivities in the membrane, the mixture can be separated (Groot, van den Oever and Kossen, 1984; Ennis, Gutierrez and Maddox, 1986). Application of pervaporation to a batch ABE fermentation has been described by Groot, van den Oever and Kossen (1984) and by Larrayoz and Puigjaner (1987). Both groups used silicone membranes and nitrogen as the gas stream, but whereas the former circulated the fermentation broth to a separate pervaporation unit, the latter placed the membranes inside the fermentation medium. The selectivity of the system for butanol and acetone is 50 and 35, respectively, but lower values are more realistic when fermentation broths are used. The system shows no selectivity for ethanol or acetic and butyric acids. Under typical batch fermentation conditions, application of pervaporation results in higher productivity values. However, the real benefit is noticed when using initial sugar concentrations higher than normal (Larrayoz and Puigjaner, 1987). With an initial sugar concentration of 100 g l^{-1}, without pervaporation, butanol and acetone production totalled 23 g l^{-1}, and not all substrate was used. With

product removal, the butanol plus acetone concentration was increased to 35 g l^{-1}, and the sugar was completely exhausted. Unfortunately, product removal by pervaporation appears not to have been applied yet to fed-batch fermentation.

Product removal by pervaporation has been applied to continuous fermentations using alginate-immobilized cells (Groot *et al.*, 1984; Groot and Luyben, 1987). Productivity increases of up to 70% have been recorded, coupled with increased sugar utilization.

A preliminary report has described the use of hydrophobic microporous membranes rather than silicone membranes (Pons and Tillier-Dorion, 1987). It is claimed that mass transfer is considerably improved, but no data were provided for butanol or acetone.

The use of a liquid membrane rather than a solid membrane has been described by Matsumura *et al.* (1988). The liquid membrane (oleyl alcohol) was supported on a microporous polypropylene flat sheet (25 μm thick), and the liquids diffused through, to be removed by a gas stream in the normal way. A selectivity for butanol of 180 was claimed, but no data were provided using fermentation broths. It was estimated that should this pervaporation technique be used as a pre-treatment process for butanol purification, the energy requirement would be only 10% of that of conventional distillation.

A recent development of pervaporation is 'perstraction'. Here, the liquid passes through a membrane (e.g. a silicone membrane), but instead of being evaporated by a gas stream, it is withdrawn by a liquid extractant (usually an organic solvent). The advantage of perstraction over conventional extraction is that the aqueous and organic phases are separated from each other, hence emulsions cannot form. The advantage over pervaporation is the high selectivity of the system. Groot, Timmer and Luyben (1987) have applied the technique to the ABE fermentation. They used a silicone rubber membrane coupled with isopropyl myristate (non-toxic to *C. acetobutylicum*), and demonstrated that product removal from the broth resulted in increased sugar utilization. It was estimated that the diffusion of butanol in the membrane using perstraction was seven times greater than with pervaporation. Although this technique appears very promising, it is clear that further work is needed to find the optimal combination of membrane and extractant.

Conclusions

At present, the ABE fermentation is uneconomic. Consequently, efforts are being made to develop a technology which satisfies the following requirements:

1. High reactor productivity;
2. High solvent concentration;
3. Process is technically simple to operate;
4. Long-term stability of reactor (for continuous fermentation).

Many reports have appeared recently describing continuous fermentation processes, including the use of immobilized cells and cell-recycle techniques. However, these technologies are little used industrially for any fermentation

products, hence there is little expertise/experience available for large-scale operation. Conversely, fed-batch culture is widely practised industrially, for a variety of fermentation products. Unfortunately, the ABE fermentation is not well suited to this technology, unless a simultaneous product removal technique is developed.

It is generally accepted that alleviation of product inhibition is the major goal in the development of an economic fermentation process. This would allow high solvent concentrations and productivities to be achieved. It is unlikely that major gains will be made in the development of solvent-tolerant bacterial strains. Hence, this goal will be reached only through the development of appropriate technology. Therefore, given the availablity of expertise in fed-batch culture, it would appear that this technology, coupled with a simultaneous product removal technique, has a good chance of success. However, the product removal technique must not be too capital-intensive or difficult to operate, since butanol remains only a low-value product. Therefore, pilot-plant data are awaited concerning the integrated fed-batch fermentation/product recovery techniques mentioned in this article, so that appropriate costings can be made.

References

AFSCHAR, A.S., BIEBL, H., SCHALLER, K. AND SCHUGERL, K. (1985). Production of acetone and butanol by *Clostridium acetobutylicum* in continuous culture with cell recycle. *Applied Microbiology and Biotechnology* **22**, 394–398.

ANDERSCH, W., BAHL, H. AND GOTTSCHALK, G. (1982). Acetone–butanol production by *Clostridium acetobutylicum* in an ammonium-limited chemostat at low pH values. *Biotechnology Letters* **4**, 29–32.

ANON (1981). In *Alcohol fuels program technical review*, US Department of Energy, Winter 1981, p. 50.

AWANG, G.M., JONES, G.A. AND INGLEDEW, W.M. (1988). The acetone–butanol–ethanol fermentation. *Critical Reviews in Microbiology* **15**(1), S33–S67.

BAHL, H. AND GOTTSCHALK, G. (1984). Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture. *Biotechnology and Bioengineering Symposium* No. 14, pp. 215–223.

BAHL, H., ANDERSCH, W. AND GOTTSCHALK, G. (1982). Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. *European Journal of Applied Microbiology and Biotechnology* **15**, 201–205.

BAHL, H., GOTTWALD, M., KUHN, A., RALE, V., ANDERSCH, W. AND GOTTSCHALK, G. (1986). Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*. *Applied and Environmental Microbiology* **52**, 169–172.

BARBEAU, J.Y., MARCHAL, R. AND VANDECASTEELE, J.P. (1988). Conditions promoting stability of solventogenesis or culture degeneration in continuous fermentations of *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **29**, 447–455.

BARBEAU, J.Y., MARCHAL, R., FAYOLLE, F. AND VANDECASTEELE, J.P. (1986). Reversibility of acids and solvents metabolism in *Clostridium acetobutylicum* grown in a two-stage continuous culture. In *Biology of Anaerobic Bacteria* (H.C. Dubourguier, G. Albagnac, J. Montreuil, C. Romond, P. Sautiere and J. Guillaume, Eds), pp. 124–129. Elsevier, Amsterdam.

BROSSEAU, J.D., YAN, J.Y. AND LO, K.V. (1986). The relationship between hydrogen

gas and butanol production by *Clostridium saccharoperbutylacetonicum*. *Biotechnology and Bioengineering* **28**, 305–310.

BRYANT, D.L. AND BLASCHEK, H.P. (1988). Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*. *Journal of Industrial Microbiology* **3**, 49–55.

CLARKE, K.G. AND HANSFORD, G.S. (1986). Production of acetone and butanol by *Clostridium acetobutylicum* in a product limited chemostat. *Chemical Engineering Communications* **45**, 75–81.

CLARKE, K.G., HANSFORD, G.S. AND JONES, D.T. (1988). Nature and significance of oscillatory behaviour during solvent production by *Clostridium acetobutylicum* in continuous culture. *Biotechnology and Bioengineering* **32**, 538–544.

DADGAR, A.M. AND FOUTCH, G.L. (1985). Evaluation of solvents for the recovery of *Clostridium* fermentation products by liquid–liquid extraction. *Biotechnology and Bioengineering Symposium* No. 15, pp. 611–620.

DAS, K., SONI, B.K. AND GHOSE, T.K. (1987). Static and column studies on selective adsorption–desorption of butanol. In *Proceedings of the 4th European Congress on Biotechnology* (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, Eds), volume 1, pp. 76–79. Elsevier, Amsterdam.

DATTA, R. AND ZEIKUS, J.G. (1985). Modulation of acetone–butanol–ethanol fermentation by carbon monoxide and organic acids. *Applied and Environmental Microbiology* **49**, 522–529.

DOREMUS, M.G., LINDEN, J.C. AND MOREIRA, A.R. (1985). Agitation and pressure effects on acetone–butanol fermentation. *Biotechnology and Bioengineering* **27**, 852–860.

DYR, J., PROTIVA, J. AND PRAUS, R. (1958). Formation of neutral solvents in continuous fermentation by means of *Clostridium acetobutylicum*. In *Continuous Cultivation of Microorganisms, A Symposium* (I. Malek, Ed.), pp. 210–226. Czechoslovak Academy of Sciences, Prague.

ECKERT, G. AND SCHUGERL, K. (1987). Continuous acetone–butanol production with direct product removal. *Applied Microbiology and Biotechnology* **27**, 221–228.

ENNIS, B.M. AND MADDOX, I.S. (1987). The effect of pH and lactose concentration on solvent production from whey permeate using *Clostridium acetobutylicum*. *Biotechnology and Bioengineering* **29**, 329–334.

ENNIS, B.M. AND MADDOX, I.S. (1989). Production of solvents (ABE fermentation) from whey permeate by continuous fermentation in a membrane bioreactor. *Bioprocess Engineering* **4**, 27–34.

ENNIS, B.M., GUTIERREZ, N.A. AND MADDOX, I.S. (1986). The acetone–butanol–ethanol fermentation: a current assessment. *Process Biochemistry* **21**, 131–147.

ENNIS, B.M., MADDOX, I.S. AND SCHOUTENS, G.H. (1986). Immobilized *Clostridium acetobutylicum* for continuous butanol production from whey permeate. *New Zealand Journal of Dairy Science and Technology* **21**, 99–109.

ENNIS, B.M., QURESHI, N. AND MADDOX, I.S. (1987). In-line toxic product removal during solvent production by continuous fermentation using immobilized *Clostridium acetobutylicum*. *Enzyme and Microbial Technology* **9**, 672–675.

ENNIS, B.M., MARSHALL, C.T., MADDOX, I.S. AND PATERSON, A.H.J. (1986). Continuous product recovery by *in-situ* gas stripping/condensation during solvent production from whey permeate using *Clostridium acetobutylicum*. *Biotechnology Letters* **8**, 725–730.

EVANS, P.J. AND WANG, H.Y. (1988). Enhancement of butanol formation by *Clostridium acetobutylicum* in the presence of decanol–oleyl alcohol mixed extractants. *Applied and Environmental Microbiology* **54**, 1662–1667.

FERRAS, E., MINIER, M. AND GOMA, G. (1986). Acetonobutylic fermentation: improvement of performances by coupling continuous fermentation and ultrafiltration. *Biotechnology and Bioengineering* **28**, 523–533.

FICK, M. AND ENGASSER, J.M. (1986). The deflocculating effect of chloride salts (ions) on *Clostridium acetobutylicum* grown in continuous cultures. *Applied Microbiology and Biotechnology* **25**, 194–196.

FICK, M., PIERROT, P. AND ENGASSER, J.M. (1985). Optimal conditions for long-term stability of acetone–butanol production by continuous cultures of *Clostridium acetobutylicum*. *Biotechnology Letters* **7**, 503–508.

FINN, R.K. AND NOWREY, J.E. (1958). A note on the stability of clostridia when held in continuous culture. *Applied Microbiology* **7**, 29–32.

FOND, O., PETITDEMANGE, E., PETITDEMANGE, H. AND GAY, R. (1984). Effect of glucose flow on the acetone butanol fermentation in fed batch culture. *Biotechnology Letters* **6**, 13–18.

FOND, O., ENGASSER, J.M., MATTA-EL-AMOURI, G. AND PETITDEMANGE, H. (1986). The acetone butanol fermentation on glucose and xylose. II. Regulation and kinetics in fed-batch cultures. *Biotechnology and Bioengineering* **28**, 167–175.

FORBERG, C. AND HAGGSTROM, L. (1985). Control of cell adhesion and activity during continuous production of acetone and butanol with adsorbed cells. *Enzyme and Microbial Technology* **7**, 230–234.

FORBERG, C., ENFORS, S.-O. AND HAGGSTROM, L. (1983). Control of immobilized, non-growing cells for continuous production of metabolites. *European Journal of Applied Microbiology and Biotechnology* **17**, 143–147.

FRICK, CH. AND SCHUGERL, K. (1986). Continuous acetone–butanol production with free and immobilized *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **25**, 186–193.

GARCIA, A., IANOTTI, E.L. AND FISCHER, J.L. (1986). Butanol fermentation liquor production and separation by reverse osmosis. *Biotechnology and Bioengineering* **28**, 785–791.

GEORGE, H.A. AND CHEN, J.S. (1983). Acidic conditions are not obligatory for onset of butanol formation by *Clostridium beijerinckii* (Synonym, *C. butylicum*). *Applied and Environmental Microbiology* **46**, 321–327.

GODIN, C. AND ENGASSER, J.M. (1988). Improved stability of the continuous production of acetone–butanol by *Clostridium acetobutylicum* in a two-stage process. *Biotechnology Letters* **10**, 389–392.

GOTTSCHAL, J.C. AND MORRIS, J.G. (1981). Non-production of acetone and butanol by *Clostridium acetobutylicum* during glucose- and ammonium-limitation in continuous culture. *Biotechnology Letters* **3**, 525–530.

GOTTSCHAL, J.C. AND MORRIS, J.G. (1982). Continuous production of acetone and butanol by *Clostridium acetobutylicum* growing in turbidostat culture. *Biotechnology Letters* **4**, 477–482.

GRIFFITH, W.L., COMPERE, A.L. AND GOOGIN, J.M. (1983). Novel neutral solvents fermentations. *Developments in Industrial Microbiology* **24**, 347–352.

GRIFFITH, W.L., COMPERE, A.L. AND GOOGIN, J.M. (1984). 1-Butanol extraction with vegetable-oil, fatty-acid esters. *Developments in Industrial Microbiology* **25**, 795–800.

GROOT, W.J. AND LUYBEN, K.CH.A.M. (1986). *In situ* product recovery by adsorption in the butanol/isopropanol batch fermentation. *Applied Microbiology and Biotechnology* **25**, 29–31.

GROOT, W.J. AND LUYBEN, K.CH.A.M. (1987). Continuous production of butanol from a glucose/xylose mixture with an immobilized cell system coupled to pervaporation. *Biotechnology Letters* **9**, 867–870.

GROOT, W.J., TIMMER, J.M.K. AND LUYBEN, K.CH.A.M. (1987). Membrane solvent extraction for *in-situ* butanol recovery in fermentations. In *Proceedings of the 4th European Congress on Biotechnology* (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, Eds), volume 2, pp. 564–566. Elsevier, Amsterdam.

GROOT, W.J., VAN DEN OEVER, C.E. AND KOSSEN, N.W.F. (1984). Pervaporation for simultaneous product recovery in the butanol/isopropanol batch fermentation. *Biotechnology Letters* **6**, 709–714.

GROOT, W.J., SCHOUTENS, G.H., VAN BEELEN, P.N., VAN DEN OEVER, C.E. AND KOSSEN, N.W.F. (1984). Increase of substrate conversion by pervaporation in the continuous butanol fermentation. *Biotechnology Letters* **6**, 789–792.

HAGGSTROM, L. (1979). Production of acetone and butanol with immobilized cells of

Clostridium acetobutylicum. In *Symposium Technische Mikrobiologie* (H. Dellweg, Ed.), pp. 271–280. Berlin Institut für Gurungswerbe und Biotechnologie, Berlin.

HAGGSTROM, L. (1981). Immobilized cells of *Clostridium acetobutylicum* for butanol production. In *Advances in Biotechnology* (M. Moo-Young and C.W. Robinson, Eds), volume 2, pp. 79–83. Pergamon Press, Toronto.

HAGGSTROM, L. AND ENFORS, S.O. (1982). Continuous production of butanol with immobilized cells of *Clostridium acetobutylicum*. *Applied Biochemistry and Biotechnology* **7**, 35–37.

HAGGSTROM, L. AND MOLIN, N. (1980). Calcium alginate immobilized cells of *Clostridium acetobutylicum* for solvent production. *Biotechnology Letters* **2**, 241–246.

HOLT, R.A., STEPHENS, G.M. AND MORRIS, J.G. (1984). Production of solvents by *Clostridium acetobutylicum* cultures maintained at neutral pH. *Applied and Environmental Microbiology* **48**, 1166–1170.

HONDA, H., MANO, T., TAYA, M., SHIMIZU, K., MATSUBARA, M. AND KOBAYASHI, T. (1987). A general framework for the assessment of extractive fermentations. *Chemical Engineering Science* **42**, 493–498.

HOSPODKA, J. (1966). Production of acetone and butanol by continuous fermentation. In *Theoretical and Methodological Basis of Continuous Culture of Microorganisms* (I. Malek and Z. Fencl, Eds), pp. 611–613. Academic Press, London.

ISHII, S., TAYA, M. AND KOBAYASHI, T. (1985). Production of butanol by *Clostridium acetobutylicum* in extractive fermentation system. *Journal of Chemical Engineering Japan* **18**, 125–130.

JOBSES, I.M.L. AND ROELS, J.A. (1983). Experience with solvent production by *Clostridium beijerinckii* in continuous culture. *Biotechnology and Bioengineering* **25**, 1187–1194.

JONES, D.T. AND WOODS, D.R. (1986). Acetone–butanol fermentation revisited. *Microbiological Reviews* **50**, 484–524.

JUNELLES, A.M., JANATI-IDRISSI, R., PETITDEMANGE, H. AND GAY, R. (1988). Iron effect on acetone–butanol fermentation. *Current Microbiology* **17**, 299–303.

KIM, T.S. AND KIM, B.Y. (1988). Electron flow shift in *Clostridium acetobutylicum* fermentation by electrochemically introduced reducing equivalent. *Biotechnology Letters* **10**, 123–128.

KIM, B.H., BELLOWS, P., DATTA, R. AND ZEIKUS, J.G. (1984). Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. *Applied and Environmental Microbiology* **48**, 764–770.

KLEI, H.E., SUNDSTROM, D.W. AND MILLER, J.E. (1984). Fermentation by *Clostridium acetobutylicum* under carbon dioxide pressure. *Biotechnology and Bioengineering Symposium* No. 14, pp. 353–364.

KROUWEL, P.G., GROOT, W.J. AND KOSSEN, N.W.F. (1983). Continuous IBE fermentation by immobilized growing *Clostridium beijerinckii* cells in a stirred-tank fermenter. *Biotechnology and Bioengineering* **25**, 281–299.

KROUWEL, P.G., VAN DER LAAN, W.F.M. AND KOSSEN, N.W.F. (1980). Continuous production of *n*-butanol and isopropanol by immobilized growing *Clostridium butylicum* cells. *Biotechnology Letters* **2**, 253–258.

KROUWEL, P.G., VAN DER LAAN, W.F.M. AND KOSSEN, N.W.F. (1981). A method for starting a fermenter with immobilized spores of the genus *Clostridium* aseptically. *Biotechnology Letters* **3**, 158–159.

KROUWEL, P.G., GROOT, W.J., KOSSEN, N.W.F. AND VAN DER LAAN, (1983). Continuous isopropanol–butanol–ethanol fermentation by immobilized *Clostridium beijerinkckii* cells in a packed bed fermenter. *Enzyme and Microbial Technology* **5**, 46–54.

LARGIER, S.T., LONG, S., SANTANGELO, J.D., JONES, D.T. AND WOODS, D.R. (1985). Immobilized *Clostridium acetobutylicum* P262 mutants for solvent production. *Applied and Environmental Microbiology* **50**, 477–481.

LARRAYOZ, M.A. AND PUIGJANER, L. (1987). Study of butanol extraction through pervaporation in acetobutylic fermentation. *Biotechnology and Bioengineering* **30**, 692–696.

LARSSON, M. AND MATTIASSON, B. (1984). Novel process technology for biotechnological solvent production. *Chemistry and Industry* **12**, 428–431.

LEVY, S. (1984). *Continuous process for producing* n-*butanol employing anaerobic fermentation*. US Patent 4, 424, 275.

LINDEN, J.C., MOREIRA, A.R. AND LENZ, T.G. (1985). Acetone and butanol. In *Comprehensive Biotechnology* (H.W. Blanch, S. Drew and D.I.C. Wang, Eds), volume 3, pp. 915–931. Pergamon Press, Oxford.

LOBOS, J.H., LAMED, R.J. AND SU, T.M. (1982). H_2 supersaturation: its effect on end product formation in anaerobic fermentations. In *Abstracts of the Annual Meeting of the American Society for Microbiology, Atlanta, 7–12 March*, p. 198.

LONG, S., JONES, D.T. AND WOODS, D.R. (1984). Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262. *Applied Microbiology and Biotechnology* **20**, 256–261.

MCNEIL, B. AND KRISTIANSEN, B. (1987). The effect of medium composition on the acetone–butanol fermentation in continuous culture. *Biotechnology and Bioengineering* **29**, 383–387.

MADDOX, I.S. (1982). Use of silicalite for the adsorption of *n*-butanol from fermentation liquors. *Biotechnology Letters* **4**, 759–760.

MADDOX, I.S. (1988). New technologies for the acetone–butanol–ethanol fermentation. *Australian Journal of Biotechnology* **1**(4), 63–66.

MADDOX, I.S., GAPES, J.R. AND LARSEN, V.F. (1981). Production of *n*-butanol from whey ultrafiltrate. In *Chemeca 81: 9th Australasian Conference on Chemical Engineering, 30 August–4 September*, pp. 535–542.

MARCHAL, R., BLANCHET, D. AND VANDECASTEELE, J.P. (1985). Industrial optimization of acetone–butanol fermentation: a study of the utilization of Jerusalem artichokes. *Applied Microbiology and Biotechnology* **23**, 92–98.

MATSUMURA, M., KATAOKA, H., SUEKI, M. AND ARAKI, K. (1988). Energy saving effect of pervaporation using oleyl alcohol liquid membrane in butanol purification. *Bioprocess Engineering* **3**, 93–100.

MATTIASSON, B. (1983). Applications of aqueous two-phase systems in biotechnology. *Trends in Biotechnology* **1**, 16–20.

MATTIASSON, B. AND LARSSON, M. (1985). Extractive bioconversions with emphasis on solvent production. *Biotechnology and Genetic Engineering Reviews* **3**, 137–174.

MEYER, C.L., MCLAUGHLIN, J.K. AND PAPOUTSAKIS, E.T. (1985). The effect of CO on growth and product formation in batch cultures of *Clostridium acetobutylicum*. *Biotechnology Letters* **7**, 37–42.

MEYER, C.L., ROOS, J.W. AND PAPOUTSAKIS, E.T. (1986). Carbon monoxide gasing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **24**, 159–167.

MILESTONE, N.B. AND BIBBY, D.M. (1981). Concentration of alcohols by adsorption on silicalite. *Journal of Chemical Technology and Biotechnology* **31**, 732–736.

MONOT, F. AND ENGASSER, J.M. (1983). Production of acetone and butanol by batch and continuous culture of *Clostridium acetobutylicum* under nitrogen limitation. *Biotechnology Letters* **5**, 213–218.

MONOT, F., ENGASSER, J.M. AND PETITDEMANGE, H. (1983). Regulation of acetone butanol production in batch and continuous cultures of *Clostridium acetobutylicum*. *Biotechnology and Bioengineering Symposium* No. 13, pp. 207–216.

MONOT, F., MARTIN, J.R., PETITDEMANGE, H. AND GAY, R. (1982). Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. *Applied and Environmental Microbiology* **44**, 1318–1324.

NIELSEN, L., LARSSON, M., HOLST, O. AND MATTIASSON, B. (1988). Adsorbents for extractive bioconversion applied to the acetone–butanol fermentation. *Applied Microbiology and Biotechnology* **28**, 335–339.

PAREKH, S.R., PAREKH, R.S. AND WAYMAN, M. (1988). Ethanol and butanol production by fermentation of enzymatically saccharified SO_2-prehydrolysed lignocellulosics. *Enzyme and Microbial Technology* **10**, 660–668.

PIERROT, P., FICK, M. AND ENGASSER, J.M. (1986). Continuous acetone–butanol fermentation with high productivity by cell ultrafiltration and recycling. *Biotechnology Letters* **8**, 253–256.

PIERROT, P., FICK, M., GODIN, C. AND ENGASSER, J.M. (1987). Improved productivity and stability of continuous acetone–butanol fermentations by two-stage and cell recycling systems. In *Proceedings of the 4th European Congress on Biotechnology* (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, Eds), volume 1, p. 258, Elsevier, Amsterdam.

PONS, M.N. AND TILLIER-DORION, F. (1987). Gas membrane removal of volatile inhibitors. In *Proceedings of the 4th European Congress on Biotechnology* (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, Eds), volume 1, p. 149, Elsevier, Amsterdam.

QURESHI, N. AND MADDOX, I.S. (1987). Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. *Enzyme and Microbial Technology* **9**, 668–671.

QURESHI, N. AND MADDOX, I.S. (1988). Reactor design for the ABE fermentation using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. *Bioprocess Engineering* **3**, 69–72.

QURESHI, N., PATERSON, A.H.J. AND MADDOX, I.S. (1988). Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. *Applied Microbiology and Biotechnology* **29**, 323–328.

RAO, G. AND MUTHARASAN, R. (1986). Alcohol production by *Clostridium acetobutylicum* induced by methyl viologen. *Biotechnology Letters* **8**, 893–896.

RAO, G. AND MUTHARASAN, R. (1987). Altered electron flow in continuous cultures of *Clostridium acetobutylicum* induced by viologen dyes. *Applied and Environmental Microbiology* **53**, 1232–1235.

RAO, G. AND MUTHARASAN, R. (1988). Directed metabolic flow with high butanol yield and selectivity in continuous cultures of *Clostridium acetobutylicum*. *Biotechnology Letters* **10**, 313–318.

ROFFLER, S.R., BLANCH, H.W. AND WILKE, C.R. (1987a). *In-situ* recovery of butanol during fermentation. Part I: batch extractive fermentation. *Bioprocess Engineering* **2**, 1–12.

ROFFLER, S.R., BLANCH, H.W. AND WILKE, C.R. (1987b). *In-situ* recovery of butanol during fermentation. Part 2: fed-batch extractive fermentation. *Bioprocess Engineering* **2**, 181–190.

ROFFLER, S.R., BLANCH, H.W. AND WILKE, C.R. (1988). *In-situ* extractive fermentation of acetone and butanol. *Biotechnology and Bioengineering* **31**, 135–143.

ROFFLER, S.R., WILKE, C.R. AND BLANCH, H.W. (1988). Design and mathematical description of differential contactors used in extractive fermentations. *Biotechnology and Bioengineering* **32**, 192–204.

ROOS, J.W., MCLAUGHLIN, J.K. AND PAPOUTSAKIS, E.T. (1985). The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. *Biotechnology and Bioengineering* **27**, 681–694.

SCHLOTE, D. AND GOTTSCHALK, G. (1986). Effect of cell recycle on continuous butanol–acetone fermentation with *Clostridium acetobutylicum* under phosphate limitation. *Applied Microbiology and Biotechnology* **24**, 1–5.

SCHOUTENS, G.H., VAN BEELEN, P.N. AND LUYBEN, K.CH.A.M. (1986). A simple model for the continuous production of butanol by immobilized *Clostridia*. *Chemical Engineering Journal (Lausanne)* **32**, B43–B50.

SCHOUTENS, G.H., GUIT, R.P., ZIELEMAN, G.J., LUYBEN, K.CH.A.M. AND KOSSEN, N.W.F. (1986a). A comparative study of a fluidised bed reactor and a gas lift loop

reactor for the IBE process: Part I. Reactor design and scale down approach. *Journal of Chemical Technology and Biotechnology* **36**, 335–343.

SCHOUTENS, G.H., GUIT, R.P., ZIELEMAN, G.J., LUYBEN, K.CH.A.M. AND KOSSEN, N.W.F. (1986b). A comparative study of a fluidised bed reactor and a gas lift loop reactor for the IBE process. Part II: Hydrodynamics and reactor modelling. *Journal of Chemical Technology and Biotechnology* **36**, 415–426.

SCHOUTENS, G.H., GUIT, R.P., ZIELEMAN, G.J., LUYBEN, K.CH.A.M. AND KOSSEN, N.W.F. (1986c). A comparative study of a fluidised bed reactor and a gas lift loop reactor for the IBE process. Part III: Reactor performances and scale up. *Journal of Chemical Technology and Biotechnology* **36**, 565–576.

SHIMIZU, K. AND MATSUBARA, M. (1987). A solvent screening criterion for multicomponent extractive fermentation. *Chemical Engineering Science* **42**, 400–504.

SONI, B.K., SOUCAILLE, P. AND GOMA, G. (1986). Effect of phosphate cycling on production of acetone–butanol by *Clostridium acetobutylicum* in chemostat culture. *Biotechnology and Bioengineering Symposium* No. 17, pp. 239–252.

SONI, B.K., SOUCAILLE, P. AND GOMA, G. (1987). Physiological effects of divalent cations in chemostat cultures of *Clostridium acetobutylicum*. In *Proceedings of the 4th European Congress on Biotechnology* (O.M. Neijssel, R.R. van der Meer and K.Ch.A.M. Luyben, Eds), volume 3, pp. 335–338. Elsevier, Amsterdam.

SPIVEY, M.J. (1978). The acetone/butanol/ethanol fermentation. *Process Biochemistry* **13**, 2–5.

STEPHENS, G.M., HOLT, R.A., GOTTSCHAL, J.C. AND MORRIS, J.G. (1985). Studies on the stability of solvent production by *Clostridium acetobutylicum* in continuous culture. *Journal of Applied Bacteriology* **59**, 597–605.

TAYA, M., ISHII, S. AND KOBAYASHI, T. (1985). Monitoring and control for extractive fermentation of *Clostridium acetobutylicum*. *Journal of Fermentation Technology* **63**, 181–187.

TAYA, M., YAGI, T. AND KOBAYASHI, T. (1986). Production of acetone and butanol using immobilized growing cells of *Clostridium acetobutylicum*. *Agricultural and Biological Chemistry* **50**, 2141–2142.

TRAXLER, R.W., WOOD, E.M., MAYER, J. AND WILSON, M.P. (1985). Extractive fermentation for the production of butanol. *Developments in Industrial Microbiology* **26**, 519–525.

VIJJESWARAPU, W.K., CHEN, W.Y. AND FOUTCH, G.L. (1985). Performance of a cell recycling continuous fermentation system for the production of *n*-butanol by *Clostridium acetobutylicum*. *Biotechnology and Bioengineering Symposium* No. 15, pp. 471–478.

WANG, D.I.C., COONEY, C.L., DEMAINE, A.L., GOMEZ, R.F. AND SINSKEY, A.J. (1979). Production of acetone and butanol by fermentation. *MIT Quarterly Report to the U.S. Department of Energy, COG–4198–9*, pp. 141–149.

WAYMAN, M. AND PAREKH, R. (1987). Production of acetone–butanol by extractive fermentation using dibutylphthalate as extractant. *Journal of Fermentation Technology* **65**, 295–300.

WELSH, F.W. AND VELIKY, I.A. (1984). Production of acetone–butanol from acid whey. *Biotechnology Letters* **6**, 61–64.

WELSH, F.W., WILLIAMS, R.E. AND VELIKY, I.A. (1987). Solid carriers for a *Clostridium acetobutylicum* that produces acetone and butanol. *Enzyme and Microbial Technology* **9**, 500–502.

WHEELER, M.C. AND GOODALE, C.D. (1932). *Continuous butyl alcohol fermentation process*. US Patent 1, 875, 536.

YAROVENKO, V.L. (1962). Principles of the continuous alcohol and butanol–acetone fermentation processes. In *2nd Symposium on Continuous Cultivation of Microorganisms* (I. Malek, Ed.), pp. 205–217. Czechoslovakian Academy of Sciences, Prague.

YERUSHALMI, L. AND VOLESKY, B. (1985). Importance of agitation in acetone–butanol

fermentation. *Biotechnology and Bioengineering* **27**, 1297–1305.

YERUSHALMI, L. AND VOLESKY, B. (1987). Culture conditions for growth and solvent biosynthesis by a modified *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **25**, 513–520.

YERUSHALMI, L., VOLESKY, B. AND SZCZESNY, T. (1985). Effect of increased hydrogen partial pressure on the acetone–butanol fermentation by *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **22**, 103–107.