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In situ recovery of fermentation products

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In situ recovery of fermentation products can increase the rate of product inhibited fermentations, reduce costs of waste-water treatment and minimize product degradation. Some methods of _in situ_ recovery show more potential than others for the production of chemicals and pharmaceuticals by fermentation.

A wide range of pharmaceuticals and chemicals can be produced by fermentation. In a number of important cases, however, the accumulation of product in the fermentation broth inhibits its further production; bulky equipment is required and often waste water treatment costs are high[1]. The effects of product accumulation in the broth can be reduced by continuously removing product as it forms. This review examines the suitability of vacuum fermentation, flash fermentation, extractive fermentation, dialysis fermentation and the use of adsorption and ion-exchange resins (Fig. 1) for the large scale production of fermentation products.

Vacuum fermentation

Volatile fermentation products can be removed during fermentation by maintaining the fermentor under vacuum so that the products boil off at the normal temperature of the fermentation. A low product concentration is maintained and product inhibition is reduced.

Cysewski and Wilke[2] operated a vacuum fermentation system in three configurations as shown in Fig. 1 (a,b,c) to produce ethanol from concentrated sugar feeds. When the system was run with continuous addition of glucose to the fermentor (Fig. 1a), productivity greatly increased at first and then rapidly declined after 48 hours of operation as shown in Fig. 2. Acetic acid accumulation was probably responsible for the loss of cell viability after 48 hours[3]. The accumulation of toxic non-volatile by-products was reduced by continuously removing some of the broth from the fermentor (Fig. 1b), thus increasing specific ethanol productivity (g ethanol g yeast cells^{-1} h^{-1}) by 38%. Feed containing 33.4% glucose was completely fermented by this method; in a conventional fermentor the concentration of glucose in the feed must be below 20% for complete fermentation. Less water is processed when a concentrated sugar feed is used and waste water treatment costs are, therefore, lower. With the addition of a yeast recycle facility during vacuum fermentation (Fig. 1c) volumetric productivities as high as 82 g l^{-1} h^{-1} were obtained, compared to 14 g l^{-1} h^{-1} in continuous fermentation. (This improved productivity resulted both from the high cell density achieved by cell recycling and from reduced ethanol inhibition of the fermentation.)

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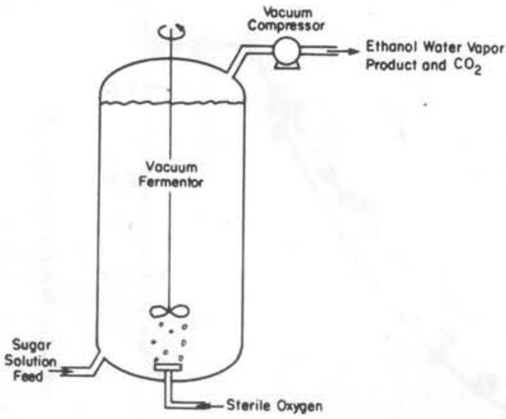
Although vacuum fermentation allows highly concentrated sugar solutions to be fermented, there are problems. Large amounts of by-product carbon dioxide must be compressed from the fermentor pressure up to atmospheric pressure using bulky expensive compressors[4]. Also, the small amount of oxygen required by the yeast must be supplied as pure oxygen because its solubility is low at the reduced fermentor pressure. Flash fermentation was developed (Fig. 1d) to overcome these difficulties.

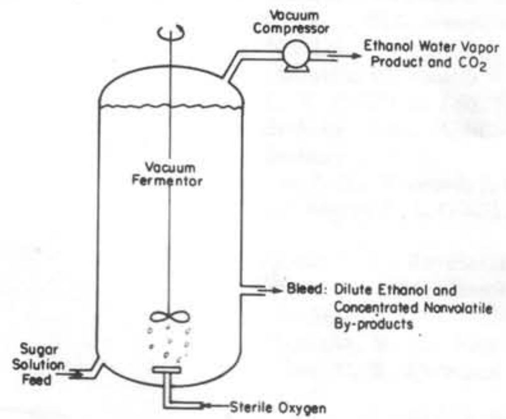
Flash fermentation

In flash fermentation, the fermentor remains at atmospheric pressure while broth is circulated to a vacuum chamber where ethanol is continuously boiled off, reducing ethanol inhibition of the yeast. By separating the vacuum chamber and the fermentor, carbon dioxide produced in the fermentor no longer has to be compressed. Compressor costs are lowered and air (rather than pure oxygen) can be used to sparge the fermentor.

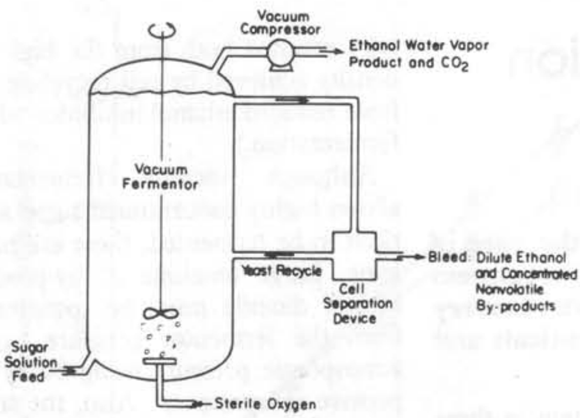
Flash fermentation has been used to produce ethanol in two studies: one using *Zymomonas mobilis*[6] and the other using a strain of *Candida acidothermophilum* and a mixed cellulase enzyme solution[6]. The latter study used vacuum cycling to periodically remove ethanol from a batch fermentor. When the concentration of ethanol approached inhibitory levels in the fermentor, the broth was circulated between a vacuum chamber at 80 mm Hg and the



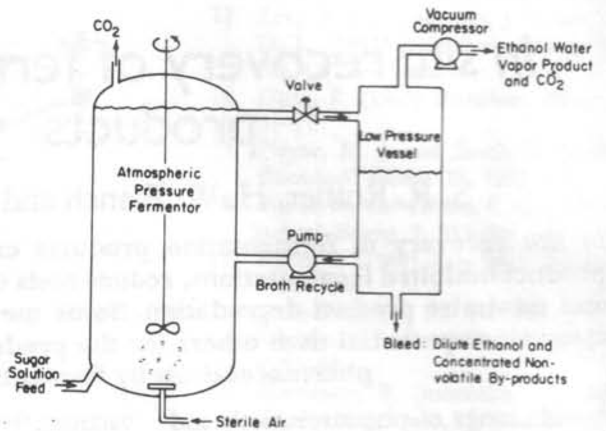
a. Continuous Vacuum Fermentation



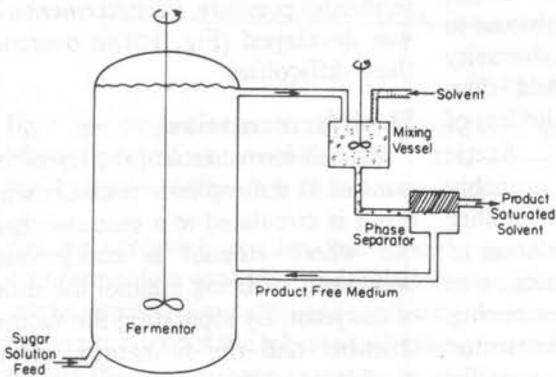
b. Continuous Vacuum Fermentation with Liquid Bleed



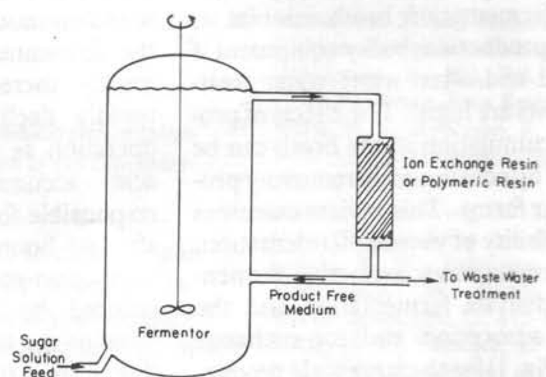
c. Vacuum Fermentation with Yeast Recycle



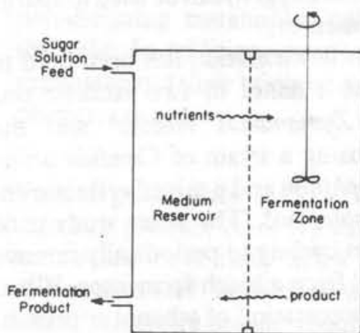
d. Continuous Flash Fermentation



e. Extractive Fermentation



f. Continuous Ion Exchange or Adsorption Fermentation



g. Dialysis Fermentation

Fig. 1. Methods for in situ *recovery of fermentation products.*

fermentor until the ethanol concentration was reduced and normal batch fermentation resumed. New feed was added periodically and the total contents of the fermentor were renewed every ten cycles because of the build-up of non-volatile compounds. Ethanol inhibition of both cellulase and fermentation activity was reduced; 97.5% of the cellulose feed (rice straw) was utilized, producing concentrated ethanol ($121\ g\ l^{-1}$).

The economics of vacuum and flash fermentation have been estimated[4]. A plant producing $9.5 \times 10^7\ l\ year^{-1}$ of 95% (w/w) ethanol by vacuum fermentation or flash fermentation was estimated to have a capital cost at 1979 prices of £13.9 million or £13.5 million and a manufacturing cost (excluding feed cost) of 7.63 or 6.97¢ l^{-1}, respectively. Although either method represents a substantial savings compared with batch fermentation (capital cost £25.4 million, manufacturing cost 13.6¢ l^{-1}), continuous fermentation with yeast recycling provided similar savings[7]. Thus, the benefits of vacuum fermentation – improved productivity and reduced volume of water that must be processed – are offset by increased capital and operating costs.

The primary disadvantage of vacuum fermentation is its limited utility. The product must be more volatile than water, limiting its use to the production of ethanol and, perhaps, acetaldehyde. While the effects of toxic non-volatile by-products accumulating in the broth can be reduced by continuously removing broth from the fermentor, dilute ethanol is also removed. This loss of ethanol in the broth ultimately limits the productivity of both vacuum and flash fermentation[3].

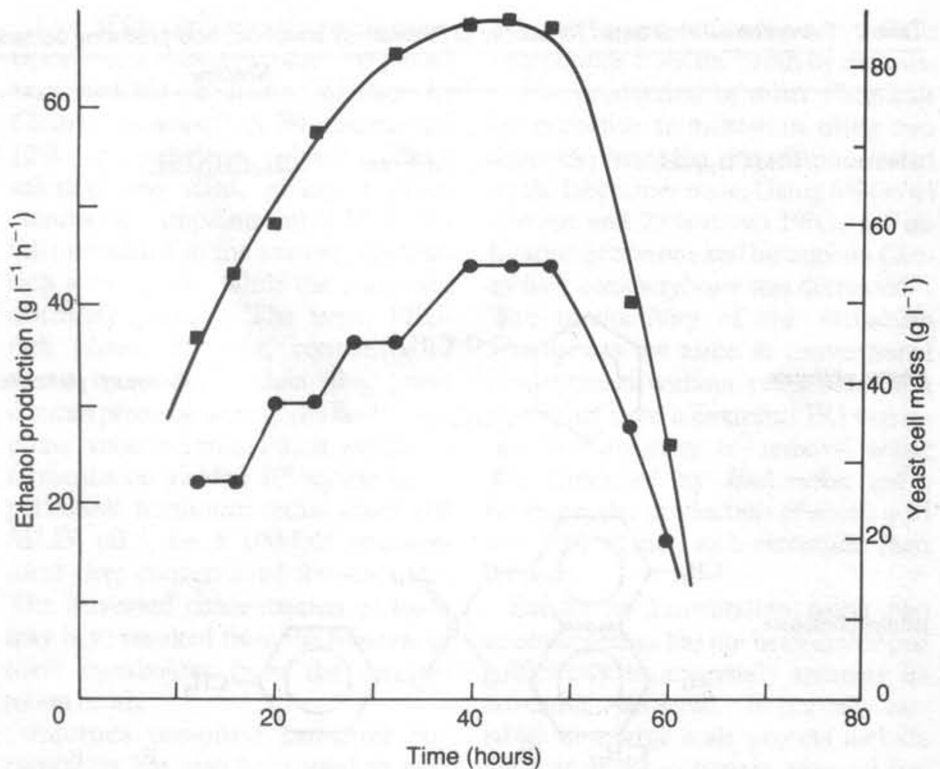


Fig. 2. Vacuum fermentation without a bleed using a 33.4% glucose feed. Ethanol production (●), yeast cell mass (■).

Extractive fermentation

Liquid–liquid extraction

Metabolites can be extracted during fermentation by contacting the broth with a suitable organic solvent which is insoluble in the broth. Products dissolving into the solvent can later be recovered by distillation or back extraction into an acid or base buffer solution. Broth and solvent can either be contacted in the fermentor or in an external extraction vessel. It is desirable to choose a solvent that has a high capacity, is selective for the fermentation product and is relatively non-toxic to the fermenting microorganisms.

In one of the first studies of extractive fermentation, Finn[8] tried to relieve the product inhibition of prodigiosin on a strain of *Serratia* by extraction into kerosene, but production was not increased. More success was achieved in studies in which ethanol produced by *Saccharomyces cerevisiae* was extracted into dodecanol[9] or dibutyl phthalate[10]. In the dodecanol studies, the fermentor consisted of a column filled with pieces of brick to which the yeast adhered. Ethanol produced by the immobilized yeast was extracted into dodecanol flowing through the fermentor. Using extractive fermentation, a feed containing 40% w/w glucose was completely fermented with a volumetric productivity five times higher than the conventional control. Dodecanol, however, has a relatively low capacity for ethanol, and the solvent flow rate was 17 times the sugar feed rate. On an industrial scale, extraction equipment would be expensive and solvent requirements large.

Butyric, valeric and caproic acids have been selectively removed from a suppressed methane anaerobic fermentation by extractive fermentation[11]. The extraction solvent, kerosene, was regenerated by back extraction into a base solution. Acid concentration in the base solution was ten times greater than that in the fermentor. In a continuous process, however, the accumulation in the broth of inhibitory levels of acetic and propanoic acids which are almost insoluble in kerosene would probably limit the fermentation productivity.

Many organic solvents are toxic to microbes, limiting the solvents that can be used in extractive fermentation. Datta[12] has investigated the effects of several solvents on anaerobic acidogenic bacteria used to produce organic acids. No toxicity was observed for saturation concentrations of diesel, toluene and amyl acetate with and without the extractants TOPO (trioctylphosphine oxide) or Alamine 336 (a tertiary amine). Playne and Smith[13] examined the effects of thirty organic solvents on a commercial inoculum of facultatively anaerobic, acid producing bacteria. At saturation concentrations, 13 solvents were nontoxic, two were partially inhibitory, and the remaining 15 were toxic (Table 1). In a study on toxicity to a lactic acid producing bacteria, *Lactobacillus delbrueckii*, solvent toxicity increased in the order: alkane = cumene < ketone < tertiary amine < secondary amine < quaternary amine (Roffler, S. R., unpublished results). The solvents that had the highest capacities for lactic acid were also the most toxic, as shown in Table 2. The toxicity of a particular solvent depends on the microbe (Refs 8 and 13 and Roffler, S. R., unpublished results), necessitating solvent screening of microbial process strains.

Table 1. Extraction solvents tested for toxicity to facultatively anaerobic, acid-producing bacteria

Solvent	Formula
Nontoxic	
n-hexane	$CH_3(CH_2)_4CH_3$
n-decane	$CH_3(CH_2)_8CH_3$
iso-octane	$(CH_3)_2CH(CH_2)_4CH_3$
dibutyl phthalate	$C_6H_4(C(=O)\text{-}O\text{-}C_4H_9)_2$
di-isoamyl phthalate	$C_6H_4(C(=O)\text{-}O\text{-}C_3H_5(CH_3)_2)_2$
tritolyl phosphate	$(CH_3C_6H_4O)_3P=O$
tributyl phosphate	$(C_4H_9O)_3P=O$
trioctyl phospine oxide	$(C_8H_{17})_3P=O$
freon 113	$CClF_2\text{-}CCl_2F$
trioctyl amine	$(C_8H_{17})_3N$
di-isoamyl ether	$(CH_3)_2C_3H_5\text{-}O\text{-}C_3H_5(CH_3)_2$
kerosene	hydrocarbon mixture
Aliquat 336	$[(C_8H_{17})_4N]^+ Cl^-$
Partially Toxic	
Primene JMT	$H_2N\ C_{14}H_{20}$
Amberlite LA-2	
mixture of highly branched secondary amines	
Toxic	
isoamyl alcohol	$(CH_3)_2C_3H_5\text{-}OH$
n-hexanol	$C_6H_{13}\text{-}OH$
n-octanol	$C_8H_{17}\text{-}OH$
2-ethylhexanol	$CH_3(CH_2)_3CH(C_2H_5)\text{-}CH_2\text{-}OH$
n-dodecanol	$C_{12}H_{25}\text{-}OH$
diethyl ketone	$C_2H_5\text{-}C(=O)\text{-}C_2H_5$
dipropyl ketone	$C_3H_7\text{-}C(=O)\text{-}C_3H_7$
methyl isobutyl ketone	$CH_3\text{-}C(=O)\text{-}CH_2CH(CH_3)_2$
benzene	C_6H_6
toluene	$C_6H_5\text{-}CH_3$
o-xylene	$C_6H_4(CH_3)_2$
isoamyl acetate	$CH_3\text{-}C(=O)\text{-}O\text{-}(CH_2)_2CH(CH_3)_2$
nitrobenzene	$C_6H_5\text{-}NO_2$
di-isopropyl ether	$(CH_3)_2CH\text{-}O\text{-}CH(CH_3)_2$
methylhexyl ketone	$CH_3\text{-}C(=O)\text{-}C_6H_{13}$

The economics of an extractive fermentation plant for the production of 10^9 l year^{-1} of 95% (w/w) ethanol have been estimated[7]. The solvent was assumed to have a distribution coefficient (concentration of ethanol in solvent/concentration of ethanol in broth) K_D = 6. Excluding the cost of the separating device and using 1981 prices, the capital cost of extractive fermentation was £10.6 million and the manufacturing cost 43.9¢ l^{-1} compared to £23.7 million and 49.1¢ l^{-1}, respectively for continuous fermentation with yeast recycling. Although the economics of extractive fermentation look attractive, a non-toxic solvent for the extraction of ethanol with a K_D = 6 has not been found.

Aqueous two phase systems

To overcome some of the problems with organic liquids in extractive fermentation, aqueous two-phase systems have been used for the *in situ* removal of products from fermentation broth. Rather than using an organic liquid as the second phase, polymers are added to the broth until two separate phases form. The phases contain 85–95% water and are normally biocompatible. Microbes often remain in one phase while low molecular weight products are distributed evenly between phases. Although the product is not concentrated by extraction, the phase volumes can be adjusted so that the phase containing the microbes is much smaller than the other phase. Most of the product will then be in the phase without microbes and can be drawn off and processed by distillation or other means.

One of the earliest extractive fermentation studies using two aqueous phases examined the production of toxin by *Clostridium tetani*[14]. A 2% dextran and 12% polyethylene glycol (PEG) solution was used, giving a phase volume ratio (top/bottom) of 15:1. The cells remained in the smaller, dextran-rich lower phase, while the toxin was distributed evenly. The large, PEG-rich phase, however, contained 15 times more toxin than the lower dextran phase because of the favourable phase volume ratio. Batch extractive fermentation yielded 10^9 mouse intraperitoneal minimum lethal doses (IP MLD) ml^{-1}, i.e. a 100-fold improvement over conventional fermentation. The increased concentration of toxin may have resulted from the removal of toxic metabolites from the fermentation broth.

Aqueous two-phase extractive fermentation has also been used to produce ethanol from glucose[15] and from cellulose[16]. When glucose was the substrate, dextran and PEG were used to give a volume ratio (top/bottom) of 9:1. The fermentor was agitated and the dextran-rich phase containing the yeast formed drops in the PEG-rich phase; yeast remained in the dextran-rich drops. After the glucose was consumed, the PEG phase (containing 90% of the ethanol) was removed and the ethanol distilled off; ethanol-free PEG phase was then returned to the fermentor and more glucose added. After ten cycles, the concentration of by-product glycerol and the accumulation of toxic non-volatile compounds greatly decreased the rate of fermentation. The fermentation rate was returned to normal by periodically adding more yeast cells and removing non-volatile compounds from the broth by dialysis.

The production of other chemicals by extractive fermentation using two aqueous phases has been demonstrated on the laboratory scale. Using 6% (w/w) dextran and 25% (w/w) PEG, the inhibition of acetone and butanol on *Clostridium acetobutylicum* was decreased[17]. The productivity of the extraction system was the same as conventional fermentation without extraction. In a study that used a dextran/PEG extractive fermentation to remove acetic acid produced by *Escherichia coli*[18], however, the production of acetic acid was 50% higher with extraction than without.

Extractive fermentation using two aqueous phases has not been developed sufficiently to accurately estimate its economic potential. Important variables in a large scale process include the cost of the polymers, renewal frequency, the ease of product recovery from the polymer mixture, and by-product accumulation problems during long-term operation. The advantages of aqueous two-phase systems include biocompatibility, possible reduction in water treatment, general applicability to many fermentation products and the possibility of by-product concentration and recovery[15].

Adsorption

Solid, porous adsorbents with extremely large surface areas, ranging from activated carbon to polymeric resins, have been used to remove metabolites from fermentation broth. They can be added directly to the fermentor or be placed in a separate vessel with

Table 2. Lactic acid extraction capacity and toxicity to Lactobacillus delbrueckii *of several extraction solvents.*

Solvent	Lactic acid distribution coefficient[a]	Solvent concentration in fermentation broth	Maximum specific growth rate (h^{-1})	Glucose conversion (%)
Control (no solvent	—	—	0.58	100
n-Heptadecane	0.01	saturation	0.55	100
Kerosene saturated with TOPO	0.6-0.8	10% of saturation	0.54	100
		saturation	0.38	100
Tributyl phosphate	0.9	10% of saturation	0.65	100
		saturation	toxic	0
30% (w/w) TOPO in cumene	1.0-1.4	10% of saturation	0.62	100
		saturation	toxic	0
Aliquat 336 (quaternary ammonium chloride salt)	1.0-4.5[b]	10% of saturation	toxic	0

(a) g l^{-1} of lactic acid in solvent phase/g l^{-1} of lactic acid in aqueous phase.
(b) 25% (w/w) Aliquat 336 in cyclo-octane at pH 5.5.

broth circulation to and from it.

Several investigators have tried to increase production of ethanol by adsorbing it with resin or carbon. In one study[19], activated carbon was added directly to a yeast fermentation and although the free-ethanol concentration in the broth decreased because of adsorption, yeast viability also decreased and the rate of ethanol production was low. Better results were obtained when resting (non-growing) yeast cells were used and direct contact between yeast and activated carbon was avoided; yeast was removed by centrifugation before contacting broth and activated carbon[20]. After ethanol was removed, broth and yeast were returned to the fermentor, more sugar was added, and fermentation continued. A maximum volumetric productivity of 25 g l^{-1} h^{-1} was obtained but the viability of the yeast gradually dropped, possibly due to the accumulation of by-products in the broth. Lenki *et al.*[21] removed ethanol from a yeast fermentation using industrially available polymers, including a cross-linked polystyrene resin, a cross-linked acrylic ester resin and silicalite pellets contain-in an alumina binder. The resins, however, either dramatically retarded or halted growth of the yeast.

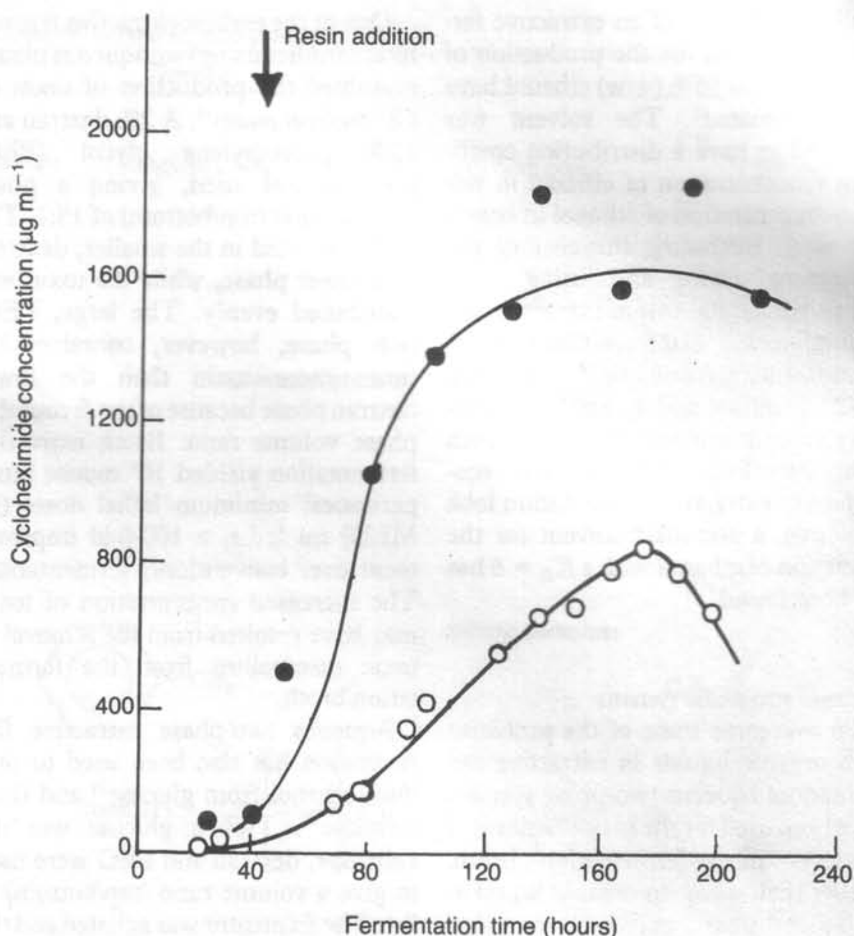


Fig. 3. Comparison between a normal cycloheximide fermentation (○) and resin addition fermentation (●) of S. griseus *using 6% (w/w) × AD-4 resin.* Reproduced from Ref. 23 with permission.

Good results were obtained in studies on the *in situ* removal of the antibiotic cycloheximide from fermentation broth[22,23]. Because the production of the antibiotic by *Streptomyces griseus* is feedback regulated by the product, a neutral polymeric resin was added directly to the broth during fermentation to reduce free cycloheximide concentration. Fig. 3 compares the results of fermentation with and without resin added to the broth. The addition of 6% (w/w) resin resulted in an increase in final product yield from 800 μg ml^{-1} to 1 600 μg ml^{-1}. When resin was wrapped in an ultrafiltration membrane before being added to the fermentor, cycloheximide yield increased to 1 800 μg ml^{-1}, indicating that the resin inhibits the microbes.

The toxicity of resins to microbes is one problem in using adsorption for *in situ* product recovery. Others include the low capacity of adsorbents, especially when contacted with whole broth[19], the non-specific adsorption of cells, feed components, or by-products, attrition of resin due to non-reversible adsorption, sugar caramelization if heat is used to regenerate the resin, and slow rates of mass transfer in the resin. Some of these problems, particularly toxicity, can be overcome by immobilizing the cells in the fermentor or by removing them by centrifugation or filtration before contacting broth and adsorbent. These methods, however, add complexity and cost to a process.

Ion exchange

Salicylic acid can be fermented from naphthalene by *Pseudomonas aeruginosa*; however, at 10 g l^{-1}, salicylic acid inhibits its further production, induces salicylic acid degradation and causes cell death. By adding anion-exchange resin directly to broth, salicylic acid production was increased to 30 g l^{-1}, over three times higher than normal[24,25]. However, when resin was wrapped in a cellophane membrane before being added to the fermentor, there was a 5.5-fold increase in salicylic acid production, indicating that direct contact between resin and cells had inhibited the microbes. Fig. 4 compares the course of fermentation with and without cellophane-wrapped resin; upon addition of the ion-exchange resin, salicylic acid concentration in the broth drops and cell viability is maintained at high levels (Fig. 4a), allowing continued production of acid (Fig 4b). In a study using *Corynebacterium renale* to produce salicylic acid, anion-exchange resin was packed in a column and broth was recirculated between the column and fermentor[26]. Salicylic acid concentration increased to 14.6 g l^{-1}, about twice the level in the control.

Ion-exchange resins have also been used to recover novobiocin and neomycin from fermentation broth[27]. Other than in these studies, however, ion-exchange has rarely been used for the *in situ* recovery of ionic products, probably because of problems in using ionic resins with whole broth. The ions that are released from an ion-exchange resin upon product adsorption may inhibit microbial growth[26], building up to toxic levels in a continuous process. Ionic compounds in the broth that are adsorbed by the resin will decrease its capacity for product adsorption while non-ionic metabolites and feed components will accumulate in the broth; ultimately this limits productivity in a continuous process. In addition, the highly polar environment around the

resin may damage sensitive molecules. Ion-exchange resins are expensive and mass transfer is slow; equipment is large and capital costs high. These factors may outweigh increased productivity obtained with ion exchange, making the use of ion-exchange resins for *in situ* product recovery uneconomical.

Dialysis fermentation

In dialysis fermentation, a selectively permeable membrane separates a culture chamber, in which fermentation takes place, from a medium reservoir. Nutrients in the reservoir diffuse to the culture chamber while metabolite products diffuse to the medium reservoir. Low product concentrations are maintained in the culture chamber, minimizing the effects of metabolic inhibition.

Friedman and Gaden studied the production of lactic acid by *Lactobacillus delbrueckii* in dialysis fermentation[28]. Figure 5 shows that cumulative specific lactic acid production and the specific growth rate were improved by the use of dialysis fermentation. The study also showed that productivity increased from 5 g l^{-1} h^{-1} for conventional fermentation to 8 g l^{-1} h^{-1} with dialysis fermentation.

The production of salicylic acid by *Pseudomonas fluorescens* in a semi-continuous dialysis system has been demonstrated[29]. The substrate, solid naphthalene, remained in the culture chamber but salicylic acid could diffuse into the medium reservoir. When salicylic acid concentration approached inhibitory levels, fresh medium was added to the reservoir. Although salicylic acid production in the dialysis fermentor was 2.6 times greater than with a conventional fermentor its concentration was necessarily lower to provide a driving force for diffusion.

In a novel dialysis system used by Kominek[30] water was pumped through cellulose dialysis tubing placed in the fermentor to extract cycloheximide as it was produced by *S. griseus*. Cycloheximide in the water was extracted into methylene chloride before the water was recycled. A two-fold increase in production was obtained with the dialysis system. This system should work with dialysable products for which there are suitable water insoluble solvents.

In general, dialysis fermentation not only relieves product inhibition but also retains cells so that high cell densi-

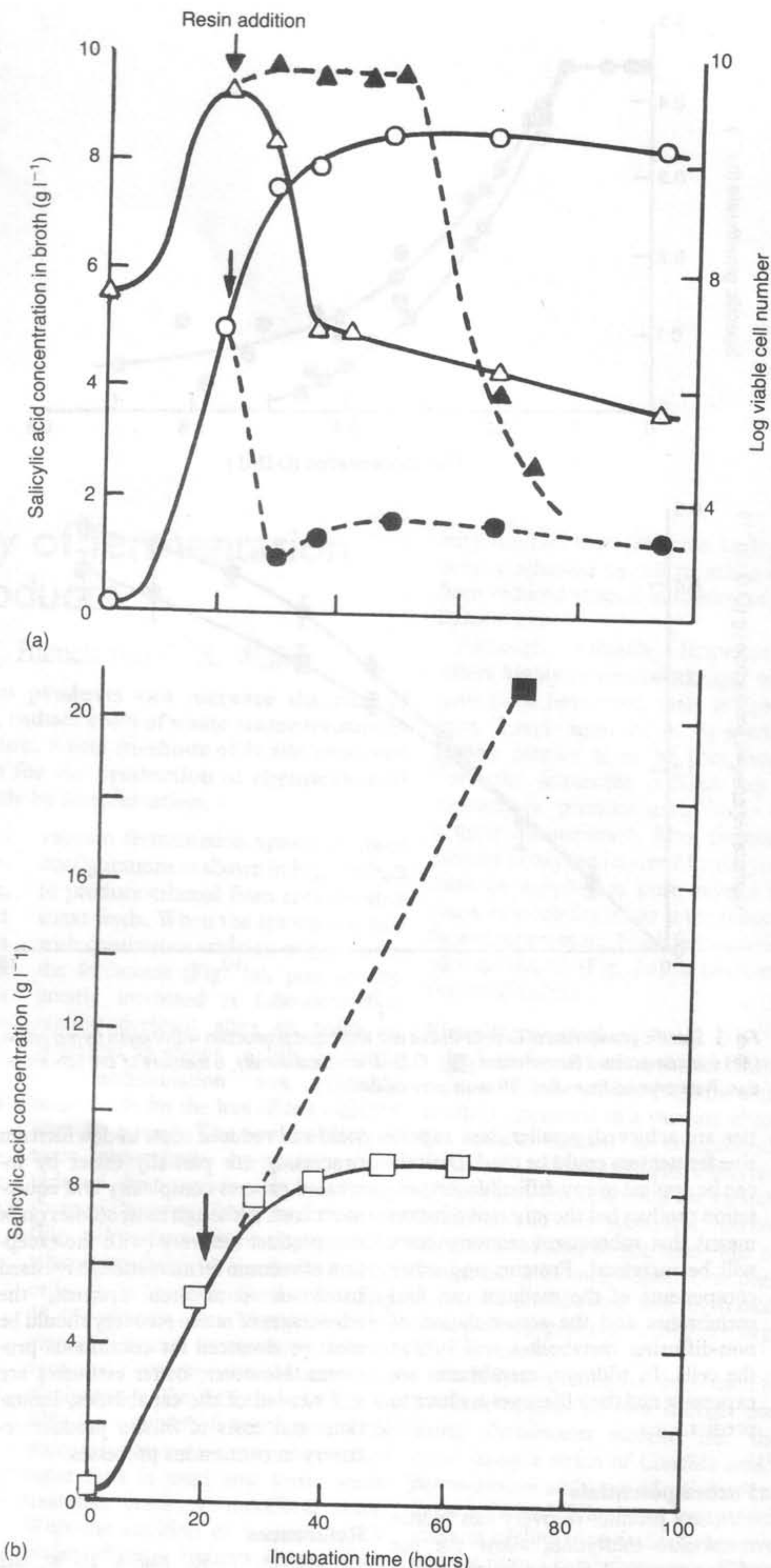


Fig. 4. Comparison between a normal salicylic acid fermentation (open symbols) and ion-exchange fermentation using anion-exchange resin wrapped in cellophane (solid symbols) of Ps. aeruginosa (a) Salicylic acid concentration in broth (○, ●) and viable cell number (△, ▲). (b) Salicylic acid concentrations in broth and resin (□, ■).

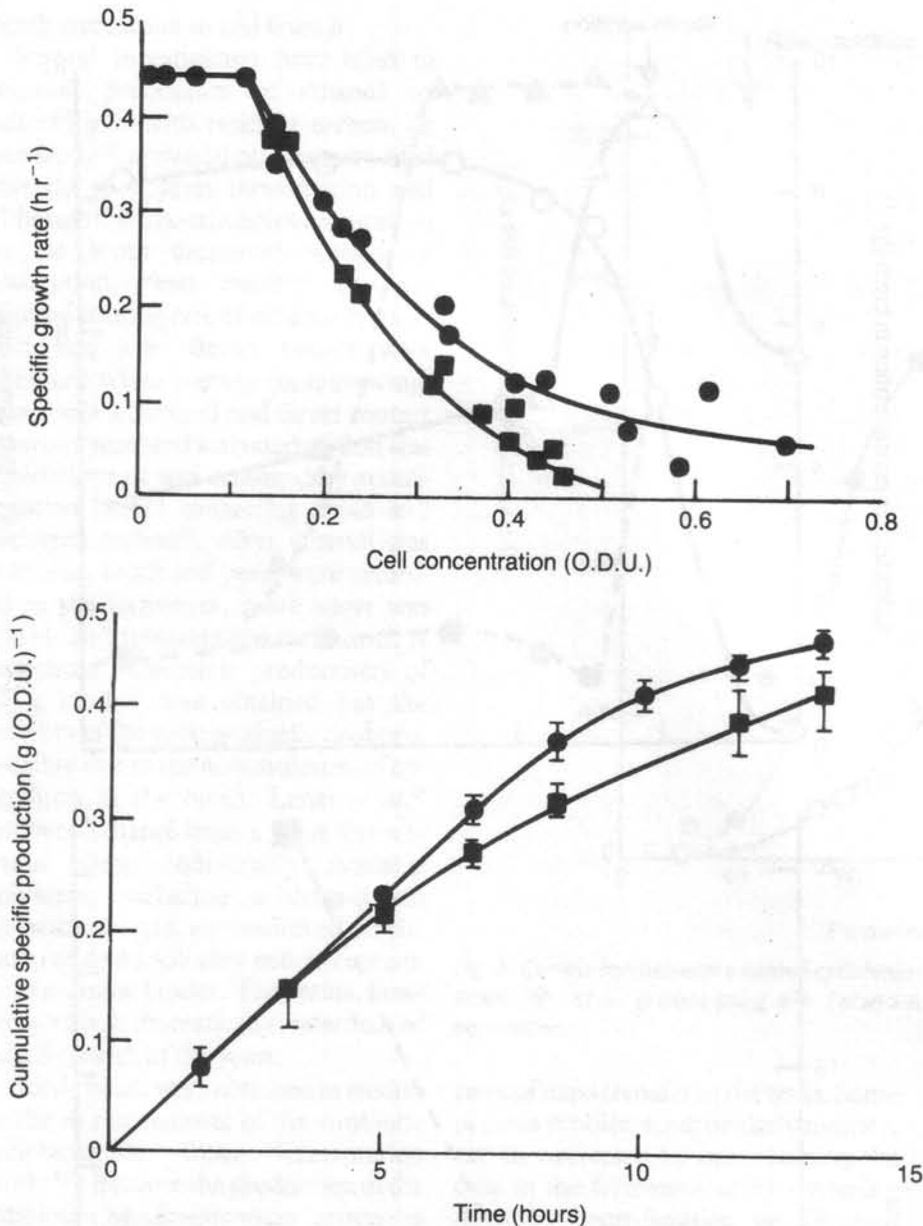


Fig. 5. Specific growth rate of L. delbrueckii *and lactic acid production with dialysis fermentation (●) and conventional fermentation (■). O.D.U. = optical density, a measure of cell concentration.* Reproduced from Ref. 28 with permission.

ties are achieved; smaller, less expensive fermentors could be used. Dialysis can be applied to any diffusible fermentation product but the inherent dilution means that subsequent recovery costs will be increased. Proteins and other components of the medium can foul membranes and the accumulation of non-diffusing metabolites will inhibit the cells. In addition, membranes are expensive and their lifetimes are hard to predict.

Future potentials

In situ product recovery can reduce metabolite inhibition, allow the use of concentrated feedstocks to reduce waste water treatment requirements, and minimize product degradation. Increases in productivity and product yield and reduced costs in downstream processing are partially offset by increased process complexity and equipment cost. Although most studies on *in situ* product recovery (with the exception of vacuum fermentation) have used batch or semi-batch systems, the advantages of *in situ* recovery should be most pronounced for continuous processes. However, better estimates are still needed of the capabilities, limitations and costs of *in situ* product recovery in continuous processes.

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