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Increased productivity of *Clostridium acetobutylicum* fermentation of acetone, butanol, and ethanol by pervaporation through supported ionic liquid membrane

Pavel Izák · Katrin Schwarz · Wolfgang Ruth · Hubert Bahl · Udo Kragl

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Abstract Pervaporation proved to be one of the best methods to remove solvents out of a solvent producing *Clostridium acetobutylicum* culture. By using an ionic liquid (IL)-polydimethylsiloxane (PDMS) ultrafiltration membrane (pore size 60 nm), we could guarantee high stability and selectivity during all measurements carried out at 37°C. Overall solvent productivity of fermentation connected with continuous product removal by pervaporation was 2.34 g l^{-1} h^{-1}. The supported ionic liquid membrane (SILM) was impregnated with 15 wt% of a novel ionic liquid (tetrapropylammonium tetracyano-borate) and 85 wt% of polydimethylsiloxane. Pervaporation, accomplished with the optimized SILM, led to stable and efficient removal of the solvents butan-1-ol and acetone out of a *C. acetobutylicum* culture. By pervaporation through SILM, we removed more butan-1-ol than *C. acetobutylicum* was able to produce. Therefore, we added an extra dose of butan-1-ol to run fermentation on limiting values where the bacteria would still be able to survive its lethal concentration (15.82 g/l). After pervaporation was switched off, the bacteria died from high concentration of butan-1-ol, which they produced.

Keywords *Clostridium acetobutylicum* · Pervaporation · Supported ionic liquid membrane · Acetone–butanol–ethanol fermentation

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Introduction

The Gram-positive bacterium *Clostridium acetobutylicum* is well known for its ability to produce under certain circumstances the solvents acetone, butan-1-ol, and, in minor amounts, ethanol (Woods 1993; Bahl and Dürre 2001). Until the middle of the last century, the clostridial acetone–butanol–ethanol (ABE) fermentation had been used in industrial-scale fermentation systems, and in total volume, it was only exceeded by ethanol fermentation (Rose 1961). However, high substrate costs and inconvenient product recovery rates caused the termination of the commercial fermentation. Due to the oil crisis of the seventies, an emerging scarcity of the oil resources, and an associated increase of the oil prices, the clostridial ABE-fermentation received attention again (Dürre 2005a, b). A lot of work was done for better understanding of the molecular mechanisms of the solvent production (Qureshi and Maddox 2005; Fischer et al. 2006; Cornillot et al. 1997; Dürre 2005a, b) as well as the optimization of it (Dürre 2005a, b). Therefore, it could be shown that the

growth of *C. acetobutylicum* in a chemostat culture with an excess of glucose, a pH below 5, and a limitation of phosphate led to the highest glucose consumption and product concentration over a long period of time (Bahl et al. 1982a, b; Bahl and Gottschalk 1984). Due to the importance of an efficient fermentation process, various product recovery methods have been tested. Their application is especially interesting in batch fermentation systems in which butan-1-ol, due to its toxicity, is one of the most important growth-limiting factors (Dürre 1998). For a long time, distillation had been the method of choice despite the high boiling point of butan-1-ol and energetic inefficiency of the process (Dürre 2005a, b). Membrane-based methods like reverse osmosis, perstraction, pervaporation, and membrane evaporation offer an alternative. These methods feature high selectivity and are, despite their high tendency for clogging and fouling, (Dürre 1998; Qureshi and Maddox 2005) still the most promising product recovery techniques coupled with fermentation. Therefore, we focused on one of the most effective downstream separation process for ABE fermentation—pervaporation.

Pervaporation is a very mild process, and hence, a very effective one for separation of those mixtures, which cannot survive harsh conditions of distillation. The liquid feed mixture is in direct contact with the upper surface of the membrane, and the permeate comes out in vapor state from the opposite side of the membrane, which is kept under low pressure by continuous pumping, i.e., vacuum pervaporation (Fig. 1). After condensation in a cold trap, the permeate is finally collected in the liquid state. Pervaporation is very effective for diluted solutions containing small amounts of a component to be removed. Yu et al. (2006) showed that ionic liquid and then polydimethylsiloxane (IL+PDMS) membranes assure a better removal of solvents than the classical polymer membrane.

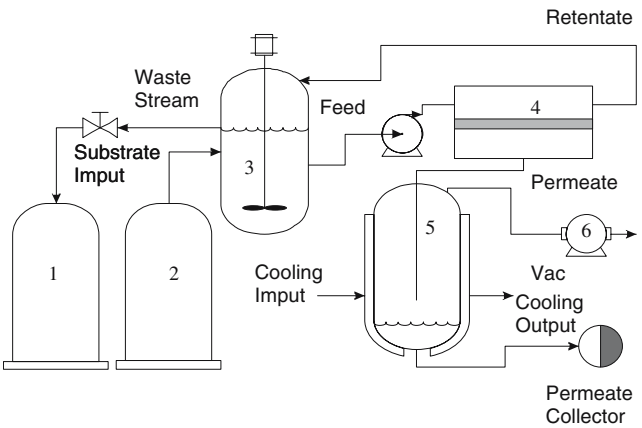

Fig. 1 Schema of continuous culture fermentation connected with pervaporation. *1* Waste tank; *2* tank with substrate; *3* culture vessel; *4* pervaporation cell; *5* cold trap; *6* vacuum pump

To achieve even more efficient ABE removal by pervaporation, we prepared the IL-PDMS membrane, which has better separation properties than classical polymer membrane (Izák et al. 2006). Ionic liquids have been recognized as a possible environmentally benign alternative to classical organic solvents mainly due to their good thermal stability and ability to solubilize a large range of organic molecules and transition metal complexes (Holbrey and Seddon 1999; Duppont et al. 2002; Welton 1999; Wasserscheid and Keim 2000). ILs are electrically conductive and have an extremely low vapor pressure (their noticeable odors are probably caused by impurities). Recent studies have shown that ionic liquids can serve as solvents for biocatalysis (Walker and Bruce 2004; Van Rantwijk and Sheldon 2007) or esterification (Izák et al. 2005). Because of their distinctive properties, ionic liquids are attracting increasing attention in many fields, including organic chemistry, electrochemistry, catalysis, physical chemistry (Heintz 2005), and engineering. Due to their non-volatility, effectively eliminating a major pathway for environmental release and contamination, ionic liquids have been considered as having a low impact on the environment and human health, and thus, recognized as solvents for green chemistry. However, this is distinct from toxicity, and it remains to be seen how "environmentally friendly" ILs will be regarded once widely used by industry (Pretti et al. 2006).

Materials and methods

Bacterial strains and growth conditions

C. acetobutylicum ATCC 824 (laboratory collection; Prof. Hubert Bahl, University of Rostock) was grown under anaerobic phosphate-limited conditions in a Biostat B 1.5-l fermenter system (BBI, Melsungen, Germany) at 37°C, pH 4.5, and 200 rpm with 4 (wt%) glucose in the minimal medium (Bahl et al. 1982a, b) of the supplying vessel (Fischer et al. 2006). For the generation of a continuing butan-1-ol surplus, a sterile solution of butan-1-ol (analytical grade; Sigma Aldrich, Germany) was injected into the supplying vessel up to concentrations of 5, 10, 15, and 18 g/l.

Measurement of optical density and phosphate concentration

Optical density (OD—bacteria quantity) at 600 nm and phosphate concentration were measured in accordance with previous description by Fischer et al. (2006). The OD was determined in a spectrophotometer (Spekol 1100 photometer; Analytic Jena, Jena, Germany) using a plastic cuvette with a light path of 1 cm. Cell suspensions out of the culture vessel

were diluted with distilled water to the maximal OD of 0.3 and measured against distilled water. Phosphate was measured colorimetrically by the formation of yellow colored molybdate–vanadate complexes (Zilversmit and Davis 1950). Therefore, 1 ml of cell suspension was collected, sedimented (16,000×*g*, 1 min, 4°C) and 0.5 ml of the cell free supernatant mixed with an equal amount of 1.2 M trichloroacetic acid followed by 15-min incubation and 15-min sedimentation (16,000×*g*) at room temperature. Subsequently, 0.5 ml of 21 mM ammonium–vanadate (in 0.28 M HNO_3) and 0.5 ml of 40 mM ammonium–molybdate (in 1.25 M H_2SO_4) were added to 0.5 ml of deproteinated culture supernatant followed by vortexing and 10-min incubation at room temperature. The extinction was measured at 405 nm in a plastic cuvette (1-cm light path) using Ultrospec 3000 photometer (Amersham Pharmacia Biotech, Freiburg, Germany). The slope (*m*), calculated from a calibration curve (0.1 and 1 mM KH_2PO_4), was used to compute the phosphate concentration ($c_{phosphate}$) by the equation $c_{phosphate}$ (in millimoles) = $E405/m$.

Analyses of fermentation products

The concentration of the fermentation products (acetate, butyrate, acetone, butanol, ethanol) in the culture vessel was detected by gas chromatographic (GC) analysis and high-pressure liquid chromatography (HPLC) at regular time intervals. For GC analysis, 2 ml of cell suspension were taken out of the culture vessel and sedimented at 16,000×*g* for 1 min at 4°C. Further treatment of the cell-free supernatant and the detection of the acids and solvents were carried out as described by Bahl et al. (1982a, b) who used gas chromatograph (CP9001; Chrompack, Frankfurt am Main, Germany) and Chromosorb 101 (80/100 mesh) column. HPLC analysis was carried out in an ion exclusion mode (Berlin, Germany) under the following operating parameters: column, Aminex HPX-87H (300×7.8 mm; Bio-Rad Laboratories, Hercules/CA, USA); mobile phase, 0.006 M sulphuric acid; temperature, 65°C; detection, refractive index. An external standard calibration was used as quantification method. HPLC analysis was also used for the determination of the product concentration in the permeate (cold trap) and GC analysis for the detection of butan-1-ol concentration in the supplying vessel.

Supported ionic liquid membrane preparation

The tetrapropylammonium tetracyano-borate ionic liquid (Fig. 2; 15 wt%) was mixed with 85 wt% of polydimethylsiloxane (PDMS). PDMS itself was prepared by mixing a solution of Elastosil M4601A and M4601B (Wacker Silicones) in a 9:1 ratio at 23°C for 1.5 h.

As a support matrix for the nonporous membrane, we used the ceramic ultrafiltration membrane made from TiO_2 with a pore size of 60 nm. The ceramic asymmetric modules were 500 mm long with an external diameter of 10 mm and an effective area of 0.011 m^2. They were made by Inopor GmbH (Germany). Subsequently, the prepared viscous blend of IL and PDMS was used to impregnate the membrane inside the burette for 0.5 h at 80°C. After impregnation, the membrane was then taken out of the burette, cooled down to the room temperature, and left to cure for 24 h. At any time, 18 ml of IL-PDMS blend was kept in the ceramic module during all the time necessary for the experiments. The stability of IL-PDMS blend inside the pores was checked by weighting, and no weight change of the module was observed.

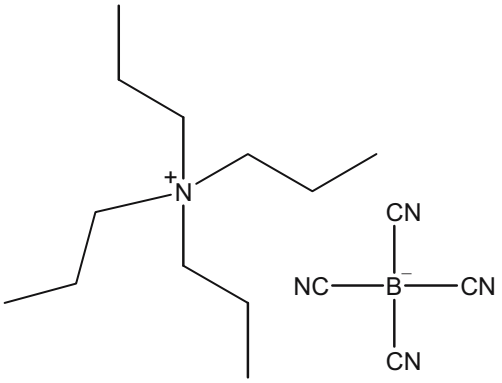

Fig. 2 The chemical structure of the tetrapropylammonium tetracyano-borate ionic liquid

Results

The previous experiments have shown that IL-PDMS membrane has significantly better separation properties than PDMS membrane itself. The enrichment factor of butan-1-ol increased from 2.2 (PDMS) up to 10.9 (IL-PDMS; Izák et al. 2008). The free radical polymerization reactions conducted in ionic liquid are faster than in classical molecular solvents, and they tend to yield polymers with higher molecular weight (Hong et al. 2002). The exact impact on the mechanism of the polymerization, caused by the replacement of traditional solvents with ionic liquids, has not been fully understood yet, but it includes reduced termination rates (partly due to solvents with higher viscosity), increased propagation constants (Harrison et al. 2002), and low chain transfer constants (Benton and Brazel 2002). To test the supported IL-PDMS membrane with a living organism, we chose *C. acetobutylicum* due to its industrial interesting skills of solvent production (Dürre 2005a, b) and its long life and stability in a continuous culture (Bahl et al. 1982a, b).

SILM was tested at different culture conditions, which had a significant influence on the amount of butan-1-ol

Table 1 The initial composition of fermentation broth at different dilution rate and phosphate concentration in supplying vessel at 37°C and pH 4.5

Dilution rate (h^{-1})	Phosphate (mM)	OD_{600}	Acetone ($g\ l^{-1}$)	Butan-1-ol ($g\ l^{-1}$)	Acetate ($g\ l^{-1}$)	Butyrate ($g\ l^{-1}$)	Ethanol ($g\ l^{-1}$)	Solvent productivity ($g\ l^{-1}h^{-1}$)
0.05	0.75	7.12	3.82	7.12	0.97	0.64	0.67	0.66
0.075	0.75	7.1	2.82	5.44	0.98	0.65	0.50	0.78
0.075	0.5	8.52	5.00	10.38	1.62	0.44	0.94	1.38
0.09	0.75	6.25	3.18	7.07	0.93	0.69	0.77	1.14

produced by the bacteria (Bahl and Gottschalk 1984). In the chosen fermentation system, especially the phosphate, concentrations as well as the dilution rates were responsible for the amount of produced solvents. Therefore, different phosphate concentrations (0.5, 0.75 mM) and dilution rates (0.05, 0.075, 0.09 h^{-1}) were tested according to the recovered solvent concentration. As it can be seen from the Table 1, the highest solvent productivity and also the highest optical density were reached at the dilution rate 0.075 h^{-1} and 0.5 mM phosphate concentration in the supplying vessel. Therefore, all our further experiments followed these conditions.

Firstly, a continuous fermentation with removal of ABE by pervaporation was measured without any butan-1-ol addition to test if the SILM was selective and stable. After successful tests, the concentration of butan-1-ol was several times (four additions marked as four arrows in the Fig. 3) increased to test the SILM under more stringent conditions and to study the effect of pervaporation on the cells.

Figure 3 shows how the concentration of the products was changing with time of fermentation in the culture vessel after several additions of butan-1-ol. It can be seen that it was able to remove more ABE by pervaporation than *C. acetobutylicum* was producing. No change in weight of the IL-PDMS inside the module during the experimental conditions was recorded in the course of 3 months.

When butan-1-ol concentration started to be lethal for the cells, the continuous ABE removal was stopped (pervaporation was switched off). Butan-1-ol amount in the culture vessel increased, and the optical density dramatically decreased (see Fig. 4). Subsequently, pervaporation was switched on again to help the cells survive the lethal butan-1-ol concentration. Pervaporation was switched off twice to prove the significance of the change in production of butan-1-ol and optical density. Between hours 396 and 444, butan-1-ol concentration increased from 9.8 to 14.5, and between hours 456 and 480 from 13.7 to 15.8 g/l, respectively, indicating the effectiveness of pervaporation. In Fig. 4 is also displayed how butan-1-ol concentration is changing in permeate with time of experiment.

To make sure if the cells after the first pervaporation disconnection (butan-1-ol in the feed reached 13.67 g/l and the optical density was 0.10) were still alive, a sample of 1 ml cell suspension was taken out of the culture vessel. It was centrifuged (5,000 rpm, 4°C, 5 min) to pellet the cells and remove them from the old medium rich in butan-1-ol. The removed cells were inoculated into 10 ml fresh anaerobic minimal medium and incubated at 37°C to make sure that they were still alive and able to grow. After a long time of convalescence (48–60 h), the cells grew again in

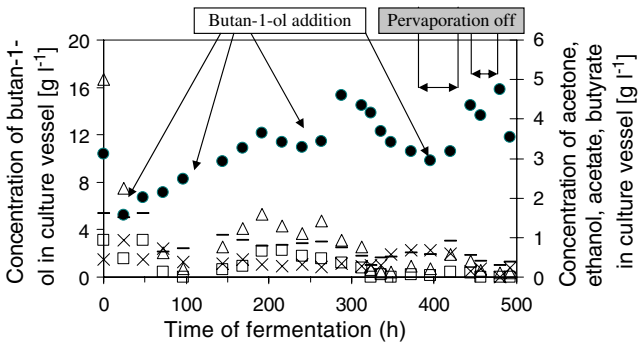

Fig. 3 Dependence of permeate concentration on fermentation time at 37°C, at dilution rate 0.075 h^{-1}, 0.5 mM phosphate concentration in supplying vessel, and pH 4.5. Butan-1-ol addition (24 h, 7 g l^{-1}; 96 h, 7 g l^{-1}; 240 h, 5 g l^{-1}; 396 h, 8 g l^{-1}); *filled circle*, butan-1-ol (summary of the produced and added butan-1-ol); *open triangle*, acetone; *open square*, ethanol; *soild line*, acetate; x, butyrate

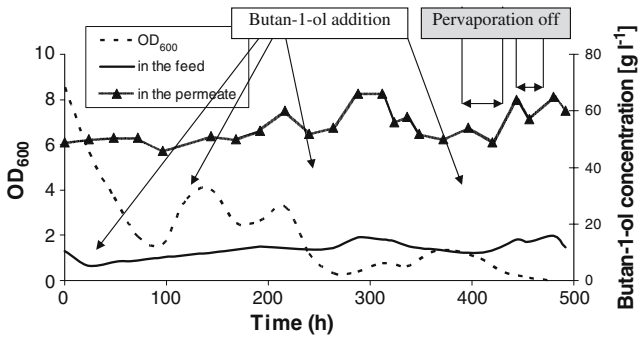

Fig. 4 Dependence of optical density and concentration of butan-1-ol in culture vessel on fermentation time. *Solid line*, butan-1-ol (summary of the produced and added butan-1-ol); *dashed line*, optical density; *filled triangle and solid line*, butan-1-ol concentration in the permeate

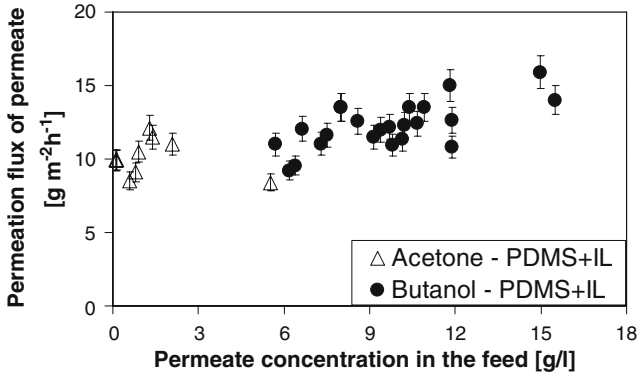

Fig. 5 Dependence of butan-1-ol and acetone permeation flux on its culture vessel concentration. *Filled circle*, butan-1-ol; *open triangle*, acetone

rich medium. Thus, it was proven that they were still able to grow after pervaporation had been stopped. When pervaporation was no longer removing any permeate, the level of butan-1-ol increased during 24 h up to 15.82 g/l and OD_{600} decreased to 0.0.

The velocity of butan-1-ol and acetone separation during pervaporation is represented by Fig. 5. Permeation fluxes of butan-1-ol and acetone (Eq. 1) slightly decreased with the reduction of their contents in the culture vessel.

$$J_i = Jw_{iP}, \tag{1}$$

where J is total permeation flux through the supported ionic liquid membrane and w_{iP} is the weight fraction of component i in the permeate. Another very important factor for the evaluation of the membrane quality is the selectivity represented by the enrichment factor. The selectivity of separation is displayed in the Fig. 6 where the enrichment factors (Eq. 2) of the permeates increased with the decrease of permeate contents in the culture vessel, which is a typical behavior during pervaporation process.

$$\beta_i = w_{ip}/w_{iF}, \tag{2}$$

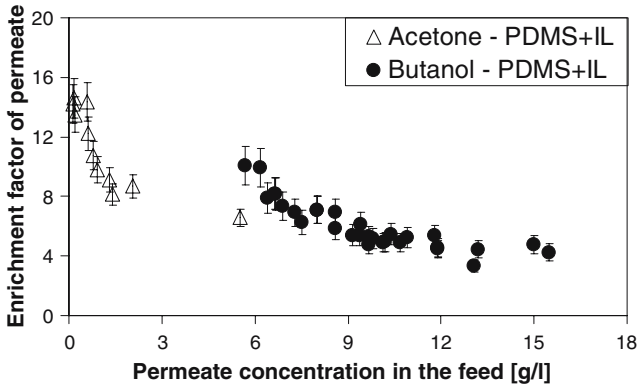

Fig. 6 Dependence of butan-1-ol and acetone concentration in the permeate on its culture vessel concentration. *Filled circle*, butan-1-ol; *open triangle*, acetone

where w_{iP} is the mol fraction of component i in the permeate and w_{iF} is the mol fraction of component i in the feed.

As it can be observed from Fig. 6, the enrichment factor of acetone increased from 6.57 at 5.25 g/l (concentration of acetone in culture vessel) to 14.6 at 0.15 g/l when 15 wt% of IL was mixed in PDMS and anchored inside 60-nm pores of ceramic ultrafiltration module. In case of butan-1-ol, the enrichment factor increased from 4.25 at 15.82 g/l (concentration of butan-1-ol in the culture vessel) to 11.23 at 5.71 g/l.

Discussion

Liu et al. (2005) studied ABE fermentation coupled with pervaporation at 23°C. They obtained similar results with Polyether block amide membrane (PEBA 2533) where the enrichment factor of butan-1-ol reached even 11.60 at 1.03 wt % of butan-1-ol in the culture vessel. However, the permeating flux of butan-1-ol was 6.60 g m^{-2} h^{-1} in PEBA 2533, which is much lower than 13.64 g m^{-2} h^{-1} in PDMS-IL at 5.71 g $l^{-1}$of butan-1-ol in the culture vessel at 37°C in our system. If we calculate solvent productivity during our fermentation conditions (see Table 1) with continuous pervaporation removal of solvents, we reach 2.34 g l^{-1} h^{-1} compared to the 2.09 g l^{-1} h^{-1} reported by Soni et al. (1987). Qureshi and Maddox (2005) compared several downstream processes integrated with ABE fermentation for product removal, namely pertraction, liquid–liquid extraction, gas stripping, and also pervaporation. The product removal allows the use of concentrated sugar solutions in the process, thereby reducing process and waste stream volumes. The relatively high salt content of whey permeate (14% of total solids) seemed to be the major reason for termination of fermentation. Qureshi and Maddox (2005) concluded that pervaporation and gas stripping appeared to be the most promising product recovery techniques coupled with fermentation.

To get even more effective ABE removal, we prepared the IL-PDMS nonporous membrane. This membrane showed high stability as well as selectivity during all experiments with fermentation broth. Using this membrane, we were able to remove butan-1-ol and acetone from the culture supernatant more effectively as described by others (Qureshi and Maddox 2005; Soni et al. 1987; Liu et al. 2005). We were able to remove more butan-1-ol and acetone than the *C. acetobutylicum* was able to produce.

As we can see from Fig. 4, butan-1-ol concentration in the permeate increased more than five times in comparison with the concentration in the culture vessel. In addition, the other products of ABE fermentation (ethanol, acetone) were enriched several times in the permeate. Distillation is a traditional technology for separating alcohols from dilute biomass fermentation broths. However, pervaporation is an

alternative, which may have energy and capital cost advantages relative to distillation, especially for smaller scale systems or at lower concentrations of the feed. Liu et al. (2005) reported that from an application point of view, the PEBA membrane can be used to extract butanol, and to a lesser extent ethanol and acetone, from the ABE fermentation broth by pervaporation.

Overall solvent productivity of fermentation connected with continuous product removal by pervaporation was 2.34 g l^{-1} h^{-1}. To prove the effectiveness of our setup, we ran the fermentation reaction on the limiting values of butan-1-ol concentration (15.82 g/l) to see if the bacteria could still be alive under these conditions. We proved experimentally that this concentration was already lethal for the bacteria. Considered together, a *C. acetobutylicum* continuous fermentation setup used together with the indicated pervaporation technique would guarantee an increase in fermentation stability and higher production of BIObutanol.

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