

Review

A Review of Process-Design Challenges for Industrial Fermentation of Butanol from Crude Glycerol by Non-Biphasic *Clostridium pasteurianum*

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Academic Editors: Badal C. Saha and Sangita Singh

Received: 5 April 2016; Accepted: 6 June 2016; Published: 15 June 2016

Abstract: Butanol, produced via traditional acetone-butanol-ethanol (ABE) fermentation, suffers from low yield and productivity. In this article, a non-ABE butanol production process is reviewed. *Clostridium pasteurianum* has a non-biphasic metabolism, alternatively producing 1,3-propanediol (PDO)-butanol-ethanol, referred to as PBE fermentation. This review discusses the advantages of PBE fermentation with an emphasis on applications using biodiesel-derived crude glycerol, currently an inexpensive and readily available feedstock. To address the process design challenges, various strategies have been employed and are examined and reviewed; genetic engineering and mutagenesis of *C. pasteurianum*, characterization and pretreatment of crude glycerol and various fermentation strategies such as bioreactor design and configuration, increasing cell density and *in-situ* product removal. Where research deficiencies exist for PBE fermentation, the process solutions as employed for ABE fermentation are reviewed and their suitability for PBE is discussed. Each of the obstacles against high butanol production has multiple solutions, which are reviewed with the end-goal of an integrated process for continuous high level butanol production and recovery using *C. pasteurianum* and biodiesel-derived crude glycerol.

Keywords: glycerol; *Clostridium pasteurianum*; butanol; PBE fermentation; *in-situ* recovery

1. Introduction

Continuous mass consumption of fossil fuels has led to high levels of greenhouse gas emissions (GHG), with little doubt in the scientific community on its dramatic impact on the world's climate [1,2]. Thus, biofuels are considered an attractive option to break dependence on petroleum-based fuels, as mobility is a major part of the world's energy system. Biobutanol could be one of the most promising alternative biofuels due to its many advantages over ethanol [3–5]. Compared to ethanol, butanol has lower solubility in water, higher energy content (27 MJ·L^{−1} vs. 19.6 MJ·L^{−1}), lower volatility and is less corrosive [5,6]. As a result, butanol can replace up to a 100% of petroleum-based fuels without structural modifications of the current engine technologies [7]. Furthermore, butanol can be blended directly at the refinery and transported through existing pipeline infrastructure [8].

Currently, 11 biobutanol fermentation plants are in operation in China (plus an additional 2 under construction) [9] and 1 in Brazil [4]. The current plants are all using either starch (corn, cassava, sweet potato) or sugars (molasses) as a carbon source. High and costly substrates that compete with human food (sugar, starch) is one of the main drawbacks of these fermentation plants. The butanol

production cost and profitability of a plant largely depend on substrate cost and are extremely sensitive to any price fluctuation [10–12]. Therefore, transition toward low-cost, non-edible, readily and reliably available feedstock at industrial scale is crucially important from a process economics perspective and can offer the biggest opportunity for cost reduction and improved sustainability [13].

Glycerol as an alternative carbon source, produced as a major byproduct of the biodiesel industry, has recently been attracting much attention as a good substrate for bio-based butanol production [14–16]. As a consequence of the expanding biodiesel production, surplus quantities of biodiesel-derived glycerol (commonly referred to as ‘crude’ glycerol) are being produced [14,17]. Disposal of crude glycerol has become a financial and environmental liability for the biodiesel industry, reducing the selling price of crude glycerol in the US to between 4 and 11 cents/kilogram in 2011 [18]. The impurities present in the crude glycerol are responsible for the greatly lowered price compared to pure glycerol [14,19]. Its abundance and cost competitiveness make glycerol an excellent alternative to other carbon substrates for butanol production [13,20]. Development of glycerol-based butanol production processes can add significant value to the biodiesel industry and presents excellent potential to establish industrial production of butanol near existing distribution infrastructure [20–23].

Although butanol has many attractive properties, ABE fermentation suffers from low productivity and high operational and capital costs [3,24,25]. Therefore, a number of researchers have tried to overcome these problems by means of genetic manipulations of *Clostridia* spp. to improve strains’ butanol titer and tolerance [1,16,26], fermentative techniques to increase the cell density as well as butanol yield and productivity [1,27,28], and *in-situ* product recovery technologies to overcome the butanol toxicity to fermentative microorganism [29–32].

This review aims to present recently published data on *Clostridium pasteurianum* as an alternative microbe for biobutanol production from crude glycerol and relevant challenges for industrial fermentative conversion.

2. Characterization of Biodiesel-Derived Crude Glycerol

Because crude glycerol is itself a waste stream which has been highly processed, the concentration of the impurities varies between and within biodiesel production plants [33,34]. This is due to variation in feedstock, the type of catalyst used, the transesterification efficiency, recovery efficiency of the biodiesel, and whether the methanol and catalysts were recovered. These impurities pose some of the greatest industrial challenges which need to be understood and addressed. Therefore, it is crucial to understand the chemical composition of crude glycerol before considering its fermentative conversion.

The crude glycerol impurities commonly are methanol, free fatty acids (FFAs), salts, moisture, ash, soap and methyl esters [34]. In one study, Rehman *et al.* reported that crude glycerol from the transesterification of sunflower oil as feedstock contained (*w/w* %): 30 glycerol, 50 methanol, 13 soap, 2 moisture, approximately 2–3 salts, and 2–3 other impurities. In another study, crude glycerol generated from biodiesel production using soybean oil contained 70% to 85% *w·w*^{−1} glycerol [35]. However, Thompson and He (2006) reported minimal variation between glycerol samples obtained from different feedstocks [36]. Although it should be noted the crude glycerol was produced in a laboratory setting, rather than industrial in all aforementioned studies.

In the case of crude glycerol from an industrial biodiesel plant, De Carvalho *et al.* investigated the chemical composition of two types of crude glycerol generated from biodiesel production, using soybean oil and a mixture made of 80% animal fat and 20% soybean oil [37]. Both samples were obtained from Biopar biofuel industry located in Brazil and contained about 55% glycerol and 4% ash. Soybean oil crude glycerol contained slightly higher amount of matter organic non-glycerol, methanol, and total fatty acids compared to crude glycerol generated from mixed substrate. Hansen *et al.* studied the chemical compositions of 11 crude glycerol samples collected from seven Australian biodiesel producers and indicated that the glycerol content ranged between 38% and 96%, with some samples including about 14% methanol and 29% ash [34]. In another study, the chemical composition of five crude glycerol samples from industry was investigated and described by eight components

including: free glycerol, methanol, water, soap, fatty acid methyl esters, glycerides, free fatty acids and ash. The compositions of these four biodiesel-derived crude glycerol samples varied significantly from each other; for example, free glycerol contents ranged from 22.9% to 63.0%.

In order to improve crude glycerol composition, heterogeneous catalysts such as solid and enzyme catalysts have been used as alternatives to homogenous alkaline catalysts. Bournay *et al.* reported 98% glycerol content in crude glycerol produced from biodiesel production using rapeseed oil as substrate with heterogeneous catalyst [38]. Neither ash, nor inorganic compounds were detected in the crude glycerol produced with the major impurities being water, methanol and other 'matter organic non-glycerol' (MONG). Therefore, characterization of crude glycerol will need to be an ongoing part of quality assurance prior to bioconversion at industrial scale.

3. Microbial Metabolism of Glycerol

A number of microorganisms are able to grow anaerobically on glycerol as the sole carbon and energy source, such as *Citrobacter freundii* [39,40], *Klebsiella pneumoniae* [41,42], *Clostridium butyricum* [43,44], *Enterobacter agglomerans* [45,46], *Enterobacter aerogenes* [47] and *Lactobacillus reuteri* [48]. However, most of them do not convert this substrate into butanol. The literature shows that the best studied organism to do so is *Clostridium pasteurianum*, a gram-positive, anaerobic and non-pathogenic bacteria [20–22]. The reported solvents produced by *C. pasteurianum* utilizing glycerol as substrate are: butanol, PDO and ethanol. By-products include acetic acid, butyric acid as well as CO₂ and H₂. In contrast to the ABE fermentation process, no acetone is produced. Therefore, fermentation of glycerol using *C. pasteurianum* could be referred to as a "PBE" process to reflect PDO production in lieu of acetone.

During anaerobic fermentation, the overall redox balance within the cell is maintained by shifting between metabolic pathways resulting in different products and reducing equivalents being formed. The highly reduced nature of glycerol results in the production of twice the amount of reducing equivalents compared to the catabolism of lignocellulosic sugars such as glucose and xylose [15]. These additional reducing equivalents provide glycerol with the natural advantage of higher theoretical product yield for reduced chemicals and fuels.

Moreover, compared to the characteristic growth pattern of acetogenesis and solventogenesis found in ABE fermentations, *C. pasteurianum* shows little biphasic behaviour when grown on glycerol [23,49]. This is a result of the regulation of the metabolic pathway leading from glycerol to butanol. This pathway has a neutral redox balance and was reported to be energetically preferred [50]. However, PDO plays an important role in maintaining glycerol fermentation of *C. pasteurianum*. In contrast to the *Clostridia* spp. used in ABE fermentation, *C. pasteurianum* has a reductive pathway for the production of PDO independent of glycolysis. The production of PDO enables *C. pasteurianum* to balance the cellular redox potential with reducing equivalents required when biomass is formed. Therefore, cellular energy can be produced in glycolysis, independent of acetic and butyric acid production, while butanol production can be maintained simultaneously to biomass formation [23,49]. A simplified pathway showing the glycerol metabolism of *C. pasteurianum* with a focus on end-products is presented in Figure 1.

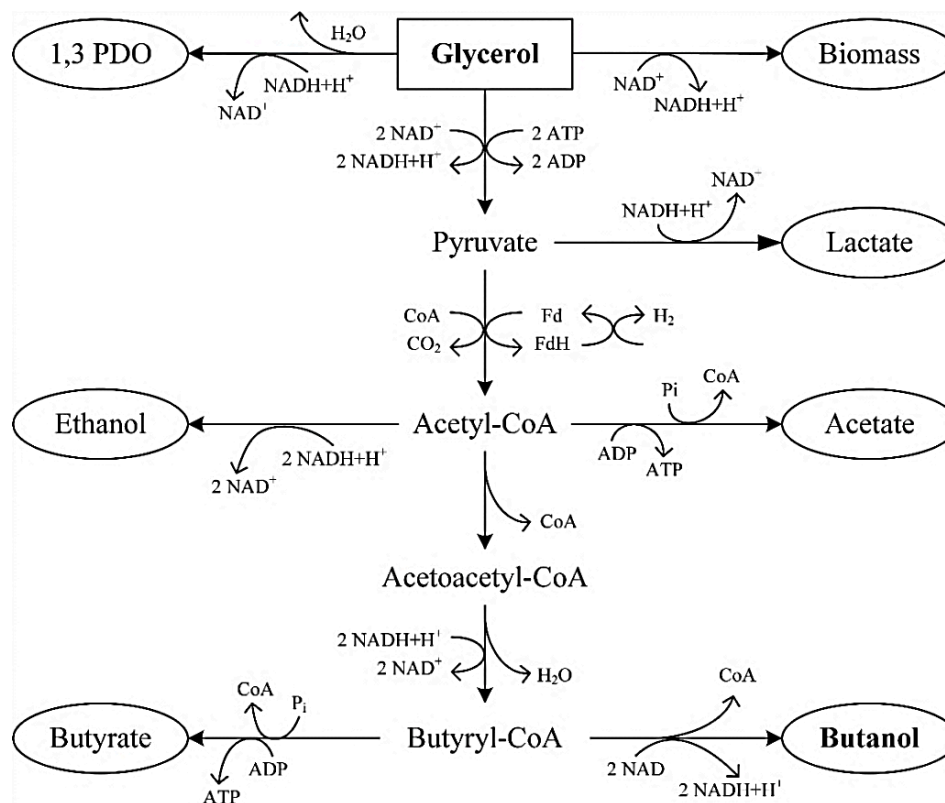


Figure 1. Possible metabolic pathway for glycerol fermentation by *C. pasteurianum*. Adapted from Biebl [50], Venkataramanan [51], and Malaviya [1].

4. Biodiesel-Derived Crude Glycerol Pretreatment

The utilization of crude glycerol in fermentations may require pretreatments due to the impurities in crude glycerol composition acting as inhibitory agents, causing microbial growth inhibition, lengthening fermentation time and lowering butanol yield and productivity. However, reports investigating the individual effects of these impurities have shown that different compounds present in the crude glycerol can have a varying effect on *C. pasteurianum* [51]. Venkataramanan *et al.* reported that the addition of methanol and salt to the media did not affect the cell growth and butanol yield. However, free fatty acids (FFAs) present in vegetable oil and thus crude glycerol had an inhibitory effect on both cell growth and butanol yield, particularly the unsaturated moieties such as oleic acid and linoleic acid. The authors found no reports on the effect of soap, glycerides or methyl esters on *C. pasteurianum* growth or butanol yield.

For example, crude glycerol can be refined by the following steps: saponification using strong alkali material to transform fatty acid methyl esters (FAMES) to soap and methanol and glycerides to soap and glycerol, acidification which converts all soap to free fatty acids (FFAs) and salts, phase separation into three layers (a top organic layer rich in FFAs, a middle layer rich in glycerol, and a bottom layer rich in inorganic salts), harvest of the glycerol rich portion, filtration, followed by neutralization. Water and salts can be removed by evaporation and centrifugation, respectively, as shown in Figure 2 [52–54].

In one study, the crude glycerol was purified following the aforementioned steps excluding vacuum distillation and the results are presented in Table 1 [54]. Potassium hydroxide was used as a strong alkali catalyst in the saponification process with 1:1 molar ratio of potassium hydroxide to ester. In the next experiment the molar ratio of potassium hydroxide to ester was enhanced to 1.2:1. As shown in Table 1, a concentrated glycerol phase with 85% purity and an organic phase with 99.5% FFAs purity were obtained, indicating the efficiency of this purification process.

However, in most studies, *C. pasteurianum* has been shown to ferment biodiesel-derived crude glycerol, requiring minimal upgrading. For example, in one study, the crude glycerol was diluted with water and filtered through a 0.2 μm filter three times to remove solids and was used as sole carbon source for butanol production. It was reported that *C. pasteurianum* was capable of converting crude glycerol ($50 \text{ g} \cdot \text{L}^{-1}$) to butanol with a maximum butanol yield of $0.27 \text{ g} \cdot \text{g}^{-1}$ after 35 h, only slightly lower than the yield on pure glycerol ($0.28 \text{ g} \cdot \text{g}^{-1}$, 30 h) [55]. In another study, Venkataramanan removed the FFAs via acid precipitation and phase separation from the crude glycerol prior to fermentation, resulting in butanol yields matching those found with pure ($0.26 \text{ g} \cdot \text{g}^{-1}$ for pure *vs.* $0.28 \text{ g} \cdot \text{g}^{-1}$ with treated crude) [56], with both fermentations taking 96 h. When compared to untreated crude glycerol, the yield was $0.21 \text{ g} \cdot \text{g}^{-1}$ over a two-week period. Some of the reported research on PBE fermentation using pure and crude glycerol as substrate is summarized in Table 2. As can be seen, volumetric productivity suffers when fermentation time is extended.

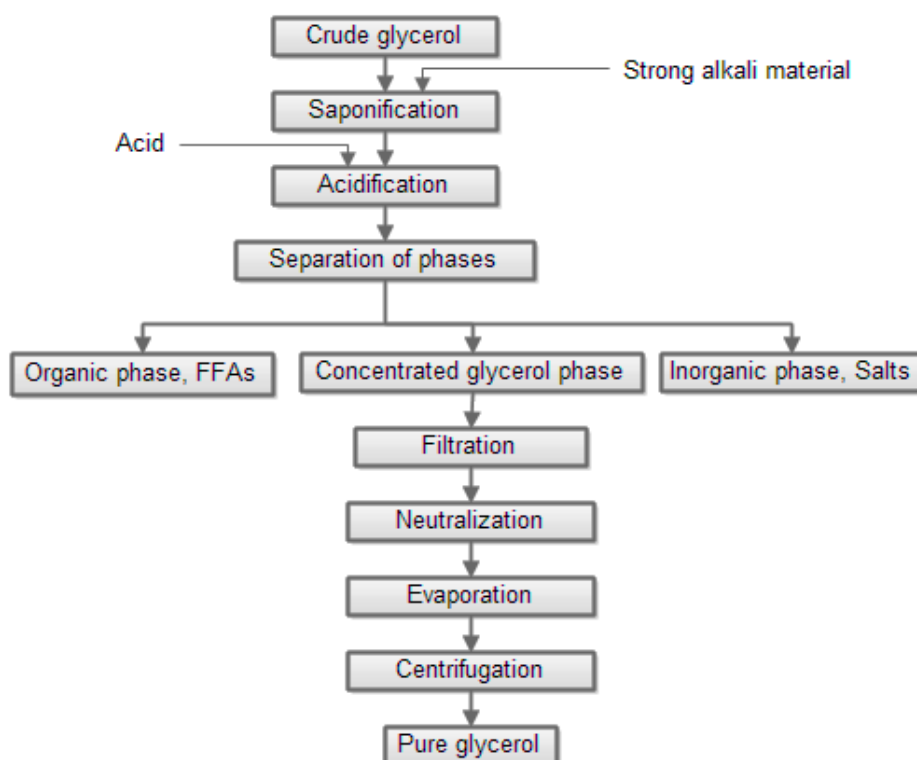


Figure 2. Diagram of possible steps of purification of crude glycerol.

Table 1. The composition of crude glycerol (Average \pm standard deviation), concentrated crude glycerol and organic acid after phase separation [54].

Compound	Crude Glycerol wt %	Concentrated Glycerol Phase		Organic Phase	
		Molar Ratio of KOH: Esters		Molar Ratio of KOH: Esters	
		1:1	1.2:1	1:1	1.2:1
Glycerol	55.5 ± 3.9	84.7	85.1	Nt	Nt
Soap	18.6 ± 2.8	Nt	Nt	Nt	Nt
Salts	1.7 ± 0.28	2.39	2.87	Nt	Nt
Water	13.3 ± 1.37	12.1	11.1	Nt	Nt
Methanol	2.9 ± 1.48	0.46	0.37	Nt	Nt
Esters	8.1 ± 1.65	Nt	Nt	4.8	0
FFAs	Nt	Nt	Nt	95	99.5
Others	Nt	0.35	0.56	0.2	0.5

Nt: Not reported.

Table 2. Comparison of bioconversion of pure and crude glycerol to butanol under identical fermentation condition by *C. pasteurianum*.

Strains	Crude Glycerol Pretreatment/ (Fermentation Time)	Culture Condition	Max. Butanol Yield ^a g · g ⁻¹ (mol · mol ⁻¹)		Overall Butanol Productivity g · L ⁻¹ · h ⁻¹		Reference
			Pure Glycerol	Crude Glycerol	Pure Glycerol	Crude Glycerol	
<i>C. pasteurianum</i> (wild type; DSM 525)	Filtration (35 h)	Batch, Free cells, Vol ~ 5 L	0.28 (0.35)	0.27 (0.34)	0.41	0.35	[55]
<i>C. pasteurianum</i> (wild type; ATCC 116)	None (120 h)	Batch, Free cells, Vol < 1 L	0.18 (0.22)	0.13 (0.16)	<0.10	<0.10	[27]
<i>C. pasteurianum</i> (wild type; ATCC 116)	None (120 h)	Batch, Immobilized cells, Vol < 1 L	0.36 (0.45)	0.23 (0.29)	<0.10	<0.10	[27]
<i>C. pasteurianum</i> (wild type; ATCC 6013)	None (14–24 days)	Batch, Free cells, Vol < 1 L	0.26 (0.32)	0.21 (0.26)	<0.1	<0.02	[56]
<i>C. pasteurianum</i> (wild type; ATCC 6013)	Acid precipitation (4 days)	Batch, Free cells, Vol < 1 L	0.26 (0.32)	0.28 (0.35)	<0.1	<0.1	[56]
<i>C. pasteurianum</i> (wild type; ATCC 6103)	None (25 days)	Batch, Free cells, Vol < 1 L	0.31 (0.39)	0.30 (0.37)	0.04	<0.02 ^{b,c}	[22]

^a yield calculated based on glycerol consumed; ^b data inferred from graphical representation; ^c productivity calculated based on active fermentation (subtracted lag phase).

5. Media Composition and Fermentation Condition

The product profile of glycerol fermentation by *C. pasteurianum* largely depends on media composition and fermentation parameters. Moon *et al.* reported that the optimal media composition for butanol production by *C. pasteurianum* was significantly different from media used for production of PDO. It was shown that iron and nitrogen limitations will favor PDO production. The influence of iron limitation matches with previous reports [16]. The optimal yeast extract concentration for butanol production was also different from concentration used for PDO production [57].

When investigating different fermentation parameters, initial glycerol concentration, the inoculum age, initial cell concentration, initial pH of medium, temperature, and agitation rate were studied as major factors that influenced butanol yield and productivity [1,55,58]. The highest butanol yield and productivity was reported to be 0.28 g · g⁻¹ and 0.41 g · L⁻¹ · h⁻¹, respectively, at optimal fermentation condition of inoculum age of 16 h, initial cell density of 0.4 g · L⁻¹_{DCW}, initial pH of 6.8, and temperature of 30 °C [55]. Sarchami *et al.* and Khanna *et al.* reported that at optimal fermentation condition, the scale of operation had no effect on butanol yield and productivity [55,58]. Some of the reported research on optimization of fermentation condition favoring butanol production by *C. pasteurianum* is summarized in Table 3.

Table 3. Summary of studies on optimization of 1,3-propanediol-butanol-ethanol (PBE) fermentation condition favoring butanol production by *C. pasteurianum*.

Strains	Culture Condition	Initial Glycerol Titer g · L ⁻¹	Inoculum Age h	Initial Cell Density g · L ⁻¹ _{DCW}	pH	Temperature °C	Agitation Rate rpm	Max. Butanol Titer g · L ⁻¹	Max. Butanol Yield ^a g · g ⁻¹ (mol · mol ⁻¹)	Overall Butanol Productivity g · L ⁻¹ · h ⁻¹	Reference
<i>C. pasteurianum</i> (wild type; DSM 525)	Batch, Free cells, Vol < 1 L	Pure Non-Sig. 50	Sig. 16	Sig. 0.4	Sig 7.0	Sig. 30	Not-studied	12.3	0.28 (0.35)	0.41	[55]
<i>C. pasteurianum</i> (wild type; ATCC 6013)	Batch, Immobilized cells, Vol < 1 L	Pure Non-Sig. 25	Not-studied	Not-stdied	Sig 7.0	Non-Sig. 30	Non-Sig. 200	7.7	0.21 (0.26)	0.04	[58]
<i>C. pasteurianum</i> (wild type; ATCC 6013)	Batch, Immobilized cells, Vol < 1 L	Crude Sig. 25	Not-studied	Not-stdied	Sig 7.0	Non-Sig. 30	Non-Sig. 200	6.8	0.17 (0.21)	0.035	[58]
<i>C. pasteurianum</i> (wild type; ATCC6103)	Batch, Free cells, Iron limitation, Vol < 1 L	Pure Not-studied 86 ^a	Sig. 18	Sig 0.42	Sig 5.5–6.0	Not-studied 37	Not-studied	10.0	0.25 (0.31)	0.27	[1]

^a yield calculated based on glycerol consumed; Sig: Significant effect on butanol production; Non-Sig: No-Significant effect on butanol production.

6. Metabolic Engineering and Mutagenesis

C. pasteurianum exhibits product inhibition at low levels ($10\text{--}15\text{ g}\cdot\text{L}^{-1}$). Therefore, mutagenesis can be applied to *C. pasteurianum* to create strains with improved product formation and tolerance [1,16,26]. In one study, batch fermentations were performed on the wild type *C. pasteurianum* ATCC 6103 and its genetically modified strain (MBEL_GLY2) [1]. A maximum butanol yield and productivity of $0.30\text{ g}\cdot\text{g}^{-1}$ and $0.31\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ were achieved, respectively, using the MBEL_GLY2 strain. Under the same experimental condition, butanol yield and productivity of $0.25\text{ g}\cdot\text{g}^{-1}$ and $0.27\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ were obtained with the wild type *C. pasteurianum* ATCC 6103. Malaviya *et al.* demonstrated significantly increased production rates in a high cell density continuous bioreactor using the MBEL_GLY2 strain [1]. In another study, the butanol yield and productivity of stored crude glycerol supplemented with activated stone carbon by *C. pasteurianum* DSM 525 and its mutants (MNO6) were investigated [26]. The maximum stored crude glycerol utilization rate attained by MNO6 was $7.59\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, whereas the wild type strain reached rates of $4.08\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$. This corresponds to an increased rate of 86% compared to the wild type. The butanol production rate was similarly increased by 38% compared to the wild type grown on stored crude glycerol. Some of reported studies on butanol production from glycerol by hyper producing mutants of *C. pasteurianum* are presented in Table 4.

Until recently, there was no information about the whole genome of *C. pasteurianum* and this restrained efforts in applying metabolic engineering to this species of bacteria. Recently, however, genomic information was revealed for these two wild-type strains *C. pasteurianum* DSM 525 and ATCC 6013 [59,60]. Now, further progress in improving strains of *C. pasteurianum* by direct genetic engineering are likely to be seen in the future.

Table 4. Summary of studies on PBE fermentation by hyper producing mutants of *C. pasteurianum*.

Strains	Process Parameters	Glycerol Consumed g·L ⁻¹	Max. Bioreactor Butanol Titer g·L ⁻¹	Max. Butanol Yield ^a g·g ⁻¹ (mol·mol ⁻¹)	Overall Butanol Productivity g L ⁻¹ h ⁻¹	Reference
<i>C. pasteurianum</i> (mutant MNO6; DSMZ 525)	Fed Batch, Free cells, <i>in-situ</i> butanol removal, Vol < 1 L	Crude 100–122	12.6	0.20 (0.25)	1.80 ^{c,d}	[26]
<i>C. pasteurianum</i> (mutant MBEL_GLY2; ATCC 6103)	Batch, Free cells, Vol < 1 L	Pure 86.0	13.7	0.30 (0.37)	0.31	[1]
<i>C. pasteurianum</i> (mutant MBEL_GLY2; ATCC 6103)	Batch, Free cells, Vol < 1 L, Optimized medium	Pure 79.3	17.3	0.30 (0.37)	0.33	[1]
<i>C. pasteurianum</i> (mutant MBEL_GLY2; ATCC 6103) High initial cell concentration	Batch, Free cells, Vol < 1 L, Optimized medium	Pure 82.0	17.8	0.30 (0.37)	0.43	[1]
<i>C. pasteurianum</i> (mutant MBEL_GLY2; ATCC 6103) High Cells/Cell Recycle	Continuous, Free cells, (D = 0.9 h ⁻¹) Vol < 1 L, Optimized medium	Pure 35 ^b	8.6	0.25 (0.31) ^b	7.8	[1]
<i>C. pasteurianum</i> (spontaneous asporogenous mutant; DSMZ 525)	Continuous, Free cells, D = 0.05 h ⁻¹ V ~ 1 L	Pure 30.85	7.45	0.24 (0.30)	0.372	[16]

^a yield calculated based on glycerol consumed; ^b data inferred from graphical representation; ^c productivity calculated based on active fermentation (subtracted lag phase); ^d corrected for by accounting for butanol removed in gas stripping.

7. Advanced Fermentative Technologies for High Productivity

Unfortunately, there is little reported on advanced fermentation technologies applied specifically to *C. pasteurianum*, however the ones used for ABE likely can be transferred. The process solutions as employed for ABE fermentation are reviewed and their suitability for PBE is discussed.

7.1. High Cell Density

Overcoming the low productivity and yield of butanol fermentation from crude glycerol sources is also a requirement in order to commercialize this process. While some fed-batch and continuous fermentations using free cells are capable of high levels of production over time, cell immobilization techniques are an option for even further increases in production, while simultaneously allowing for easier downstream recovery of products [20,61]. Various methods of cell immobilization have been successfully employed to enhance butanol productivity in the ABE and PBE processes, including adsorption of cells onto a solid surface, immobilization of cells within a porous matrix, encapsulation of cells within a permeable membrane, and cell recycling using ultrafiltration [6,27,28,62].

The adsorption of cells onto a solid surface is relatively easy and inexpensive compared to other immobilization techniques, as it uses natural cellular adhesion and biofilm formation to attach to the support [63,64]. Glutaraldehyde is commonly used as a linking agent to facilitate cellular adhesion, though other agents (metal oxides, aminosaline) can also be used [20]. Research using *C. pasteurianum* cells immobilized on a silica support and using biodiesel-derived crude glycerol as the carbon source reported higher productivity of butanol, ethanol, and PDO than with suspended cells, though productivity was still higher when using immobilized cells and pure glycerol [27]. These results were similar when the same group immobilized *C. pasteurianum* cells on Amberlite, an ion-exchange resin. In this case, the bacteria was able to tolerate and metabolize waste glycerol to butanol and PDO at a higher production rate than suspended cells [20]. Unfortunately, there was no comparison with pure glycerol to assess if immobilization on Amberlite helped cells overcome the inhibitory compounds present in crude glycerol. However, other groups have also reported that even with cell adsorption, the impurities cause delayed growth of cells when using waste glycerol with *Clostridia* spp. [22,63].

Immobilizing cells within a matrix of materials is advantageous in that low-cost, environmentally friendly materials may be used to form the matrix, while cells are also protected from shear forces within the reactor. Survase *et al.* screened several lignocellulosic materials for their efficacy as a support matrix for repeated batch and continuous ABE fermentation using *C. acetobutylicum*, finding that immobilizing the cells within a matrix of wood pulp allowed for the highest increases in solvent titer (18.88 g·L⁻¹ total solvents produced, compared to 8.18 g·L⁻¹ when using suspended cells) [65]. Using lignocellulosic materials, specifically corn stover, as a support matrix, *C. pasteurianum* was found to metabolize glycerol to butanol at a much higher rate in continuous cultures *vs.* suspended cells, 4.2 g·L⁻¹·h⁻¹ butanol *vs.* 0.1 g·L⁻¹·h⁻¹ butanol, respectively [66].

Encapsulation of the cells has been shown to reduce susceptibility of cells to end-product inhibition and making them more tolerant to the inhibitory effects of crude glycerol [61]. Cells are typically grown to high densities in rich media, then mixed with the encapsulation material, completely separating the cells from the fermentation medium behind a semi-permeable membrane. This allows the substrate to enter the micro-bead while products (both desirable and inhibitory) are removed, allowing for higher substrate concentration to be tolerated and less end-product inhibition to be observed [67]. However, encapsulation can affect the rate of transport into and out of the cell and impact rates of reaction. To the best of the authors' knowledge, no reports of encapsulated *Clostridium* spp. using crude glycerol have been reported. However, based on results with ABE fermentative organisms, this is an interesting area for future exploration with *C. pasteurianum*. Rathore *et al.* demonstrated that *C. acetobutylicum* encapsulated in gellan gum could be used in up to five cycles of fermentations, though the encapsulated cells produced less butanol than free cells in the first cycle (7.66 g·L⁻¹ *vs.* 9.79 g·L⁻¹, respectively). However, the butanol yield from free cells in a second fermentation cycle was greatly diminished, down to 2.9 g·L⁻¹, while encapsulated cells did not see a significant drop in

butanol production until the fifth cycle [68]. Considering the success of microencapsulation techniques for ABE fermentations, this technique could feasibly be successfully applied to PBE fermentations with *C. pasteurianum*.

Cell recycling is a technique used to simultaneously increase the concentration of cells and reaction rate in a fermentation and separate the fermentation broth from the cells for collection. The fermentation broth is passed over a porous membrane through which cells cannot pass, separating the permeate from the cells. The cells can then be cycled back into the fermenter, while cell-free permeate can be collected and the desirable end-products recovered [1]. By matching the substrate feed rate to the permeate outflow allows for high cell density continuous culture fermentations. This strategy has been successfully applied by groups to increase cell concentrations in fermentations using a variety of species and substrates, butanol fermentation using glycerol included [1,69]. Using a *C. pasteurianum* mutant and pure glycerol, butanol productivity as high as $7.8 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ has been obtained, compared to $0.43 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ when grown without cell recycling [1]. A recent study demonstrated the possibility of using this technology as a purification technique to remove cells and proteins prior to extraction of PDO with great success, however in this case, the cell retentate was discarded rather than reintroduced to the fermenter [70].

While the majority of the studies done using immobilization have been conducted on ABE fermentation processes, the technologies could be applied to fermentation processes producing primarily butanol using *C. pasteurianum*. The few studies using *C. pasteurianum* found that similar results could be expected; higher cell densities, productivity, and tolerance to inhibitors present in the fermentation medium. Table 5 demonstrates the effects of cell recycling and immobilization on cells in PBE fermentations, namely to increase the productivity of the cultures.

Table 5. Summary of studies for high cell density of PBE fermentation by *C. pasteurianum*.

Strains	Culture Condition	Carbon Source	Max. Butanol Yield ^a g · g ^{−1} (mol · mol ^{−1})		Butanol Productivity g · L ^{−1} · h ^{−1}		Reference
			Free Cells	Cell Immobilization	Free cells	Cell Immobilization	
Cell Immobilization							
<i>C. pasteurianum</i> (wild type; DSM 525)	Continuous (D = 0.44 h ^{−1} for immobilized cells and D = 0.01 h ^{−1} for free cells) Vol ~ 400 mL	Pure glycerol	0.4 (0.50)	0.33 (0.41)	0.1	4.2	[66]
<i>C. pasteurianum</i> (wild type; MTCC 116)	Batch Vol < 1 L	Pure glycerol	0.18 (0.22)	0.36 (0.45)	<0.10	<0.10	[27]
<i>C. pasteurianum</i> (wild type; MTCC 116)	Batch Vol < 1 L	Crude glycerol	0.13 (0.16)	0.23 (0.29)	<0.10	<0.10	[27]
<i>C. pasteurianum</i> (wild type; MTCC 116)	Batch Vol < 1 L	Crude glycerol	Nt	0.35 (0.43)	Nt	<0.10	[20]
<i>C. pasteurianum</i> (wild type; MTCC 6013)	Batch Vol < 1 L	Pure glycerol	Nt	0.21 (0.26)	Nt	0.04	[58]
<i>C. pasteurianum</i> (wild type; MTCC 6013)	Batch Vol < 1 L	Crude glycerol	Nt	0.17 (0.21)	Nt	0.035	[58]
Cell Recycling			Free cells	Cell Recycling	Free cells	Cell Recycling	
<i>C. pasteurianum</i> (mutant MBEL_GLY2; ATCC 6103)	Continuous (D = 0.9 h ^{−1}), Vol < 1 L Optimized medium	Pure glycerol	0.3 (0.37) ^b	0.25 (0.31)	0.43 ^b	7.8	[1]

^a yield calculated based on glycerol consumed; ^b Batch Vol < 1 L; Nt: Not reported.

7.2. Continuous Bioreactors for High Productivity

The high cell density continuous bioreactor creates a static metabolic state for a stable production culture, eliminating the unproductive phases of cell proliferation (*i.e.*, lag or growth phase) and downtime to clean and restart, with its associated extra costs (labour, water, chemicals, *etc.*) [71,72]. Continuous reactors allow controlling the product concentration by manipulating the feed concentration and dilution rate so that product inhibition is avoided. Thus continuous fermentation would appear to be the best choice for scale-up of butanol production at industrial scale for these reasons. Continuous industrial bioreactors for the bioethanol industry have been as large as 2 million litres or more and typically are simple fluidized tanks, mixed by external recirculation loops aided by large eductors (jet pumps) (GreenField Specialty Alcohols Inc. Chatham, ON, Canada, 2010, verbal communication). At industrial scale cell recycle is common in continuous systems and is typically accomplished using large centrifuges (Iogen Corp., Ottawa, ON, Canada, 2005, verbal communication). On the other hand, in the lab or pilot plant the bioreactor design and configuration may look different, however it is intended to mimic the ideal design for industrial scale. For continuous regime, the bioreactor is initiated in a batch regime, inoculated from seed cultures typically 5–10 *v/v* %. When the cell growth reaches a desired phase of exponential growth, the bioreactor is continuously fed with medium, while the product stream is withdrawn to keep constant volume in the reactor. For cell recycle in the lab or pilot plant, ultrafiltration units can be used such as that shown in Figure 3a, where an internal loop for recirculation (feed and bleed mode) can be used to achieve high velocity in the UF membrane and reduce fouling. For ABE fermentation, research has focused on multi-staged types of bioreactors that can accommodate the physical separation of the environments required for biphasic metabolism, the sequential phases of acetogenesis followed by solventogenesis, typically requiring different pH and residence times. These multistage bioreactors typically have individual stage-wise parameter controls for pH, temperature, feeding, cell recycle, temperature, *etc.* An example of a three-stage continually stirred Tank Reactor (CSTR) in series is shown in Figure 3b. Tank volume can be used to alter the residence time without interrupting flow. Series stirred tanks and plug flow packed bed bioreactors have dominated in recent years. One disadvantage of continuous systems is that cell degeneration can occur especially with lower pH, requiring re-seeding with inoculum at various stages [73]. The design and configuration of multi-staged systems are usually more complex and more difficult to control as can be seen by non-steady state data. Two popular biofilm reactors are the packed bed bioreactor (PBB) and the fibrous bed bioreactor (FBB) with the trickle bed bioreactor (TBB) being less popular in recent years (Figure 3c). The PBB and FBB are vessels in which the immobilization support material remains in the tank and liquid flows through, usually co-currently to the gas phase. The biofilm bioreactors always require a pre-production growth phase where medium and inoculum recirculate until biofilm is formed on the support material. Packed beds are generally operated in a plug flow regime in order to achieve the separate physical environments, where the first zone has a higher pH and lower volume for acetogenesis, followed by solventogenesis. The PBB tends to suffer from head loss because of excessive cell growth. The main difference between the FBB and the PBB is that the FBB is packed with spiral wound highly porous fibrous material for support of biofilm (usually hydrophilic) such that the majority of cells in the bioreactor are present in the void space between the fibrous matrix and as such they can continually be sloughing off and renewed. Also, there is a gap between the sheets of the fibrous material, allowing liquid and solids to flow and gas to be released and thus reducing the risk of plugging. The FBB has been used for cell immobilization, often in multi-staged systems to achieve very high cell densities (up to 100 g·L⁻¹), and in applications of extractive fermentation. The FBB can be operated as a trickle bed, a packed bed, or as an air lift where gas is sparged through the hollow core area and liquid circulates through the matrix. TBBs are fed at the top of the reactor thus obtaining product at the bottom. Stagnant pockets can form in the TBB and may affect the efficiency of the reactor. Table 6 summarizes the more successful examples of uses in research for these bioreactors while Table 7 summarizes the advantages and disadvantages of these bioreactors.

Very high productivities have been reached in either case (CSTR with cell recycle *vs.* biofilm bioreactors), however the long term operation of biofilm bioreactors has been plagued by plugging and multi-staged bioreactors with degeneration and lack of control and product consistency. With regard to biobutanol production from crude glycerol, there is an advantage to using the simple mixed tank (CSTR in lab-scale) with cell recycle design, as is the case in the bioethanol industry, which could be conceivable if the metabolism is non-biphasic.

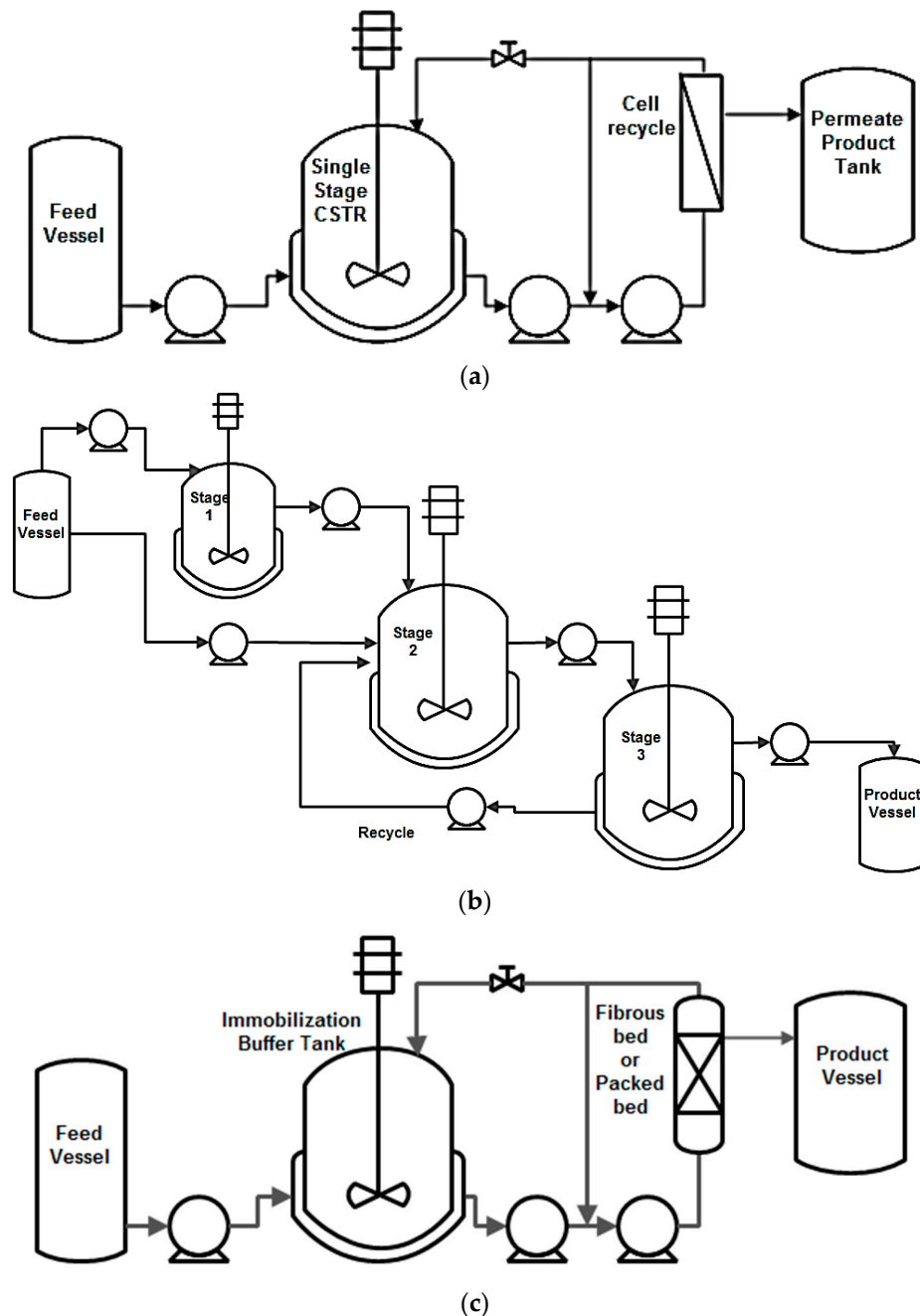


Figure 3. (a) Continuous single stage continually stirred tank reactor (CSTR) with cell recycle; (b) Continuous multi-staged CSTRs in Series; (c) Continuous Biofilm Reactor.

Table 6. Types of continuous high cell density bioreactor used in research.

Bioreactor Type	Fermentation Mode	Cell Configuration/Support	Bacteria	Dilution Rate h ⁻¹	Hours of Operation h	Substrate	Max Prod (g·L ⁻¹ ·h ⁻¹)	Reference
<i>CSTR with cell recycle</i>								
3-stage CSTR (600 mL)	Continuous single pass	Immobilized on corn stover	<i>C. acetobutylicum</i> ABE 1201	0.04 overall	~400	Corn stover juice	0.45	[73]
Single stage CSTR (400 mL)	Continuous single pass	Cell recycle with ultrafiltration	<i>C. saccharoper-butylacetonicum</i> N1-4 ATCC 13564 (DCW = 18.0 g/L)	0.78	~100	xylose	3.32	[28]
Single stage CSTR (400 mL)	Continuous single pass	Cell recycle with ultrafiltration	<i>C. pasteurianum</i> ATCC 6013	0.9	~50	glycerol	7.8	[1]
<i>Packed Bed Bioreactor</i>								
Single stage PBB (200 mL)	Continuous single pass	Immobilized on corn cob residue	<i>C. pasteurianum</i> NRRL B-598	0.12	~700	glucose	0.48	[74]
Single stage PBB (180 mL)	Continuous single pass	Immobilized on corn stover pieces (1 cm ³)	<i>C. pasteurianum</i> DSM 525	0.44	~300	glycerol	4.2	[66]
Single stage PBB (250 mL)	Continuous single pass	Tygon ring carriers (ID = 3.2 mm)	<i>C. acetobutylicum</i> DSM 792 (DCW = 74 g/L)	0.97	~750	lactose	4.4	[75]
Single stage PBB (100 mL)	Continuous single pass	Immobilized on corn stover (5–8 mm)	<i>C. beijerinckii</i> ATCC 55025 on corn stock	1.00	~480	glucose	5.06	[76]
<i>Fibrous Bed Bioreactor</i>								
Two-stage FBB (2 L)	Continuous single pass	Immobilized on spiral wound fibrous material	Co-culture <i>C. tyrobutyricum</i> ATCC 25755 <i>C. beijerinckii</i> ATCC 55025	0.144	~100	cassava starch	0.96	[77]
Single stage FBB (150 mL)	Continuous single pass	Immobilized on spiral wound fibrous cotton sheets	<i>C. beijerinckii</i> ATCC 55025 DCW = 100 g/L, 70% viable	1.88	~350	glucose/butyric acid	17.29	[78]
Single stage FBB (200 mL)	Continuous single pass	Immobilized on spiral wound fibrous sheets	<i>C. acetobutylicum</i> ATCC 55025	0.90	~1100	glucose/butyric acid	4.6	[79]

Table 7. Advantages and disadvantages of common types of bioreactors for continuous high cell density fermentation.

Advantages		Disadvantages
(a)	CSTR w/cell recycle <ul style="list-style-type: none"> • high cell density • high reaction rate • well mixed, therefore no gradients in pH, temperature or pressure • easy to operate, model, sample • no risk of plugging • easy to scale up • simple mechanically 	<ul style="list-style-type: none"> • increased heat production • physical zone separation not possible • Higher viscosity of liquid
(b)	Biofilm Reactors <ul style="list-style-type: none"> • high cell density • high reaction rate • plug flow regime/physical zone separation • simple mechanically 	<ul style="list-style-type: none"> • increased heat production • pH, temperature, pressure gradients (mixing problems) • low substrate utilization on single pass • difficult to sample for biomass quantification/viability • immobilization of biofilm growth phase required • lack of control of biofilm overgrowth issues with plugging • gas hold up pockets, channeling • PBB has higher risk of plugging • TBB has poor solid-liquid-gas contact, lower substrate utilization and mass transfer, pH gradients, sporulation and difficult to achieve plug flow regime

PBB: Packed Bed Bioreactor; TBB: Trickle Bed Bioreactor.

8. One Stage *In-Situ* Butanol Recovery Technologies

Distillation remains the standard industrial method of recovery and concentration of butanol from dilute aqueous solutions, due to advantages such as its ease of scale-up, high recovery efficiency, and high concentration factors. However, the very low concentration of butanol, its high boiling point (118 °C), and the presence of other fermentation products in the broth make butanol recovery by distillation energy intensive [80–83]. There are many studies in the literature where modeling has been used to evaluate and optimize energy usage in distillation, however there is a great deal of discrepancies [82,84,85]. At a concentration of 10 g·L⁻¹ butanol, distillation requires 1.5 times the energy contained in the resulting butanol (36 MJ·kg⁻¹_{butanol}). If fermentation could result in a butanol concentration of 40 g·L⁻¹ this ratio would decrease to 0.25 [81]. Therefore, it is crucial to develop techniques for simultaneous butanol fermentation and *in-situ* product recovery to mitigate toxicity and enhance productivity. The high concentration of butanol resulting from *in-situ* product removal would potentially and substantially lower the energy consumption in distillation, which would follow in a commercial process [86,87].

Over the years many relatively economic and feasible techniques have been developed for *in-situ* butanol removal including gas stripping, vacuum stripping, pervaporation, liquid-liquid extraction, perstraction, and adsorption [32,80,82,86,88,89]. Table 8 summarizes the principles, advantages, and disadvantages of these techniques.

Due to high butanol productivity and less labor and maintenance cost of continuous fermentation, the main emphasis of this section is being placed on the review of *in-situ* butanol recovery integrated with continuous fermentation. To the authors' knowledge there are very limited reports on integrated PBE fermentation with *in-situ* product recovery, none of which used a continuous mode. Therefore, we first report on those few studies found in the literature on integrated PBE fermentation with the aforementioned *in-situ* recovery techniques. Next we report on those for continuous ABE fermentation coupled with *in-situ* recovery and their potential for application to PBE fermentation. Table 9 summarizes studies found on integrated PBE fermentation with *in-situ* butanol recovery while Tables 10 and 11 summarize *in-situ* product removal techniques coupled with continuous ABE fermentation.

8.1. Integrated PBE Fermentation with *In-Situ* Butanol Recovery

8.1.1. Gas Stripping

In recent years, gas stripping has been attracting much attention as an alternative for butanol removal from fermentation broth [90–93]. The studies found in the literature on integrated PBE fermentation with gas stripping used only fed-batch mode [26,94]. In one study, Jensen *et al.* evaluated butanol production from biodiesel-derived crude glycerol using *C. pasteurianum* DSMZ 525 in a fed-batch pH-controlled fermentor integrated with gas stripping [26]. The crude glycerol was pretreated using a combination of addition of activated stone carbon and storage of the crude glycerol for 10 months at 20 °C. Using pretreated glycerol resulted in a productivity of 1.3 g·L⁻¹·h⁻¹, whereas using technical grade glycerol without gas stripping resulted in the productivity of 1.21 g·L⁻¹·h⁻¹. It should be noted that the productivities were calculated based on 'active fermentation time' by eliminating the lag phase time from calculations. In the next study, Jensen *et al.* repeated the same experiment but using the mutant *C. pasteurianum* (MNO6). Under the same experimental conditions as the first study higher butanol productivity of 1.8 g·L⁻¹·h⁻¹ was achieved (Table 9). From the results of these two studies it is clear that the application of gas stripping resulted in reduced butanol inhibition and enhanced productivity, however a considerable lag phase still existed.

8.1.2. Liquid-Liquid Extraction

Liquid-liquid extraction (LLE) is another separation technique that can be applied for butanol *in-situ* recovery during fermentation or as a separate step after fermentation. The authors could find only one study in the literature on integrated PBE fermentation with LLE which used batch mode [95].

Zhang *et al.* investigates the capability of the *C. pasteurianum* SE-5 to produce butanol using crude glycerol as the sole carbon source and biodiesel as the extractant [95]. This resulted in $89.1 \text{ g} \cdot \text{L}^{-1}$ of crude glycerol consumption and $24.6 \text{ g} \cdot \text{L}^{-1}$ of butanol production with more than 50% of the butanol extracted into the biodiesel phase. A butanol yield of $0.3 \text{ g} \cdot \text{g}^{-1}$ and productivity of $0.34 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ were obtained, whereas using pure glycerol as substrate without extraction resulted in a butanol yield and a productivity of $0.29 \text{ g} \cdot \text{g}^{-1}$ and $0.27 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, respectively. The results suggested that the application of LLE resulted in reduced butanol inhibition, thereby improving butanol productivity and yield in a fermentation process directly in biodiesel solution.

8.2. Integrated Continuous ABE Fermentation with In-Situ Butanol Recovery

8.2.1. Gas Stripping

Ezeji *et al.* studied a single-stage fermentation integrated with gas stripping using *C. beijerinckii* BA101. A concentrated glucose solution ($250\text{--}500 \text{ g} \cdot \text{L}^{-1}$) was fed as substrate to the bioreactor and a continuous bleed of bioreactor contents to reduce [91]. The bioreactor produced $461.3 \text{ g} \cdot \text{L}^{-1}$ ABE from 1125.0 g total glucose as compared to a control batch process in which $18.4 \text{ g} \cdot \text{L}^{-1}$ ABE was produced from 47.3 g glucose. This resulted in an ABE productivity of $0.92 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ with no change in yield. These results demonstrated that *in-situ* butanol removal improved the ABE fermentation, however there remained some inhibitory by-products that had to be bled from the reactor for stable operation and producing very noisy product data. In another study, Qureshi and Maddox (1990) investigated continuous ABE fermentation with gas stripping using immobilized cells of *C. acetobutylicum* [96]. A single-stage fluidized bed bioreactor was used for butanol production from whey permeate. The integrated system was operated for 380 h and was improved over the non-integrated system for ABE yield and productivity, however if the bioreactor substrate concentration (lactose) fell below a critical level, the reactions reverted to an acetogenesis phase leading to a loss of substrate (Table 10). Figure 4 shows a schematic diagram of a typical gas stripping process integrated with fermentation.

8.2.2. Vacuum Stripping

To the authors' knowledge there are no studies in the literature on PBE or continuous ABE fermentation coupled with vacuum stripping. Therefore, we report on an integrated batch ABE fermentation with vacuum stripping. This technique is in the early stages of its development but seems to be a promising method for butanol *in-situ* removal.

Mariano *et al.* investigated simultaneous ABE fermentation and *in situ* product recovery using a vacuum process. Vacuum was applied continuously or intermittently with 1.5 h vacuum sessions separated by 4, 6, and 8 h intervals [88]. Fermentation coupled with *in situ* recovery by both continuous and intermittent vacuum modes resulted in a decrease in fermentation time, complete utilization of glucose, greater cell growth, and more concentrated product stream. The fermentation under continuous vacuum resulted in ABE yield and productivity of $0.22 \text{ g} \cdot \text{g}^{-1}$ and $0.28 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, whereas solvent yield and productivity of $0.35 \text{ g} \cdot \text{g}^{-1}$ and $0.26 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ were achieved from a control experiment without *in-situ* recovery. Operation of the vacuum in intermittent mode with vacuum sessions of 1.5 h at intervals of 4 h resulted in the shortest fermentation time and highest ABE productivity ($0.34 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$) compared to control experiment, continuous vacuum, and 6 and 8 h intervals.

The high level of productivity achieved by vacuum stripping is an important factor that can turn this process into a promising technology for the fermentative butanol production. Figure 5 shows a schematic diagram of a vacuum stripping process coupled with continuous fermentation.

Table 8. Alternative separation techniques for butanol recovery from fermentation broth.

Method	Principle	Advantages	Disadvantages
Gas stripping	<ul style="list-style-type: none"> • Volatile solvents being stripped out by oxygen-free nitrogen or fermentation gases (H₂ and CO₂) and then condensed • Stripping gas can be recycled back into the process • Can be integrated with fermentation in the bioreactor, or performed in an individual stripping column 	<ul style="list-style-type: none"> • Easy to operate • No harm to the culture • Strips only the volatiles • Ability to operate under fermentation temperature 	<ul style="list-style-type: none"> • Low selectivity • Low efficiency • Requires high gas flow rate
Vacuum stripping	<ul style="list-style-type: none"> • Volatile solvents being stripped out by vacuum and then condensed • Can be integrated with fermentation in the bioreactor, or performed in an individual stripping column 	<ul style="list-style-type: none"> • Easy to operate • No harm to the culture • Strips only the volatiles • Ability to operate under fermentation temperature • No need for extra volume in the fermentation tank for gases compared to gas stripping 	<ul style="list-style-type: none"> • Low selectivity
Pervaporation	<ul style="list-style-type: none"> • Using membrane to selectively let the vaporous solvents pass through, driven by a chemical potential gradient • Vacuum pervaporation: Permeate side is under vacuum • Thermal pervaporation: the permeate is condensed on a cold wall at atmospheric pressure • Can be selective due to differences in membrane properties affecting sorption and diffusion • Diffusion is governed by the molecule size, shape, molecular weight, and inter/intra molecular free space in the membrane 	<ul style="list-style-type: none"> • Low operating temperature • Low operating cost • No harm to the culture • Reduced energy demand • No loss of substrate or nutrients from fermentation broth • High selectivity 	<ul style="list-style-type: none"> • Membrane fouling • Require high liquid flow rates • Redundancy for batch wise cleaning
Liquid-liquid extraction	<ul style="list-style-type: none"> • Using the soluble differences of solvents in fermentation broth and water-insoluble organic extractant for separation • Extractant can be recycled back into the process • Can be integrated with fermentation in the bioreactor, or performed in an individual extractor column 	<ul style="list-style-type: none"> • High selectivity, efficient 	<ul style="list-style-type: none"> • Forming emulsion • Toxic to the culture • High extractant recovery cost and loss
Perstraction	<ul style="list-style-type: none"> • Membrane-based extraction, separating the fermentation broth from the extractive solvents • Extractant can be recycled back into the process 	<ul style="list-style-type: none"> • High selectivity • Low toxicity to the culture compared to liquid-liquid extraction 	<ul style="list-style-type: none"> • Forming emulsion • Membrane fouling
Adsorption	<ul style="list-style-type: none"> • Adsorption of solvents onto the surface of adsorbent • Adsorbent can be regenerated for reuse • Can be integrated with fermentation in the bioreactor, or performed in an individual adsorption column 	<ul style="list-style-type: none"> • Low energy requirement • Fully immiscible and unsusceptible to emulsification 	<ul style="list-style-type: none"> • Adsorbent regeneration

Table 9. Summary of studies on integrated PBE fermentation with *in-situ* butanol removal.

Bacteria	Fermentation Mode	Substrate	Max Butanol Yield g·g ⁻¹	Overall Butanol Productivity g·L ⁻¹ ·h ⁻¹	Hours of Operation h	Comment	Reference
<i>Gas Stripping</i>							
<i>C. pasteurianum</i> (mutant MNO6; DSMZ 525)	Fed Batch, Single-stage, Free cells, Vol < 1 L	Crude glycerol	0.20	1.8 (1.2)	~96–120	H ₂ and CO ₂ , Stripping temperature 37 °C, Condensation temperature 0 °C	[26]
<i>C. pasteurianum</i> (wild type; DSMZ 525)	Fed Batch, Single-stage, Free cells, Vol < 1 L	Crude glycerol	0.225	1.3 (1.2)	~96–120	H ₂ and CO ₂ , Stripping temperature 37 °C, Condensation temperature 0 °C	[94]
<i>Liquid-liquid extraction</i>							
<i>C. pasteurianum</i> SE-5	Batch, Single-stage, Free cells, Vol = 1 L	Crude glycerol	0.30 (0.29)	0.34 (0.27)	~72	Biodiesel was used as extractant	[95]

Table 10. Summary of studies on integrated continuous acetone-butanol-ethanol (ABE) fermentation with gas stripping, vacuum stripping, and pervaporation.

Bacteria	Fermentation Mode	Substrate	ABE Yield g·g ⁻¹	ABE Productivity g·L ⁻¹ ·h ⁻¹	Hours of Operation h	Comment	Reference
<i>Gas stripping</i>							
<i>C. beijerinckii</i> BA101	Continuous, Single-stage, Free cells	glucose	0.41 (0.39)	0.92 (0.29)	~504	H ₂ & CO ₂ , Stripping temperature 35 °C, Condensation temperature 1 °C	[91]
<i>C. acetobutylicum</i> P262	Continuous, Single-stage, Immobilized cells in a fluidized bed reactor	Whey permeate	0.4 (0.33)	5.1 (1.66)	~380	N ₂ , Stripping temperature 65–67 °C, Condensation temperature 3–4 °C	[96]
<i>Clostridium</i> sp. DSM 2152	Continuous, Single-stage, Free cells	Glucose	0.34 (0.37)	0.18 (0.17)	~300	N ₂ , 10 L·L ⁻¹ min, Stripping temperature 30 °C, Condensation temperature –5 to –40 °C	[97]
<i>C. acetobutylicum</i> P262	Continuous, Single-stage, Free cells	Whey permeate	0.35 (0.32)	0.62 (0.15)	~52	N ₂ , 2.0 L·min ⁻¹ , Stripping temperature 34 °C, Condensation temperature 4 °C	[98]
<i>Vacuum stripping</i>							
<i>C. beijerinckii</i> 8052	Batch ^a , 7 L fermentation volume, Free cells	Glucose	0.29	0.43	~44	Continuous vacuum	[89]
<i>C. beijerinckii</i> P260	Batch ^a , 14 L Bioreactor (7 L fermentation volume), Free cells	Glucose	0.22	0.28	~48	Continuous vacuum	[88]
<i>C. beijerinckii</i> P260	Batch ^a , 14 L Bioreactor (7 L fermentation volume), Free cells	Glucose	0.26	0.34	~63	Intermittent vacuum, 1.5 h vacuum sessions were separated by 4 h time periods	[88]

Table 10. Cont.

Bacteria	Fermentation Mode	Substrate	ABE Yield g·g ⁻¹	ABE Productivity g·L ⁻¹ ·h ⁻¹	Hours of Operation h	Comment	Reference
<i>Pervaporation</i>							
<i>C. acetobutylicum</i> (CICC 8012)	Continuous, Single-stage, Free cells	glucose	0.24	0.23 ^b	~192	PDMS (800 cm ²)	[99]
<i>C. acetobutylicum</i> DP 217	Continuous, Single-stage	glucose	0.37	0.97	~268	PDMS (240 cm ²), $\alpha_{\text{butanol}} = 31.6$	[100]
<i>C. acetobutylicum</i> ATCC 824	Continuous 2 stage, Free cells	glucose	0.28	0.88	~475	PDMS (180–270 cm ²), $\alpha_{\text{butanol}} = 17.67\text{--}19.81$	[101]
<i>C. isopropyllicum</i>	Continuous, Single-stage, Immobilized cells	molasses	0.29 ^b	Nt	~370	Liquid (1500 cm ²), Butanol flux of 3.3 g·m ⁻² ·h ⁻¹ , $\alpha_{\text{butanol}} = 66$	[102]

Values in parenthesis were from the control experiments or fermentation without integrated product removal. ^a Authors could not find any continuous study on butanol fermentation integrated with vacuum stripping; ^b butanol yield or productivity; Nt: Not-reported.

Table 11. Summary of studies on integrated continuous ABE fermentation with liquid-liquid extraction, perstraction, and adsorption.

Bacteria	Fermentation Mode	Substrate	ABE Yield g·g ⁻¹	ABE Productivity g·L ⁻¹ ·h ⁻¹	Hours of Operation h	Comment	Reference
<i>Liquid-liquid extraction</i>							
<i>C. acetobutylicum</i> DSM 792	Continuous, 2 stage immobilized column reactor, Free cells, D = 1.0 h ⁻¹	Sugar mixture	0.38 (0.33)	10.85 (12.14)	~1152	oleyl alcohol and decanol (4:1)	[103]
<i>C. acetobutylicum</i> B5313	Continuous, two stage, Free cells, chemostat system, D = 0.05 h ⁻¹	glucose	0.35 (0.25)	2.5 (2.12)	~720	oleyl alcohol and decanol (4:1)	[31]
<i>C. acetobutylicum</i> P262	Continuous, Single-stage, Immobilized cells	Whey permeate	0.23 (0.36) 0.39 (0.36) 0.36 (0.35)	1.5 (3.5) 1.9 (3.6) 1.9 (3.0)	Nt	Dibutyl phthalate Benzyl benzoate Oleyl alcohol	[104]
<i>C. acetobutylicum</i> P262	Continuous, Single-stage, Free cells	Whey permeate	0.35 (0.32)	0.14 (0.07)	~170	Oleyl alcohol	[105]
<i>Perstraction</i>							
<i>C. acetobutylicum</i> P262	Continuous, Single-stage, Free cells, 1 L culture	Whey permeate	0.37 (0.32)	0.24 (0.07)	~290	Oleyl alcohol, Silicone membrane	[105]
<i>Adsorption</i>							
<i>C. acetobutylicum</i> ATCC 824	Fed-batch ^a , Free cells, 1 L culture, expanded bed adsorption	Glucose	0.28 (0.17)	0.72 (0.63)	~38.5	hydrophobic polymer resin Dowex Optipore L-493	[30]
<i>C. acetobutylicum</i>	Repeated Fed-batch ^a , Free cells, Cell recycle	Glucose	0.32 (30.9)	1.69 (0.4)	~250	Polyvinylpyridine	[106]
<i>C. acetobutylicum</i>	Fed-batch ^a , Free cells	Glucose	0.32 (30.9)	1.33 (0.4)	~250	Polyvinylpyridine	[106]

Values in parenthesis were from the control experiments or fermentation without integrated product removal. ^a Authors could not find any continuous study on butanol fermentation integrated with adsorption.

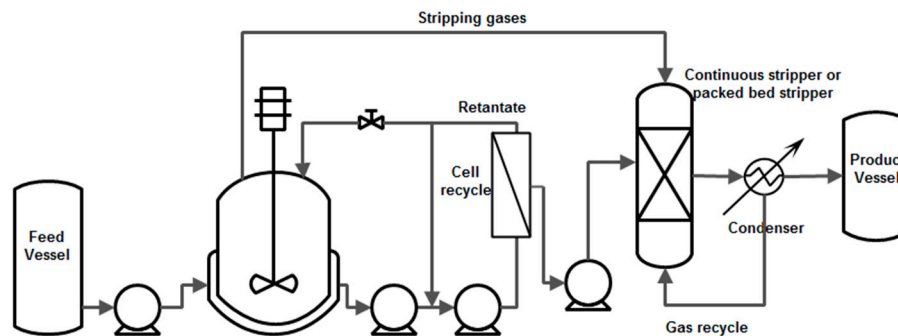


Figure 4. Continuous butanol fermentation integrated with gas stripping.

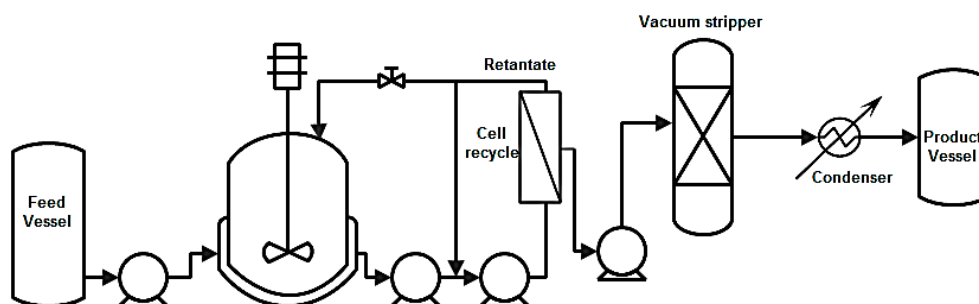


Figure 5. Continuous butanol fermentation integrated with vacuum stripping.

8.2.3. Pervaporation

There were no reports on PBE fermentation integrated with pervaporation. Most of the studies found in the literature on integrated continuous ABE fermentation-pervaporation lacked stable fermentation operation, likely due to the biphasic nature of the *Clostridia* spp. used [102,107]. Figure 6 shows a schematic diagram of a pervaporation process coupled with continuous fermentation.

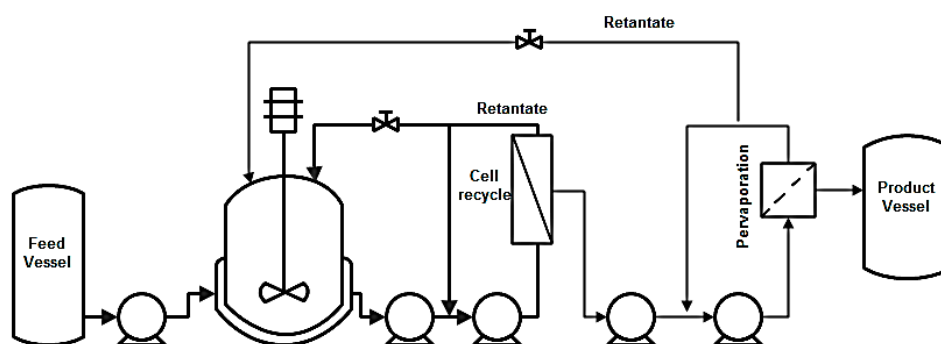


Figure 6. Continuous butanol fermentation integrated with pervaporation.

In one study, Van Hecke *et al.* ran a continuous 2-stage CSTR fermentation integrated with pervaporation in the second fermentor using freely suspended cells (*C. acetobutylicum*) and PMDS composite membrane for a duration of 825 h, however this time was broken into five phases with different operating parameters [101] ran. In the phase with the highest stable operation, they achieved an overall productivity of $0.88 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ with an average total ABE flux of $621 \text{ g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ and a permeate enriched to $202 \text{ g} \cdot \text{L}^{-1}$ total solvents. In another study, Li *et al.* obtained steady state conditions at a very low dilution rate (0.0117 h^{-1}) in an ABE fermentation integrated with pervaporation for 268 h and achieved an ABE productivity of $0.97 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ [100]. This resulted in a total flux of $486 \text{ g} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ and a separation factor of 31.6. More recently, Van Hecke *et al.*

prepared a Chemcad simulation for a conceptual plant design which resulted in a 50% energy savings when pervaporation was integrated. The process involved a two-stage continuous ABE fermentation (dilution rate 0.109 h^{-1} , lignocellulosic hydrolysate as substrate). The productivity of $0.65\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and $185\text{ g}\cdot\text{kg}^{-1}$ solvent in the permeate resulted [108]. The details of the studies on continuous ABE fermentation integrated with pervaporation can be found in Table 10.

Also a recent review of the literature on butanol removal using pervaporation can be found in Kujawska *et al.* [109].

8.3. Liquid-Liquid Extraction (LLE)

Bankar *et al.* studied a two stage immobilized column bioreactor system integrated with LLE using immobilized *C. acetobutylicum* B 5313 [31]. The extraction module and the settling tank consisted of two glass jacketed bioreactors with a total volume of 1 L. Glucose was used as a substrate for continuous ABE production. The integrated system was operated for 720 h without any technical problems. This resulted in ABE productivity of $2.5\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and yield of $0.35\text{ g}\cdot\text{g}^{-1}$ at a dilution rate of 0.2 h^{-1} , whereas solvent productivity and yield of 2.12 and 0.25 were achieved from a single stage system without *in-situ* recovery at a dilution rate of 0.6 h^{-1} . Maximum total ABE solvent concentration of $25.32\text{ g}\cdot\text{L}^{-1}$ was achieved at a dilution rate of 0.05 h^{-1} . Bankar *et al.* went on to study the sugar mixture (glucose, mannose, galactose, arabinose, and xylose) representative to the lignocellulose hydrolysates as a substrate for continuous ABE production [103]. The experiments were carried out using the same system as the first study however the cells were immobilized on wood pulp (Table 11) and the ABE productivity of $10.85\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and yield of $0.38\text{ g}\cdot\text{g}^{-1}$ were achieved. The integrated system was operated for 1152 h (48 days) at seven different dilution rates and maximum total ABE solvent concentration of $20.30\text{ g}\cdot\text{L}^{-1}$ was achieved at a dilution rate of 0.2 h^{-1} . Figure 7 shows a schematic diagram of an integrated continuous fermentation with LLE.

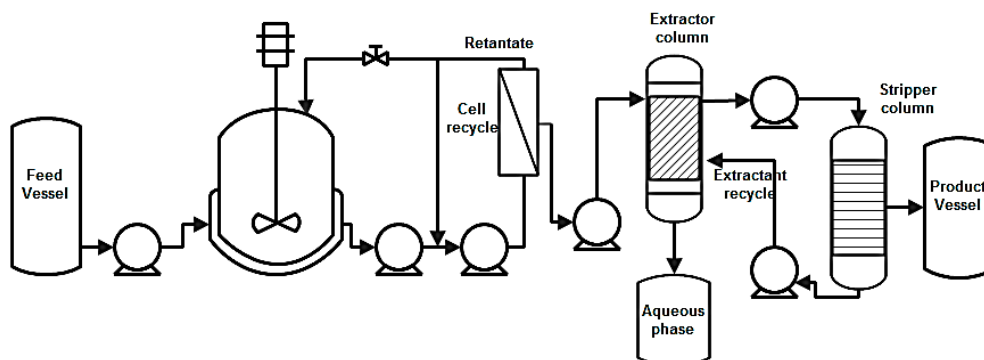


Figure 7. Continuous butanol fermentation integrated with liquid-liquid extraction (LLE).

8.4. Perstraction

Perstraction is a membrane based LLE technique that was developed to overcome problems associated with LLE. There are very limited reports in the literature on perstraction coupled with ABE fermentation.

Qureshi *et al.* studied ABE fermentation in an integrated continuous one-stage fermentation and perstraction product recovery system using a silicone membrane and oleyl alcohol as the perstraction solvent [105]. The continuous system was operated for about 290 h and the bioreactor produced $57.8\text{ g}\cdot\text{L}^{-1}$ ABE with a maximum concentration $9.8\text{ g}\cdot\text{L}^{-1}$ of ABE in the oleyl alcohol. This resulted in an ABE productivity of $0.24\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and a yield of $0.37\text{ g}\cdot\text{g}^{-1}$, whereas an ABE productivity of $0.07\text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and yield of $0.32\text{ g}\cdot\text{g}^{-1}$ were obtained from batch fermentation without product recovery.

8.5. Adsorption

To the authors' knowledge there are no studies in the literature on continuous ABE fermentation integrated with adsorption. The studies found in the literature on integrated ABE fermentation with adsorption used fed-batch fermentation, some used batch mode. The details of the fed-batch studies on ABE fermentation integrated with adsorption can be found in Table 11. Figure 8 shows a schematic diagram of an adsorption process coupled with continuous fermentation.

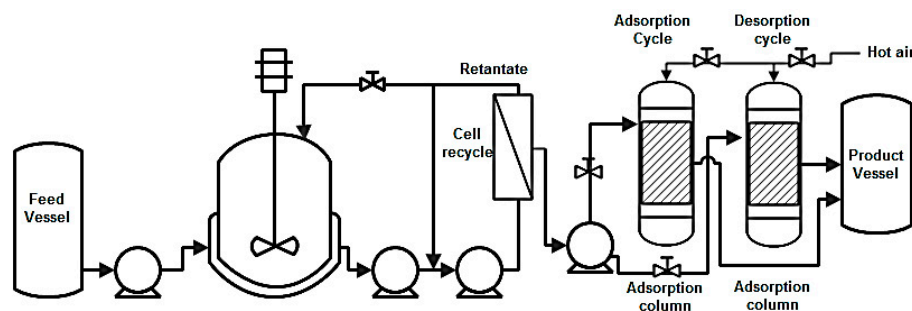


Figure 8. Continuous butanol fermentation integrated with adsorption.

Of note, Wiehn *et al.* investigated the application of expanded bed adsorption on butanol productivity. In this case, the expanded bed consisted of 0.17 L glass column containing 75 g of Dowex® Optipore L-493. This resulted in ~55% free head space (column volume unoccupied) in the column for bed expansion [30]. The contents of the 1 L culture were continuously re-circulated between the bioreactor and adsorption bed at a rate of about $100 \text{ mL} \cdot \text{min}^{-1}$. This integrated system was operated for 38.5 h with maximum butanol and total solvent production of $27.2 \text{ g} \cdot \text{L}^{-1}$ and $40.7 \text{ g} \cdot \text{L}^{-1}$, respectively. The butanol concentration in the cold trap reached as high as $85.8 \text{ g} \cdot \text{L}^{-1}$ and an average 81% butanol recovery was obtained via adsorbent regeneration. Yang and Tsaot studied integrated repeated fed-batch fermentation with adsorption and cell recycle [106]. Glucose was fermented by *C. acetobutylicum* and Polyvinylpyridine were used as adsorbent. This integrated system was operated for about 250 h and $47.2 \text{ g} \cdot \text{L}^{-1}$ of ABE was produced. Also ABE yield of $0.32 \text{ g} \cdot \text{g}^{-1}$ and productivity of $1.69 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ were obtained compared to ABE yield of $30.9 \text{ g} \cdot \text{g}^{-1}$ and productivity of $0.4 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ in conventional batch fermentation (Table 11).

8.6. Transferring In-Situ Recovery Techniques from ABE Fermentation to PBE Fermentation

From the results reported by various authors it is clear that the application of *in-situ* recovery techniques resulted in reduced butanol inhibition, thereby improving butanol productivity. Also, due to simultaneous product removal, the microorganism can utilize concentrated substrates in an integrated fermentation process, which would otherwise cause substrate inhibition. However, most of these studies have short operation periods, especially if using batch or fed batch fermentation. Many did not provide sufficient experimental details to compare or did not analyze the *in-situ* recovery technique fully, likely due to a lack of steady state operation. It would appear that steady state operations were rarely achieved as can be seen from some of the data, thus there appears to be some issues with unstable operation of integrated ABE systems and it is not clear if this stems from unstable fermentation or unstable *in-situ* removal or both. It is also noted that with continuous ABE fermentation of biphasic *Clostridia* spp. with free cells, dilution rates have been very low for single stage bioreactors, whereas two-stage bioreactors have been able to achieve higher dilution rates. It is suggested that by using *C. pasteurianum* in a continuous PBE fermentation, unstable fermentation due to biphasic behavior would at least be eliminated from the other challenges, as reported by Johnson and Rehmann (2016), however that has yet to be demonstrated [49].

It should also be noted that no acetone (boiling point 56°C) is produced in PBE fermentation, but instead PDO whose boiling point is between 211 and 217°C . Thus PDO is much less volatile than

butanol and will likely remain in the fermentation broth *vs.* be removed by *in-situ* recovery processes, accumulating if the dilution rate is not greater than or equal to the production rate. However, there is a lack of information in the literature on the toxicity of PDO to *C. pasteurianum*. Therefore, more research is needed to investigate the effect of the by-products in PBE fermentation broth, more specifically the effect of glycerol and PDO on the performance and efficiency of *in-situ* recovery techniques.

Table 12 summarizes a brief assessment of the technologies. It should be noted that pervaporation and perstraction, both involving membranes, will likely require batch-wise switching and cleaning and adsorption, involving ion exchange resin would require batch-wise switching and regeneration.

Therefore, vacuum stripping, pervaporation and adsorption appear to be promising technologies for *in-situ* butanol removal for PBE fermentation.

Table 12. Summary of brief assessment of different *in-situ* recovery technologies.

Technology	Green	Energy Demand	Efficiency
Gas stripping	Yes	High	High
Vacuum stripping	Yes	Low	High
Pervaporation	Yes	Low	High
Liquid-liquid extraction	No	Low	Low
Perstraction	No	Low	High
Adsorption	Yes	Low	High

9. Hybrid *In-Situ* Butanol Recovery Processes

In order to remove butanol toxicity from the fermenter, *in-situ* butanol removal is necessary, however, a single stage of *in-situ* butanol recovery is not efficient enough. A hybrid process therefore is needed to compliment technologies for the purpose of energy savings as well as to increase fermenter titers and productivity for commercialization. Single separation technologies have their inherent weaknesses but when coupled they can enhance each other. As well there is an advantage to run the fermenter at the highest butanol concentration possible. Also it is noted that there have been very little reports of the implementation of hybrid *in-situ* butanol removal with demonstrated successful steady-state continuous operation.

As already mentioned, due to the much higher boiling point of PDO compared to acetone, PDO will remain in the fermentation broth. Thus, downstream purification will be different in PBE compared to ABE fermentation.

Unfortunately, the authors' could not find any reports on hybrid *in-situ* butanol recovery coupled to PBE fermentation, however we report on those for ABE fermentation using glucose as substrate and their potential for application to PBE fermentation.

9.1. Two-Stage Gas Stripping

Xue *et al.* tested a two-stage gas stripping *in-situ* removal process coupled with ABE fed-batch fermentation (*C. acetobutylicum*) in a fibrous bed bioreactor [110]. The first stage removed ABE *in-situ* from the fermenter and the second stage concentrated the aqueous portion of the condensate from the first stage. After process optimization, overall effective 48.5 g·L⁻¹ butanol (73.3 g·L⁻¹ ABE) was produced from the coupled hybrid process from 270.8 g glucose in 201 h, as a result of reduced butanol inhibition on cells. The resultant butanol yield and productivity was 0.27 g·g⁻¹ and 0.24 g·L⁻¹·h⁻¹ respectively. The first-stage condensate contained 147.2 g·L⁻¹ butanol (199.0 g·L⁻¹ ABE), while the second stage condensate contained 515.3 g·L⁻¹ butanol (671.1 g·L⁻¹ ABE).

9.2. Gas Stripping-Pervaporation

One advantage of using gas stripping *in-situ* prior to pervaporation (GS-PV) is that the condensate from stripping will be void of salts, cell debris, residual sugars and other fermentation media components and remediate membrane fouling. A fed-batch fermentation with immobilized

C. acetobutylicum, coupled to *in-situ* gas stripping (stripper external to fermenter) followed by pervaporation (GS-PV) relieved inhibition in the fermenter and producing a permeate from pervaporation with high concentration of ABE ($706.68 \text{ g} \cdot \text{L}^{-1}$) and butanol ($482.55 \text{ g} \cdot \text{L}^{-1}$). The high concentration of butanol (98.8 *w·v*⁻¹%) would potentially lower the energy consumed in distillation, which would follow in a commercial process [111]. Unfortunately the pervaporation stage was only operational for 11 h.

A similar lab scale hybrid system was studied by [112] using fed batch fermentation with *C. acetobutylicum* in a fibrous bed bioreactor for cell immobilization for 224 h coupled to *in-situ* gas stripping and a second stage of pervaporation. Fermentation resulted in a butanol yield and productivity of $0.24 \text{ g} \cdot \text{g}^{-1}$ and $0.34 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ respectively. In this study, the condensate from gas stripping was separated into an organic and an aqueous phase, where only the aqueous phase ($85.6 \text{ g} \cdot \text{L}^{-1}$ butanol) went to pervaporation. The organic phase from gas stripping was reunited with the permeate from pervaporation. The process resulted in a butanol selectivity of 97.8 and a final product concentration of $521.3 \text{ g} \cdot \text{L}^{-1}$ butanol after combination. Gas stripping was relatively stable, however the pervaporation (second stage) was operated batch-wise in unsteady state for only 28 h. Fed-batch fermentation is not a steady state operation and has limits of operation, unlike continuous fermentation.

9.3. Gas Stripping—Gas Permeation

Vane and Alvarez (2013) studied an experimental hybrid *in-situ* butanol removal process including vapor stripping, vapor compression, and a vapor permeation membrane separation in series, referred to as ‘membrane assisted vapor stripping’ (MAVS); however, the process was not coupled to fermentation, rather processed batch-wise [113]. The separation of solvents from ABE fermentation was benchmarked by a conventional distillation-decanter process. In the MAVS, feed liquid containing a solvent was fed into the top of a vapor stripping column. Solvent was stripped from the water in the column and the overhead vapor leaving the column was enriched in solvent, relative to the feed liquid, owing to favorable vapor liquid equilibria (VLE). The overhead vapor was compressed and the resulting higher pressure vapor was fed to a vapor permeation membrane module with a water-selective (hydrophilic) membrane. Pilot unit demonstrations were carried out on actual bacterial ABE fermentation broth (1.3 wt % butanol) produced in an 80 L batch fermentation using *C. acetobutylicum*. The fermentation proceeded in a biphasic behavior taking 96 h to finish followed by cell separation by centrifugation and down-stream batch-processing in the MAVS system. A product of 95 wt % butanol resulted using approximately 54% less energy compared to a distillation-decanter system.

9.4. Extraction-Gas Stripping

Lu and Li (2014) investigated an integrated *in-situ* extraction-gas stripping butanol removal process coupled with batch fermentation (*C. acetobutylicum*) in a 500 mL serum bottle [114]. The non-volatile solvent oleyl alcohol acted as the extraction solvent and nitrogen was used for gas stripping. At first butanol was extracted by oleyl alcohol during ABE fermentation and gas stripping was initiated after 48 h of fermentation in the oleyl alcohol phase. The butanol yield and productivity of $0.226 \text{ g} \cdot \text{g}^{-1}$, $0.28 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ was obtained respectively, after 96 h of fermentation. $121 \text{ g} \cdot \text{L}^{-1}$ glucose was consumed during fermentation and butanol concentration of 93–113 $\text{g} \cdot \text{L}^{-1}$ was achieved in the condensate.

In summary, more research is needed to study hybrid *in-situ* butanol removal for PBE fermentations, more specifically the effect of having PDO and glycerol but not acetone and glucose in the fermentation broth. This is required for achieving higher productivity in fermentation by implementing high cell density and high gravity feeds, all of which require *in-situ* butanol removal to keep the fermenter butanol titer below toxic levels. Finally, it would appear that this technology has many benefits that should be transferrable from ABE to PBE.

10. Conclusions

The production of butanol using *C. pasteurianum* is an attractive option, given the possible use of crude glycerol as the feedstock and the non-biphasic nature of *C. pasteurianum* allowing for a single-stage continuous fermentation process. However, several obstacles still must be addressed before economic large scale butanol production can be implemented.

Most of the reports on butanol production in the literature implement ABE fermentation. As mentioned earlier, these *Clostridia* spp. cannot utilize glycerol as sole carbon and energy source. Therefore a considerable portion of this review, especially concerning technologies for *in-situ* butanol removal and high cell density come from studies from ABE fermentation. These technologies can be transferred to fermentation with *C. pasteurianum* and glycerol as substrate.

However, impurities and the variable nature of the crude glycerol must be overcome. Therefore, a consistent, inexpensive and broad-reaching pretreatment method to allow for efficient use of the crude glycerol from any source is required. *C. pasteurianum* is then able to convert crude glycerol into butanol by what appears to be a non-biphasic fermentation. However, the fermentation can still be improved in terms of yield and productivity and needs to be validated at larger scale. Successful applications of mutagenesis and metabolic engineering towards improved butanol production with *C. pasteurianum* were demonstrated and suggest even further advances are in the near future, while higher reaction rates have been achieved using high cell density via cell recycling or immobilization in CSTRs, packed bed and fibrous bed bioreactors in single or multi-staged. Most important is that the process design and configuration be scaled to industrial size and perform with long term stable operation without plugging from biofilm overgrowth, while for a non-biphasic production host such as *C. pasteurianum* a multi-staged bioreactor design may not be necessary and the bioethanol industry could be used as the standard, with large mixed submerged culture tanks easy to control and operate. Multi-staged bioreactor design tends to be more difficult to control with product variability and cellular metabolic inconsistency.

To date, most research has been performed on very small volume systems. More research is needed at a larger scale and for longer duration at constant operating parameters, with additional focus on the downstream. Distillation systems are energy-intensive due to the low solvent titers in the fermentation broth. Various *in-situ* butanol removal technologies can alleviate butanol inhibition, improve productivity and mitigate energy consumption of the butanol purification system, where the lack of acetone will allow for simplified design and lowered costs, both operational and capital, involved in downstream distillation. More research is needed to study *in-situ* butanol removal (one-stage and hybrid) for PBE fermentations, more specifically the effect of having PDO and glycerol in the fermentation broth [115].

Acknowledgments: The authors are grateful to the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, BioFuelNet Canada, the Ernest Solvay Foundation and the Alexander von Humboldt Foundation for financial support.

Author Contributions: Tahereh Sarchami coordinated the study and contributed to the writing, editing, and correction of the manuscript; Garret Munch contributed to the writing, editing, and correction of the manuscript; Erin Johnson contributed to the writing, editing, and correction of the manuscript; Sascha Kießlich contributed to the writing, editing, and correction of the manuscript; Lars Rehmann assisted in the editing and correction of the manuscript. He is also the corresponding author of this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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