Biobutanol production in a *Clostridium acetobutylicum* biofilm reactor integrated with simultaneous product recovery by adsorption

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**Abstract**

**Background:** *Clostridium acetobutylicum* can propagate on fibrous matrices and form biofilms that have improved butanol tolerance and a high fermentation rate and can be repeatedly used. Previously, a novel macroporous resin, KA-I, was synthesized in our laboratory and was demonstrated to be a good adsorbent with high selectivity and capacity for butanol recovery from a model solution. Based on these results, we aimed to develop a process integrating a biofilm reactor with simultaneous product recovery using the KA-I resin to maximize the production efficiency of biobutanol.

**Results:** KA-I showed great affinity for butanol and butyrate and could selectively enhance acetoin production at the expense of acetone during the fermentation. The biofilm reactor exhibited high productivity with considerably low broth turbidity during repeated batch fermentations. By maintaining the butanol level above 6.5 g/L in the biofilm reactor, butyrate adsorption by the KA-I resin was effectively reduced. Co-adsorption of acetone by the resin improved the fermentation performance. By redox modulation with methyl viologen (MV), the butanol-acetone ratio and the total product yield increased. An equivalent solvent titer of 96.5 to 130.7 g/L was achieved with a productivity of 1.0 to 1.5 g · L⁻¹ · h⁻¹. The solvent concentration and productivity increased by 4 to 6-fold and 3 to 5-fold, respectively, compared to traditional batch fermentation using planktonic culture.

**Conclusions:** Compared to the conventional process, the integrated process dramatically improved the productivity and reduced the energy consumption as well as water usage in biobutanol production. While genetic engineering focuses on strain improvement to enhance butanol production, process development can fully exploit the productivity of a strain and maximize the production efficiency.

**Keywords:** Biofilm reactor, *Clostridium acetobutylicum*, Simultaneous product recovery, Acetoin, Adsorption, Redox modulation

**Background**

Butanol fermentation, commonly known as ABE (acetone, butanol, and ethanol) fermentation, once the second largest biotechnological industry in the world [1], has attracted renewed interest in recent years for several economic and environmental reasons [2,3]. However, low alcohol yield (<25% w/w), low reactor productivity (approximately 0.3 g · L⁻¹ · h⁻¹), and high energy-consumption and water usage due to low solvent titer (approximately 20 g/L) are major challenges of conventional ABE fermentation, limiting the development of economically viable biobutanol production [2,3].

The low butanol yield in ABE fermentation is largely attributed to the fact that considerable amounts of glucose are converted to acetone with concomitant release of CO₂ and H₂. Genetic engineering technologies, for example, disrupting the *adc* gene [4] or reinforcing the direct butanol-forming route [5], have been applied to improve butanol yield. Introducing CO₂ fixation pathways into a butanol-producing host may also be a promising
approach to improve the product yield [6]. To address the low solvent-productivity, immobilized cell systems have been developed that maintain a high cell-activity and reduce the downtime of a repeated batch fermentation mode or continuous fermentation mode [7]. The highest ABE productivity, 15.8 g · L⁻¹ · h⁻¹ was obtained by continuous fermentation of immobilized *Clostridium beijerinckii* (C. beijerinckii) cells adsorbed to clay brick; this productivity was approximately 50 times the typical productivity of planktonic cell fermentation [8]. However, the butanol titer in this process was only about 5 g/L. A more elaborate process should be designed to avoid the loss of substrate in the effluent and to increase the solvent productivity.

Product toxicity to the producing strain at low solvent-concentrations is another critical limitation of biobutanol production. Due to the susceptibility of *Clostridia* to butanol, the solvent titer during ABE fermentation is low. Until now, the highest butanol concentration achieved in the fermentation broth without simultaneous product recovery was only 20.9 g/L, which was obtained in a batch fermentation using *C. beijerinckii* BA101 generated by nitrosoguanidine (NTG) mutagenesis [9]. Solvent recovery using conventional distillation is energy intensive and expensive. Low butanol titers increase recovery costs and water usage. Numerous processes have been developed for simultaneous recovery of solvent from the broth during fermentation. The most common solvent recovery techniques are liquid-liquid extraction, gas stripping, pervaporation, and adsorption. In particular, gas stripping has been extensively explored in recent years, and attractive results have been obtained in terms of solvent concentration and productivity. By fed-batch culture of a genetically engineered strain with gas-stripping recovery, 585.3 g of butanol was produced from 1,861.9 g of glucose, with a productivity of 1.32 g · L⁻¹ · h⁻¹ [5]. Similarly, by applying gas stripping intermittently in fed-batch fermentation with a butanol tolerant strain, 172 g/L ABE (containing 113.3 g/L butanol) were produced with an overall ABE productivity of 0.53 g · L⁻¹ · h⁻¹ [10]. Although gas stripping is a favorable choice due to its operation simplicity, it usually requires a higher energy-input because of the intensive product-capturing step [11]. Since the energy density of butanol is only 36 kJ/g, it is essential to minimize the energy consumed for butanol recovery in order to recover the highest net energy increase. The energy requirements for butanol recovery by conventional distillation, gas stripping, extraction, pervaporation, and adsorption-desorption have been estimated at 24, 21, 14, 9, and 8 kJ/g butanol, respectively [12,13], highlighting the superiority of adsorption-desorption with respect to energy return. An assessment conducted by Oudshoorn and his co-workers also demonstrated that adsorption- and pervaporation-based techniques are the most attractive recovery options for the recovery of butanol from aqueous solution [11]. Silicalite, bonopore, polyvinylpyridine, zeolite, activated carbon, and polymeric resins have been exploited for butanol adsorption. Being solid phase, the adsorbents have the advantages of complete immiscibility and ease of regeneration and reuse [13]. However, application of adsorbents in butanol fermentation has been limited by their low adsorption selectivity and capacity [12].

In our preliminary work, *Clostridium acetobutylicum* (*C. acetobutylicum*) cells were immobilized as a biofilm on a fibrous matrix, which increased the butanol tolerance and dramatically improved the ABE productivity of the cells [14]. A novel macroporous resin, KA-I, was also synthesized and used for the recovery of butanol from the model solution, with high selectivity and capacity [15,16]. Based on these results, we aimed to develop an integrated process to maximize the butanol production efficiency with minimal resource consumption. First, the adsorption behavior of KA-I during fermentation was investigated and controlled. Second, a system integrating a biofilm reactor with fixed-bed adsorption using KA-I was constructed and optimized for the production of ABE and acetoin with simultaneous product recovery.

**Results**

**In situ product removal using the macroporous resin KA-I**

Effects of resin amount

The biocompatibility of the KA-I resin and the effects of the resin on the metabolism of the cells were first investigated. Various amounts of resin (40 to 80 g/L) were directly added to planktonic cultures. The fermentation times were shortened from 80 h to between 45 and 70 h depending on the resin amounts (Table 1). When the fermentation was ended, the adsorbed solvents were eluted with methanol and quantified (see Methods). The solvent adsorption capacities of the resin were calculated as solvents adsorbed (mg) divided by the amount of resin (g) used in the fermentation. The KA-I resin had the highest adsorption capacity for butanol (maximum: 110 mg/g, obtained when 40 g/L resin was used), followed by adsorption of acetone. In contrast, no ethanol, acetic acid, acetoin, or glucose were detected in the eluent, indicating KA-I exhibited no adsorption capacity for these compounds at the end of the fermentation, which was consistent with the previous study using model solutions [15]. However, relatively high amounts of butyric acid were also adsorbed by the KA-I resin. Butyric acid and acetic acid were predominantly generated during the exponential growth of *C. acetobutylicum* and reached peak concentrations before being re-assimilated for ABE production. Adsorption of butyric acid led to an increased residual concentration, reducing the solvent yield. The greater the amount of resin added to the culture, the higher the residual levels of acids (including acetate)
at the end of the fermentation. When an excessive amount (80 g/L) of resin was initially added to the culture, 5.46 g/L acids remained in the culture, which was 117% and 475% higher than the concentration of acids obtained after the addition of 50 g/L resin and in the control, respectively.

The amounts of residual acids could be reduced by adding the resin at a later time point. An optimal time point for adding the resin was found to be 40 h, when the acids started to be re-assimilated. Addition of 50 g/L of resin at 40 h, rather than at 0 h, led to a 43% reduction in the residual acids, and thus a higher butanol production was achieved. Based on the solvent production and fermentation time, 50 g/L was considered the optimal amount of resin to be added at 40 h.

**Inverse relationship between the production of acetoin and acetone**

An inverse relationship between acetoin and acetone production was observed in the adsorptive fermentation. Addition of resin significantly increased acetoin production and decreased acetone production (Table 1). An increase in the resin amount or addition at an earlier time led to a higher acetoin production and lower acetone production. When 60 g/L resin was initially added to the culture, acetoin production was as high as 3.8 g/L, whereas acetone production was only 1.9 g/L. Interestingly, an inverse relationship between acetoin and acetone production was also observed in the study by Doremus et al., who investigated the effects of pressure and agitation on ABE fermentation [17]. Thus, it appears that acetoin can be overproduced at the expense of acetone without negatively affecting butanol production. However, acetoin production by *C. acetobutylicum*, as reported in early studies, was usually not higher than 1.0 g/L [18]. To the best of our knowledge, 3.8 g/L is the highest yield of acetoin produced by *C. acetobutylicum*.

**Fermentation kinetics**

As shown in Figure 1A, when 50 g/L resin was added to the culture at 40 h, the rates of cell growth and glucose consumption were significantly accelerated. Glucose was completely consumed in less than 65 h, with higher butanol productivity (0.30 g · L⁻¹ · h⁻¹) than in the control (0.25 g · L⁻¹ · h⁻¹). The aqueous concentrations of butanol and butyric acid decreased quickly (Figure 1B), and optical density (OD)₆₀₀ increased by 80.4% (from 3.17 to 5.72), instead of continuing to decline as observed in the control. Thus, it can be concluded that additional butyric acid and acetic acid were produced during the stimulated growth. The pH decreased throughout this period to 3.63, a low value that was not observed in the control. Despite the low pH value, the *C. acetobutylicum* cells still exhibited efficient fermentation, suggesting that butyric acid and butanol exerted more dominant effects on *C. acetobutylicum* than other acids affecting pH. In conclusion, *in situ* extractive fermentation with KA-I resin improved the cell growth and accelerated the fermentation process, indicating good biocompatibility of the KA-I resin.

**Batch fermentation in biofilm reactors coupled with fixed-bed adsorption**

**Biofilm reactor**

Different materials such as resins, activated carbon, silk, cotton, or polyester, were used to support biofilm formation. Generally, *C. acetobutylicum* B3 biofilms on these carriers were visible after 48 h in culture, suggesting a good ability of *C. acetobutylicum* B3 cells to form a biofilm on solid surfaces. However, fermentation rates and product patterns of the biofilms differed from each other (data not shown). In the present study, a fibrous matrix (cotton towel, Skyshow Textiles Co. Ltd. Shanghai, China) was selected after experimental examinations as the support for *C. acetobutylicum* B3 biofilm formation. A thick, sticky layer of biofilm stacked on the matrix surface was observed. Scanning electron microscopy

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**Table 1 Effects of resin quantity on the final product yield**

| Resin Amount (g/L) | Timea (h) | Butanol (g/L) | Butyric acid (g/L) | Acetic acid (g/L) | Acetoin (g/L) | Acetone (g/L) |
|-------------------|-----------|---------------|-------------------|------------------|---------------|--------------|
|                   | Aqueous   | Adsorbed      | Total             | Aqueous          | Adsorbed      | Total         |
| 0                 | 0 to 80   | 11.8          | 11.8              | 0.29             | 1.81          | 4.63          |
| 40                | 0 to 70   | 6.87          | 4.42              | 11.3             | 0.50          | 0.37          | 0.87          | 1.23          | 1.28          | 3.61          | 2.02          |
| 50                | 0 to 60   | 6.25          | 4.76              | 11.0             | 0.77          | 0.46          | 1.23          | 1.28          | 3.61          | 2.02          |
|                   | 40 to 65  | 6.42          | 5.09              | 11.5             | 0.35          | 0.29          | 0.64          | 0.79          | 3.44          | 2.58          |
| 60                | 0 to 45   | 5.11          | 4.62              | 9.73             | 0.98          | 0.94          | 1.93          | 1.51          | 3.83          | 1.90          |
|                   | 40 to 60  | 5.55          | 4.81              | 10.4             | 0.58          | 0.54          | 1.12          | 0.77          | 3.20          | 2.92          |
| 80                | 0 to 45   | 2.51          | 5.31              | 7.81             | 1.26          | 2.27          | 3.53          | 1.93          | 3.09          | 1.78          |

aFrom the time the resin was added to the time the fermentation ended. For example, 40 to 65 means that resin was added to the culture at 40 h and the fermentation ended at 65 h. Data for ethanol are not shown. The experiments were performed in triplicate, and the mean value was calculated.
(SEM) revealed that the cells formed aggregates and were effectively immobilized by the extracellular polymeric substance that they produced (Figure 2). The performance of the biofilm reactors is summarized in Table 2. Batch fermentations with a stable biofilm reactor could be completed in approximately 12 h, with a 5-fold higher productivity compared with a planktonic cell reactor. Furthermore, the cell density of the bulk medium in the biofilm reactors was about 10-fold lower than that of the planktonic cell bioreactors, indicating that the cells preferred to propagate on the fibrous matrix rather than in the bulk medium. While there have been many efforts to increase the cell density in a traditional planktonic cell reactor to achieve an increased production rate [19,20], in the present study, a minimum turbidity of the bulk medium in the biofilm reactors is desired to lower the burden of the cell separator. Apparently, this low cell-density was beneficial for the long runtime of the integrated fermentation process. Furthermore, in the biofilm reactor, fermentations can be performed in a repeated batch mode or a continuous fermentation mode. Interestingly, contrary to the substitution of acetone by acetoin in the in situ adsorption fermentations, production of acetoin in the biofilm reactors was much lower, whereas the production of acetone was higher.

**Importance of the butanol level**

The online fixed-bed adsorption system was constructed by packing the KA-I resin in glass columns and connecting the glass columns to the biofilm reactor (Figure 2). The butanol titer was maintained at different levels to investigate the effects of the titer on the fermentation. As shown in Table 2, lowered butanol titers in the biofilm reactor did not improve the productivity but increased the cell density of the bulk medium as well as the residual butyric acid level at the end of the fermentation, resulting in a low butanol yield. In addition, low butanol titers also significantly decreased the adsorption capacity of the KA-I resin (data not shown). Hence, the butanol titer was maintained above 6 g/L in the subsequent experiments.

**Figure 1** Kinetics of batch fermentation by planktonic culture. (A) pH, optical density (OD)\(_{600nm}\) and glucose consumption; (B) product concentrations during the fermentation process. Initially, experiments were performed in six Duran bottles under the same condition. At 40 h, three of the bottles were supplemented with KA-I resin (final concentration 50 g/L) (open symbol and dashed line), with the other ones left as control (solid symbol and solid line). The mean value (± SD) was calculated from the results of parallel runs. The vertical dashed lines represent the equivalent concentrations adsorbed by the resin (determined by dividing the adsorbed amounts by the volume of the fermentation broth) at the end of the fermentation.
Fed-batch fermentation in biofilm reactors coupled with fixed-bed adsorption

**Fermentation kinetics**

As shown in Figure 3, after 13 h of batch culture, concentrated P2 medium (500 g/L glucose) was fed into the reactor via a peristaltic pump. Glucose was first maintained at approximately 30 g/L for 10 h and then at approximately 15 g/L for 10 h to investigate its effect on fermentation. Whenever the glucose concentrations in the reactor fell below the pre-determined levels, the feed pump was turned on at a flow rate of 15 mL/h until the predetermined levels were reached. The butanol titer in the fermentation broth was also maintained between 6.5 and 8.5 g/L with fixed-bed adsorption. Acetone, ethanol, and acetoin gradually accumulated owing to the selective adsorption of the KA-I resin. In batch fermentations, low glucose concentrations led to increased residual acids [21]. However, in the fed-batch mode, under a controlled butanol titer, 15 and 30 g/L glucose did not significantly affect the acid production or the cell density in the medium. Both the level of butyrate and the OD<sub>600</sub>nm were relatively constant, except for a sudden increase observed when the butanol level was below 6.5 g/L (Figure 3). Hence, it was important to maintain butanol at a higher level to reduce the butyrate level and the cell density. The pH was also constant at 4.2, in contrast to low value of 3.6 observed in the in situ adsorption experiment. The fermentation was stopped when the residual glucose was completely used owing to depletion of the resin columns. The results obtained are

| Fermentation process | Ethanol (g/L) | Butanol (g/L) | Acetone (g/L) | Acetoin (g/L) | Butyrate (g/L) | Yield (g/g) | Productivity (g · L<sup>-1</sup> · h<sup>-1</sup>) | Maximum OD<sub>600</sub> nm |
|---------------------|--------------|---------------|--------------|--------------|---------------|-------------|-----------------------------|--------------------------|
| Biofilm reactor, fixed-bed adsorption 2.5 to 3.2 g/L butanol | 1.37 | 10.6 | 5.73 | 1.51 | 1.88 | 0.32 | 1.47 | 7.88 |
| Biofilm reactor, fixed-bed adsorption 4.0 to 5.5 g/L butanol | 1.44 | 11.1 | 6.32 | 1.23 | 0.95 | 0.33 | 1.60 | 3.46 |
| Biofilm reactor, fixed-bed adsorption 5.2 to 6.4 g/L butanol | 1.49 | 11.7 | 6.43 | 1.25 | 0.49 | 0.35 | 1.53 | 1.55 |
| Biofilm reactor, without adsorption | 1.48 | 11.6 | 6.70 | 0.98 | 0.17 | 0.35 | 1.66 | 0.30 |
| Traditional planktonic cell fermentation, without adsorption | 2.1 | 11.8 | 4.6 | 1.8 | 0.29 | 0.34 | 0.25 | 3.2 |

The data represent the averages of two independent fermentations. Data for acetate are not shown. Typically, acetate displayed a change trend similar to that of butyrate. OD, optical density.
summarized in Table 3. An equivalent solvent concentration of 59.8 g/L was achieved, which was about three times higher than that obtained in batch fermentation.

Kinetics of fixed-bed adsorption
Figure 4 shows the dynamic process of the fixed-bed adsorption. Although the in situ adsorption experiments showed that the KA-I resin had no adsorption capacity for ethanol at the end of the batch fermentation, the fixed-bed adsorption curve revealed that initially, ethanol, acetone, and butanol could be simultaneously adsorbed by the KA-I. However, ethanol, followed by acetone, was subsequently eluted, whereas butanol continued to be adsorbed, which indicated that ethanol was a weakly retained component and butanol was a strongly retained component; acetone retention was intermediate. Similar to the in situ adsorption experiments, butyrate was also significantly adsorbed by the fixed-bed columns, and it appeared that butyrate could not be competitively eluted by butanol. The KA-I resin, with a cross-linked polystyrene framework, is a weakly polar resin. The adsorption affinity for ABE showed a correlation with the polarity from the sorbate, that is, the affinity for the sorbate decreased with the increase in the sorbate polarity, from butanol to acetone to ethanol [22]. However, the adsorption behavior of butyrate was not in line with this. The high affinity for butyrate might be attributed to the hydrophobic interactions between the alkyl chain of butyrate and the aromatic groups of the KA-I resin [15].

Fermentation with co-adsorption of acetone
In the fermentation process by C. acetobutylicum, acetone was not as toxic as butanol. However, selective adsorption of butanol by the KA-I resin could make acetone a predominant inhibitory product in view of its quantity. More importantly, high acetone levels in the fermentation broth could result in serious mass loss of acetone due to its high volatility, leading to a low solvent yield (Table 3). Considering the competitive adsorption behavior of butanol with respect to acetone, as indicated by the fixed-bed adsorption dynamics, the resin columns were displaced properly before being saturated by butanol to enhance acetone adsorption and lower the level of acetone in the fermentation broth. As shown in Figure 5, butanol was maintained at a higher level (7 to 9 g/L) to further reduce the cell density and butyrate level in the medium. When the fixed-bed adsorption was stopped owing to the maintenance of the filtration unit, residual glucose was completely consumed, and the final aqueous butanol level reached 14.7 g/L. Three different levels (42, 23 and 13 g/L) of glucose were maintained during the fermentation to investigate the effect of glucose again. Similar to the previously observed results, the butanol titer rather than the glucose concentration, significantly affected butyrate production. With co-adsorption
of acetone, the aqueous acetone level in the fermentation broth was successfully maintained at approximately 5 g/L. Although the adsorbed butanol level was reduced from 101 to 83.8 mg/g resin, the adsorbed acetone level was increased from 29.4 to 43.9 mg/g resin. The final equivalent solvent concentration was 96.5 g/L, which was approximately five times higher than that of the batch fermentation. The solvent yield was also partly recovered and thus higher than that of the fermentation with selective adsorption of butanol (Table 3).

**Redox modulation for improved product yield**

During ABE fermentation by *C. acetobutylicum*, the release of H$_2$ causes a deficiency in nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) reducing equivalents used for ethanol and butanol production. Therefore, a large quantity of oxidation products (especially acetate and acetone) is commonly produced, resulting in a low alcohol yield [14]. In fact, acetone was produced in greater quantities in the biofilm reactor than in the planktonic cell reactor due to the increased H$_2$ release. Methyl viologen (MV) was used to enhance butanol production because MV can divert the electron flow from H$_2$ to NAD(P)H [23]. The results are summarized in Table 3. With addition of 1 mM MV, butanol production was markedly increased whereas acetone was reduced. Thus, the butanol-acetone molar ratio increased from 1.9 to 3.2. More importantly, when one glucose molecule is converted to the C4 compound butanol instead of the C3 compound acetone, the mass yield is expected to increase. Indeed, the total solvent yield (ABE and acetoin) upon MV addition increased by 9%. Redox modulation by MV addition also stimulated butyrate production, consistent with a previous report [4]. The butyric acid was maintained at approximately 1.5 g/L throughout the fermentation, which was a much higher level than the average of 0.5 to 0.7 g/L observed when no MV was added. Due to the adsorption by the KA-I resin, the final butyrate concentration reached 10.8 g/L. Therefore, the total product yield (ABE, acetoin, and butyrate) was further increased by 16%. This increased product yield will reduce carbon emission in the ABE fermentation. In addition, MV was not adsorbed by the KA-I resin, suggesting that MV could be reused in the fermentation. However, the use of MV resulted in a slower fermentation rate, likely due to the inhibition of cell growth [23] or through a toxic effect of butyric acid on metabolism [24] during the prolonged fermentation.

**Comparison with other studies**

Although adsorption of butanol from model solutions onto various resins has been extensively studied, there have been few reports of the application of resins for butanol fermentation, perhaps due to relatively low

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Table 3 Results of fed-batch fermentation in biofilm reactors coupled with fixed-bed adsorption

|                     | Fermentation with selective adsorption of butanol | Fermentation with co-adsorption of acetone | Fermentation with redox modulation |
|---------------------|--------------------------------------------------|-------------------------------------------|-----------------------------------|
| Fermentation time (h) | 57                                               | 64                                        | 135                               |
| Glucose fermented (g/L) | 198                                              | 297                                       | 367                               |
| Resin used (g)       | 418                                              | 812                                       | 910                               |
| Adsorbed ethanol (g) | 1.8                                              | 0.82                                      | 2.2                               |
| Adsorbed acetone (g) | 12.3                                             | 35.7                                      | 23.6                              |
| Adsorbed butanol (g) | 42.1                                             | 68.1                                      | 84.7                              |
| Adsorbed acetoin (g) | 0.40                                             | 2.4                                       | 1.2                               |
| Adsorbed butyrate (g) | 1.9                                               | 3.1                                       | 8.7                               |
| Equivalent ethanol concentration (g/L) | 5.1                                               | 4.8                                       | 6.8                               |
| Equivalent acetone concentration (g/L) | 14.8                                              | 30.9                                      | 28.8                              |
| Equivalent butanol concentration (g/L) | 38.2                                              | 58.3                                      | 92.6                              |
| Equivalent acetoin concentration (g/L) | 1.7                                               | 2.6                                       | 2.6                               |
| Equivalent butyrate concentration (g/L) | 1.7                                               | 2.4                                       | 108                               |
| Solvent concentration (g/L) | 59.8                                              | 96.5                                      | 130.7                             |
| Solvent productivity (g · L$^{-1}$ · h$^{-1}$) | 1.05                                              | 1.51                                      | 0.97                              |
| Solvent yield (g/g) | 0.302                                             | 0.325                                     | 0.356                             |
| Total product yield (g/g) | 0.311                                             | 0.333                                     | 0.386                             |
| Butanol-acetone molar ratio | 2.6$^*$                                           | 1.9                                       | 3.2$^*$                           |

$^*$Solvent: ABE and acetoin. $^*$Total product: ABE, acetoin, and butyrate. Acetate is not considered to be a product. $^*$The increased butanol-acetone ratio was probably due to the evaporation of acetone.
adsorption capacities of previously studied adsorbents (Table 4). In small-scale batch fermentations, poly(styrene-co-divinylbenzene)-derived materials were demonstrated to be excellent adsorbents for butanol with high adsorption capacity [12,25]. However, continuous or fed-batch fermentation with this resin has not yet been reported. Yang and Tsao used polyvinylpyridine as an adsorbent for ABE in repeated fed-batch fermentations [26]. The equivalent solvent concentration and the productivity obtained in this integrated process increased by 140% and 320%, respectively, compared with the solvent concentration and productivity obtained through traditional batch fermentation. In this study, fermentations with fixed-bed adsorption using KA-I were conducted in a biofilm reactor. The biofilm could maintain its metabolic activity during a long period of feeding fermentation; thereby, a high solvent concentration was achieved. The equivalent solvent concentrations and the productivities obtained in the fermentation were four to six times and three to five times higher, respectively, than those obtained by conventional fermentation. In the present study, by redox modulation using MV, a relatively high yield of solvent was also achieved. However, similar to many other resins, KA-I also showed a high adsorption affinity for butyrate, which could affect butanol yield. A genetically engineered strain with a modified product spectrum (for example, diminished acetone, acetate, and butyrate) may compensate for the imperfect selectivity of the adsorbents and make adsorption more competitive than other recovery methods.

Discussion

Acetoin: a value-added product

Interestingly, an inverse relationship was observed between the production of acetoin and acetone. Acetoin (3-hydroxybutanone) is a fine chemical widely used for food flavoring and as a fragrance. Acetoin is also used to synthesize novel optically active α-hydroxyketone derivatives and liquid crystal composites [30]. Recently, the ratio of D-acetoin to L-acetoin produced by Clostridium strain ATCC 824 was found to be 12:1 [31]. In contrast, acetoin produced by butanediol-producing strains are often racemic. The inverse relationship suggests that ABE fermentation may be switched to acetoin-butanol-ethanol fermentation, which is a promising approach to improve the product value of ABE fermentation. It was

Figure 4 Kinetics of the fixed-bed adsorption. The fixed-bed adsorption was started at 13 h. A sample before the adsorption (influent, black square) and a sample after the adsorption (effluent, red circle) were withdrawn simultaneously every time to determine the extent of resin saturation. A higher sorbate titer in the effluent meant that the sorbate initially adsorbed onto the KA-I resin was subsequently eluted in the butanol adsorption process. The resin column was replaced with a new resin column when saturated by butanol.
Figure 5 Kinetics of fed-batch fermentation in the biofilm reactor with co-adsorption of acetone. After 8 h of batch culture, concentrated medium was fed into the reactor to maintain desired glucose levels and the fixed-bed adsorption was started, as is indicated by arrows. By co-adsorption of acetone using KA-I resin, the acetone titer in the fermentation broth was also maintained at a relatively stable level. OD, optical density.

Table 4 Performance of different types of resin used as adsorbents during butanol fermentation

| Fermentation | Resin Type | Selectivity | Capacity | Equivalent | Solvent | Solvent |
|--------------|------------|-------------|----------|------------|---------|---------|
|              |            |             | (mg/g)   | concentration (g/L) | productivity (g · L⁻¹ · h⁻¹) | yield (g/g) | Reference |
| Batch fermentation, in situ adsorption | XAD-8 | Mainly butanol, isopropanol, glucose | 30 | 12.6 | Approx. 0.13 | - | [27] |
| Two-stage continuous fermentation, in-line adsorption | XAD-16 | Butanol, acetone, acetate, and butyrate; no lactose | 64 | 11.01 | Approx. 1.5 | 0.30 to 0.36 | [28] |
| Repeated batch fermentation, batch adsorption | Bonopore, copolymers of divinylbenzene, and styrene | Solvent and acids | 43 | - | - | - | [29] |
| Batch fermentation, in situ adsorption | Dowex Optipore SD-2ploy (styrene-co-DVB) derived | Mainly butanol and butyrate | 264 | Approx. 37 | Approx. 0.51 | 0.27 to 0.40 | [12] |
| Repeated fed-batch fermentation, fixed-bed adsorption | Reillex 425, polyvinylpyridine | ABE and acids; no glucose | Approx. 61 | 47.2 | 1.69 | 0.32 | [26] |
| Fed-batch fermentation, fixed-bed adsorption | KA-I, cross-linked polystyrene framework | Butanol, butyrate, and acetone; no glucose | 84 | 96.5 | 1.51 | 0.33 | This work |
| Fed-batch fermentation, fixed-bed adsorption, MV addition | KA-I, cross-linked polystyrene framework | Butanol, butyrate, and acetone; no glucose or MV | 93 | 130.7 | 0.97 | 0.36 | This work |

*aCalculated as butanol adsorbed (mg) divided by the amount of resin (g) used in the fermentation process. MV, methyl viologen; ABE, acetone-butanol-ethanol; approx., approximately.
presumed that acetoin is a structural analog of acetoacetate and could not be decarboxylated by acetoacetate decarboxylase, which would impede acetone production [17]. However, exogenous addition of 1, 2, or 4 g/L acetoin to the fermentation broth failed to lower the acetone production (data not shown). Recent insights from molecular studies indicate that acetoin is the product of \(als\) (encoding an \(α\)-acetolactate synthase and an acetolactate decarboxylase), which is regulated by the transcription factor \(alsR\) in response to acetate accumulation and a pH change in \(Bacillus subtilis\) [32]. Study of disruption of \(alsR\) in \(C. acetobutylicum\) is ongoing to better understand the mechanisms underlying the inverse relationship.

**High productivity of the biofilm reactor**

Our previous study demonstrated that \(C. acetobutylicum\) cells exhibited improved butanol tolerance and production rate in biofilm compared with planktonic cells [14]. Thus, the \(C. acetobutylicum\) B3 biofilm was used for butanol fermentation in this study. The solvent production rate was increased dramatically, from 0.25 to 0.30 to 0.97 to 1.66 g·L\(^{-1}\)·h\(^{-1}\). Meanwhile, in the biofilm reactor, fermentation can be performed in a repeated batch mode or a continuous mode, which can overcome the drawbacks of batch operation, such as down times and long lag periods. Thus, the reactor productivities were greatly improved, which can significantly reduce capital investment and operational costs [2]. Furthermore, the cells can remain in the biofilm during a prolonged feeding fermentation or repeated batch fermentations, and the already established solvent-producing capacity of the cells would increase the carbon flux to solvent production rather than biomass synthesis.

**Conservation of energy and water by simultaneous product removal**

Due to product toxicity, \(C. acetobutylicum\) fermentation rarely produces >13 g/L butanol [4]. Product recovery from the dilute fermentation broth by conventional distillation is energy intensive because water is the major component, with a boiling point below that of butanol (100 versus 117°C). When the fermentation was coupled to a simultaneous adsorption process using KA-I, the product was removed, and the levels of butanol and acetone were maintained below the inhibitory threshold. Thus, the fermentation could be prolonged with a continuous feed of highly concentrated nutrients, and a high equivalent-solvent titer was achieved. When the resin columns were eluted with methanol, the average concentration of butanol in the eluent could be increased by approximately 6-fold to 50 to 60 g/L. Distillation technology showed that if the butanol concentration could be raised from 12 to 19 g/L, the energy consumption of butanol recovery from the fermentation broth could be cut in half [16]. Together with the low methanol boiling point (64°C), the high butanol titer in methanol would significantly reduce the energy requirement for butanol separation. Moreover, the high equivalent solvent concentration obtained in the integrated process meant that the fermentation was performed with a highly concentrated medium, which reduced the water usage and resulted in significant savings in sterilization and wastewater treatment.

**Improvement of yield by redox modulation**

Theoretically, butanol can be produced from glucose with 100% molar yield. However, release of \(H_2\) causes a deficiency of the reducing equivalents NAD(P)H, thus resulting in a large quantity of oxidation products, especially acetone [14]. Except for acetoin, butanol is the preferred product, attracting the highest price of the solvents. Addition of MV diverted the electron flow from \(H_2\) to NAD(P)H [23], which facilitated acetoacetyl-CoA reduction to butanol rather than decarboxylation to acetone. Thus, the butanol-acetone ratio was improved, and the emission of \(H_2\) and \(CO_2\) was reduced, leading to increased product yield. Hence, redox modulation of \(C. acetobutylicum\) metabolism by small-molecule effectors can be used to improve both the butanol-acetone ratio and the total solvent yield in ABE fermentation, which has been rarely achieved by genetic manipulations [4,33,34].

In summary, the process developed in this study could potentially (1) reduce capital investment and operational costs due to high reactor productivity, (2) reduce energy consumption and wastewater treatment due to the highly concentrated medium and high solvent titer, and (3) increase product value and reduce gas emission due to the improved butanol ratio and solvent yield. Genetically engineered strains with modified product spectra (for example, diminished acetone and acid production, or selective butanol production) will make this process more economically favorable.

**Conclusions**

KA-I resin can be used as a good adsorbent for product recovery in ABE fermentation. Use of KA-I resin enhanced acetoin production at the expense of acetone production during the fermentation. To reduce butyrate adsorption by KA-I, the butanol titer should be maintained at a relatively high level (>6.5 g/L). The biofilm reactor dramatically enhanced the fermentation productivity compared with the planktonic reactor. In fed-batch production with simultaneous product recovery, the equivalent solvent concentration was much higher than in a conventional fermentation. Redox modulation was effective for improving the butanol-acetone ratio and the
total product yield. While molecular technologies focusing on strain construction are becoming mainstream in the development of economically viable biobutanol production, process technologies should not be overlooked because they can be an effective means of fully exploiting the productivity of a strain and maximizing the production efficiency.

Methods
Organism and culture conditions
*C. acetobutylicum* B3 (CGMCC number 5234) was used in all of the fermentation experiments. The strain was stored in 30% (v/v) glycerol at −80°C and was grown in solid reinforced clostridia medium (RCM) at 37°C in an anaerobic chamber (Bug Box, Ruskinn Technology, Leeds, UK). Cultures of *C. acetobutylicum* B3 were grown in modified P2 medium (containing 10 g/L glucose as the sole carbohydrate source) for seed culture. Unless otherwise stated, the fermentation experiments were performed in P2 medium (comprising glucose, 60 g/L; K₂HPO₄, 0.5 g/L; KH₂PO₄, 0.5 g/L; CH₃COONH₄, 2.2 g/L; MgSO₄·7H₂O, 0.2 g/L; MnSO₄·H₂O, 0.01 g/L; NaCl, 0.01 g/L; FeSO₄·7H₂O, 0.01 g/L; p-aminobenzoic acid, 1 mg/L; thiamine, 1 mg/L; and biotin, 0.01 mg/L) [35] at 37°C with 10% inoculum (v/v).

Biofilm formation and repeated batch fermentations
For biofilm formation, a 2-L stainless steel column was packed with a spiral fibrous matrix (90 g). The columns were sterilized at 121°C for 30 minutes before use. Subsequently, 150 mL of 12-h-old seed culture was inoculated into 1.5 L of P2 medium and allowed to grow statically for 20 h at 37°C. Next, the culture was transferred to the stainless steel column and circulated through the fibrous matrix at a pumping rate of 25 mL/minute to allow the cells to immobilize onto the fibrous matrix and form a biofilm. The temperature was controlled via connection of the jacketed column to a warm water bath at 37°C. After 20 h of continuous circulation, the broth was replaced with fresh P2 medium to start the ABE fermentation. The medium circulation rate was maintained at 35 mL/minute via a peristaltic pump during fermentation. When the butanol titer reached the predetermined threshold, the fermentation broth was transferred to a microporous filtration unit (Tianjin MOTIMO Membrane Technology Co., Model MOF-1b, Tianjin, China) to separate the cells before being loaded onto the resin bed. The effluent was transferred back to the reactor for further fermentation. In the fed-batch mode, 1 L of P2 medium was initially fed into the reactor. Subsequently, P2 medium containing a high glucose concentration (500 g/L) was fed into the reactor using a peristaltic pump to maintain the glucose concentration within the desired range. When the resin column was saturated, it was replaced with a new resin column.

Redox modulation
MV was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) as the dichloride hydrate. It was prepared as a sterile stock solution and added to the fermentation broth as necessary to achieve a final concentration of 1 mM. The MV concentration was determined spectrophotometrically as described previously [36] to determine whether it had adsorbed to the KA-I resin.

Solvent desorption and resin regeneration
The resin in the *in situ* adsorption experiment was collected at the end of the fermentation and washed gently with 100 mL of deionized water. Subsequently, the resin was soaked in 500 mL of methanol and agitated at 200 rpm for 30 minutes at 25°C. The supernatant liquids were filtered and analyzed by gas chromatography. The solvents adsorbed to the resin packed in the glass columns were desorbed in a fixed-bed mode using one bed volume of 85% (v/v) methanol. The solvent recovery
from the KA-I resin was >99%. The resin could be regenerated using two bed volumes of water (for details, see [15]).

**Metabolite analyses and calculations**

The cell concentration was determined spectrophotometrically as the OD_{600nm}. The concentrations of glucose, acetate, and other organic acids were determined by HPLC analysis (Agilent 1100 series, Hewlett-Packard, California, USA), using an Aminex HPX-87H ion exclusion column (300 x 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) heated to 50°C. The analytes were separated with a mobile phase of 5.0 mM H_2SO_4 at 0.6 mL/minute and detected using a refractive index detector. Acetone, ethanol, butanol, butyric acid, and acetoin were analyzed using gas chromatography (7890A, Agilent, Wilmington, DE, USA) equipped with a flame ionization detector and an Agilent HP-INNOWAX column (0.25 mm x 60 m).

The equivalent solvent concentration was calculated as the total amount of solvent produced, including the aqueous volume and the adsorbed mass (g) divided by the total volume of medium (L) in the fermentor, cell separator, and samples. The solvent productivity was calculated as the equivalent solvent concentration (g/L) divided by the fermentation time (h). The yield was calculated as the total mass of solvent (ABE and acetoin) produced (g) divided by the total mass of glucose utilized (g).

**Abbreviations**

ABE: Acetone-butanol-ethanol; HPLC: High performance liquid chromatography; MV: Methyl viologen; NAD(P)H: Reduced nicotinamide adenine dinucleotide (phosphate); NTG: Nitrosoguanidine; OD: Optical density; PBS: Phosphate-buffered saline; SEM: Scanning electron microscopy.

**Competing interests**

The authors have declared that no competing interests exist.

**Authors' contributions**

DL, YC, and HY designed experiments. DL, FD, TZ, and HC performed experiments. BL, HN, ZC, XL, JX, and XC contributed materials and sample analysis. DL and YC analyzed data. DL and HY wrote the manuscript. All authors have read and approved the final manuscript.

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**References**

1. Jones DT, Woods DR. Acetone–butanol fermentation revisited. *Microbiol Rev* 1986, 50:494–524.
2. Green EM. Fermentative production of butanol—the industrial perspective. *Curr Opin Biotechnol* 2011, 22:1–7.
3. Tracy BP. Improving butanol fermentation to enter the advanced biofuel market. *mBio* 2012, 3:e00019-12.
4. Jiang Y, Xu C, Dong F, Yang Y, Jiang W, Yang S. Disruption of the acetocetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. *Metab Eng* 2009, 11:284–291.
5. Jiang YS, Lee JY, Lee J, Park JH, Im JA, Eom MH, Lee J, Lee SH, Song H, Cho JH, Seung DY, Lee SY. Enhanced butanol production obtained by reinforcing the direct butanol-forming route in *Clostridium acetobutylicum*. *mBio* 2012, 3:e00314-12.
6. Papoutsakis ET, Al-Hinai MA, Jones SW, Indurthi DC, Mitchell DK, Fast A. Recombinant *Clostridia* That Fix CO2 and CO and Uses Thereof; U.S. Patent 2012/0065827 A1, 15 March.
7. Doele J, Rebrus M, Rosenberg M. Immobilisation of clostridium spp. for production of solvents and organic acids. *Chemical Papers* 2014, 68:1–14.
8. Qureshi N, Schripsema J, Li, Lienhardt J, Blaschek HP. Continuous solvent production by Clostridium beijerinckii BA101 immobilized by adsorption onto brick. *World J Microb Biot* 2000, 16:377–382.
9. Chen CX, Blaschek HP. Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. *Appl Microbiol Biotechnol* 1999, 52:170–173.
10. Xue C, Zhao JB, Lu CC, Yang ST, Bai FW, Tang J. High-titer n-butanol production by *Clostridium acetobutylicum* J8200 in fed-batch fermentation with intermittent gas stripping. *Biotechnol Bioeng* 2012, 109:2746–2756.
11. Oudshoorn A, Van der Welken LM, Straathof AJ. Assessment of options for selective 1-butanol recovery from aqueous solutions. *Ind Eng Chem Res* 2009, 48:7325–7336.
12. Nielsen DR, Prather KJ. In situ product recovery of n-butanol using polymeric resins. *Biotechnol Bioeng* 2009, 102:811–821.
13. Ranjan A, Moholkar VS. Biobutanol: science, engineering, and economics. *Int J Energy Res* 2012, 36:277–323.
14. Liu Q, Chen Y, Li A, Ding FY, Zhou T, He Y, Li BB, Niu HQ, Lin XQ, Xie JJ, Chen XC, Wu J, Ying HJ. Enhanced butanol production by modulation of electron flow in *Clostridium acetobutylicum* B3 immobilized by surface adsorption. *Bioresour Technol* 2013, 129:321–328.
15. Lin XQ, Wu JL, Jin XH, Fan JS, Li RJ, Wen QS, Qian WB, Liu D, Chen XC, Xie JJ, Bai JX, Ying HJ. Selective separation of biobutanol from acetone–butanol–ethanol fermentation broth by means of sorption methodology based on a novel macroporous resin, *Biotechnol Prog* 2012, 28:962–972.
16. Lin XQ, Wu JL, Fan JS, Qian WB, Zhou XQ, Qian C, Jin XH, Wang LL, Bai JX, Ying HJ. Adsorption of butanol from aqueous solution onto a new type of macroporous adsorption resin: studies of adsorption isotherms and kinetics simulation. *J Chem Technol Biootechnol* 2012, 87:924–931.
17. Ooremus MG, Linden JC, Moreira RA. Agitation and pressure effects on acetone-butanol fermentation. *Biotechnol Bioeng* 1984, 27:852–860.
18. Collas F, Kuit W, Clément B, Marchal R, López-Conteras AM, Morot F. Simultaneous production of isopropanol, butanol, and 2,3-butanol by *Clostridium acetobutylicum* ATCC 824 engineered strains. *AMB Express* 2012, 245.
19. Tashiro Y, Takeda K, Kobayashi G, Sonomoto K. High production of acetone-butanol-ethanol via microcell culture by cell-recycling and bleeding. *J Biotechnol* 2005, 120:197–206.
20. Pierrut P, Fick M, Engasser JM. Continuous acetone butanol fermentation with high productivity by cell ultrafiltration and recycling. *Biotechnol Lett* 1988, 10:253–256.
21. Fond O, Engasser JM, Matta-El-Amouri G, Petitdemange H. The acetone butanol fermentation on glucose and xylose. II. Regulation and kinetics in fed-batch cultures. *Biotechnol Bioeng* 1986, 28:167–175.
22. Oudshoorn A, van der Welken LM, Straathof AJ. Adsorption equilibria of bio-based butanol solutions using zeolite. *Biochem Eng J* 2009, 48:59–103.
23. Peggini S, Gorra G, Delomez P, Soucaille P. Metabolic flexibility of *Clostridium acetobutylicum* in response to methyl viologen. *Appl Microbiol Biotechnol* 1994, 42:511–616.
24. Maddox IS, Steiner E, Hirsch S, Wessner S, Gutierrez NA, Gapes JR, Schuster KC: The cause of "Acid crash" and "Acidogenic fermentations" during the batch Acetone-Butanol-Ethanol (ABE) fermentation process. J Mol Microbiol Biotechnol 2000, 2:95–100.

25. Nielsen DR, Amarasiriwardena GS, Prather KLJ: Predicting the adsorption of second generation biofuels by polymeric resins with applications for in situ product recovery (ISPR). Bioresour Technol 2010, 101:2762–2769.

26. Yang X, Tiao GT: Enhanced acetone-butanol fermentation using repeated fed-batch operation coupled with cell recycle by membrane and simultaneous removal of inhibitory products by adsorption. Biotechnol Bioeng 1995, 47:444–450.

27. Groot WJ, Luyben K: In situ product recovery by adsorption in the butanol/isopropanol batch fermentation. Appl Microbiol Biotechnol 1986, 25:29–31.

28. Ennis BM, Qureshi N, Maddox IS: In-line toxic product removal during solvent production by continuous fermentation using immobilized Clostridium acetobutylicum. Enzyme Microb Technol 1987, 9:672–675.

29. Nielsen L, Larsson M, Holst G, Mattiasson B: Adsorbents for extractive bioconversion applied to the acetone butanol fermentation. Appl Microbiol Biotechnol 1988, 28:335–339.

30. Xiao Z, Lv C, Gao C, Qin J, Ma C, Liu Z, Liu P, Li L, Xu P: A novel whole-cell biocatalyst with NAD + regeneration for production of chiral chemicals. PLoS One 2010, 5:e8860.

31. Siemerink MA, Kuit W, López-Contreras AM, Eggink G, van der Oost J, Kengen SW: D-2,3-Butanediol production due to heterologous expression of an acetoin reductase in Clostridium acetobutylicum. Appl Environ Microbiol 2011, 77:2582–2588.

32. Fradrich C, March A, Fiege K, Hartmann A, Jahn D, Hartig E: The transcription factor AlsR binds and regulates the promoter of the alsSD operon responsible for acetoin formation in Bacillus subtilis. J Bacteriol 2012, 194:1100–1112.

33. Kuit W, Minton NP, López-Contreras AM, Eggink G: Disruption of the acetate kinase (ack) gene of Clostridium acetobutylicum results in delayed acetate production. Appl Microbiol Biotechnol 2012, 94:729–741.

34. Lehmann D, Hönicke D, Ehrenreich A, Schmidt M, Weuster-Botz D, Bahl H, Lütke-Eversloh T: Modifying the product pattern of Clostridium acetobutylicum. Appl Microbiol Biotechnol 2012, 94:743–754.

35. Baer SH, Blaschek HP, Smith TL: Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant Clostridium acetobutylicum. Appl Environ Microbiol 1987, 53:2854–2861.

36. Tunuli MS, Fendler JHJ: Aspects of artificial photosynthesis. photosensitized electron transfer across bilayers, charge separation, and hydrogen production in anionic surfactant vesicles. Am Chem Soc 1981, 103:2507–2513.

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