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Chapter 3

Extractive Fermentation Employing Ion-Exchange Resin to Enhance Cell Growth and Production of Metabolites Subject to Product or By-Product Inhibition

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76879

Abstract

In recent years, commercial production of proteins and metabolites from microbial fermentation for industrial applications has increased significantly. Innovative approaches are directed towards the improvement of the conventional batch fermentation method and the segregated downstream processing of target product to improve the overall process efficiency and to ensure that the process is economically viable. Feedback inhibition is a common problem faced during fermentation process when the concentration of end-product/by-product reaches a certain level. The excessive accumulation of end-product/by-product in the culture may inhibit the growth of cell and represses the secretion of target metabolite. In the production of many fermentative products such as antibiotics, amino acids, and fungal metabolites, a serious problem of feedback inhibition is often encountered. Cultivation of lactic acid bacteria and recombinant bacteria is usually subjected to by-product inhibition. Hence, extractive fermentation via *in situ* ion-exchange-based adsorptive technique is a possible approach to be used industrially to mitigate feedback inhibition, aimed at enhancing fermentation performance. In this chapter, advances in this area were presented. Strategies to overcome problem related to product/by-product inhibitions by this technique via dispersed, external, and internal resin system, and the general methodology in the implementation of the technique were also discussed.

Keywords: extractive fermentation, ion-exchange resin, product/by-product inhibition, feedback inhibition, *in situ* extraction

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1. Introduction

Fermentation generally is a metabolic process, in which a microorganism (e.g. yeast or bacteria) converts a substrate (e.g. starch or sugar) to microbial metabolic products, whether in a shake flask or bioreactor system. These metabolic products can be divided into: (1) primary metabolites, that is, compounds that are produced during the logarithmic phase of microbial growth, and they are essential for growth (e.g. alcohol, lactic acid, and amino acids) and (2) secondary metabolites, that is, compounds that are produced during the stationary phase of microbial growth, and they generally do not have any function in growth except maybe for survival function (e.g. antibiotics, anticancer agents, pigments, and organic acids).

Under normal circumstances, in the context of industrial biotechnology, there is usually only one target product in fermentation; therefore, this means that the rest of the simultaneously produced metabolites (as highlighted above) are instead considered as by-products. For example, during the fermentation of recombinant *Escherichia coli* to produce a targeted product periplasmic interferon alpha-2b, the acetate ion (which is also commercially valuable) produced is considered to be the by-product of the fermentation [1]. The primary concern on the accumulation of products/by-products in culture medium is the possibility of inhibiting cell growth and subsequently suppressing the production of targeted product.

The examples of products/by-products that exert feedback inhibition and their general mechanism of inhibition are:

i. Alcohols

Feedback inhibition is best exemplified by acetone-butanol-ethanol (ABE) fermentation, where butanol concentration as low as 1% w/w severely inhibit cell growth, resulting in low titers [2]. Alcohol like ethanol does not cause protons to leak through membrane; however, it does cause structural and function alterations of cellular membrane. The rate of substrate utilisation in alcohol-treated cells is lower relative to control. This directly points to the theory that the inhibition is a result of irreversible denaturation of membrane-associated glycolytic enzymes [3].

ii. Organic acids

In most of the *in situ* organic acid removal studies reported so far, the organic acids can be either the desired product or by-product of fermentation for producing recombinant protein or enzyme. The accumulation of organic acids in cultivation medium generally has an adverse effect on productivity [3]. Organic acids act as uncouplers that allow proton (H^+) to enter cell from medium and counteract the action of proton pump [4]. This results in the interference in the difference between internal and external pH (ΔpH). For every molecule of acid entering the cell, one H^+ is internalised, as the species permeating to the inside is neutral acid (HA) and the one leaving the cell is anion (HA_{2^-}). The attempt of cell to restore this imbalance by expending more energy (in the form of ATP) at membrane level rather than for biosynthesis thus could explain the mechanism behind the inhibition by organic acids.

iii. Antibiotics

Antibiotic is a type of secondary metabolite that can also be lethal towards its own producer strain [4]. In comparison with other types of inhibitors mentioned above, which are multi-target inhibitors, antibiotic usually interacts specifically towards cell, owing to the unique inhibitor-receptor structural complementation.

iv. Others

Besides the above-mentioned inhibitors, the accumulation of some metabolites such as pigment [5], anticancer agent [6] and so on, up to a certain concentration threshold, has also been reportedly suppressed their own production. Nevertheless, their mechanisms of inhibition are still not very clear.

In view of this, in order to improve fermentation performance (yield, productivity, and final concentration of biomass or secondary metabolites), beside the requirement to optimise fermentation conditions (e.g. inoculum density, pH, temperature, aeration and mixing, and concentration of nutrients) and to alleviate substrate inhibition effect (if any)—where process control via fed-batch operation and change of substrate type can be implemented, one must also address the problem of product/by-products inhibition.

So far, various strategies have been used to address the problem. Genetic engineering is one of the approaches used to overcome the feedback inhibition. The primary aim of this technique is to develop a product/by-product tolerant strain, which may be achieved, specifically, via the modification of the product receptor on the cell membrane, overexpression of protein, deletion of metabolic pathway, and so on [4, 7]. Besides, extractive fermentation (interchangeably known as *in situ* removal/recovery), which basically is an integration of fermentation with product/by-product removal, has been extensively studied in order to mitigate the problem of feedback inhibition, to improve the productivity of bioprocesses by converting the conventional multi-step methods to one-pot process and to address product decomposition after its formation [8]. **Table 1** lists some of the techniques (which have been categorised based on the basis of product extraction) that have been employed previously in extractive fermentation. The selection of the techniques is basically depending on the nature of the target product/by-product. In addition, Hecke et al. designed a heuristics for selecting the appropriate technique based on the properties of target compound (inhibitory, stability, volatility, and charge) [7] (**Figure 1**). For volatile products such as the fermentatively produced acetone-butanol-ethanol, techniques such as gas stripping, distillation, and membrane-based technology, pervaporation have been proposed [2]. For organic acids, separation based on adsorption, electrodialysis, and solvent extraction has been widely reported [3]. Moreover, the overall developments in these extractive fermentation techniques have also been extensively reviewed earlier [2, 7, 9, 10].

On a separate note, ion-exchanger is an inert support medium that is covalently coupled to positive/negatively charged functional groups, of which oppositely charged ions are bounded. The counter ions will be exchanged with like-charge ions in a sample (provided that their charge magnitude is larger than the one possessed by the bounded-ions). Extractive fermentation can be performed either by adding an ion-exchange resin into a fermentation broth or by passing the broth through an adsorbent-filled column, where inhibiting product/by-product

No.	Basis of *in situ* extraction	Specific techniques	Examples of product
1	Target product immobilisation	Adsorption onto polymeric matrices such as ion-exchangers, activated carbon, zeolites, and cellulose	Ethanol, salicyclic acid, cycloheximide, anthraquinone, alkaloids, monoterpenes, tissue plasminogen activator
2	Extraction into a second liquid phase	Solvent extraction, aqueous two-phase system, and supercritical fluid	Alcohol, organic acids
3	Size selective permeation	Membrane-based techniques	Microbial, mammalian, and hybridoma cells
4	Evaporation	Vacuum fermentation, gas stripping, and evaporation	Ethanol, butanol
5	Change in solubility of target product	**i.** Formation of complex via the addition of soluble reagent	Acetaldehyde
		ii. Crystallisation of target product Schiff's base formation	Carboxylic acids, antibiotics, amino acids, steroids

Table 1. Categorisation of *in situ* extraction techniques made by freeman et al. but with slight modification [11].

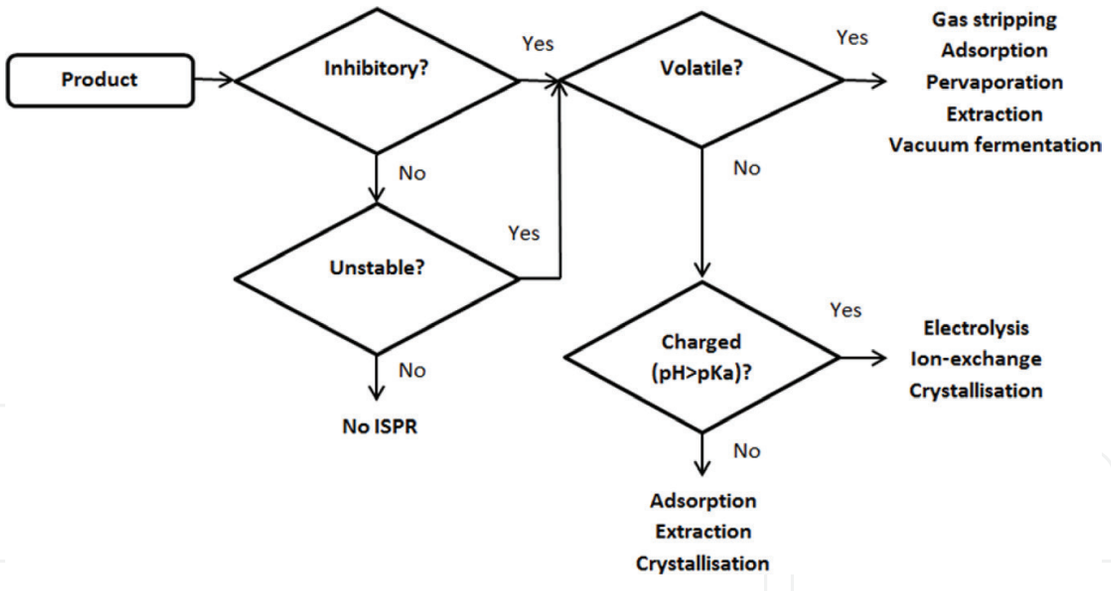


Figure 1. Heuristics for selecting suitable *in situ* product removal technique based on the nature of target product [7].

will be captured by the resin, thereby maintaining the optimal culture condition for cell growth or production of metabolites (**Figure 2**). The advantages of using ion-exchange-based separation have been extensively elaborated previously [6]. The sequestration of product/by-product helps to mitigate the feedback inhibition, improving fermentation performance. Besides, for fermentation products that are prone to degradation (e.g. fragrances and flavours), their stability in the culture medium can also be improved. In comparison with other organic solvent-based extraction, ion-exchange-based techniques may also increase the stability of product by reducing the length and scale of exposure of such products to organic solvent.

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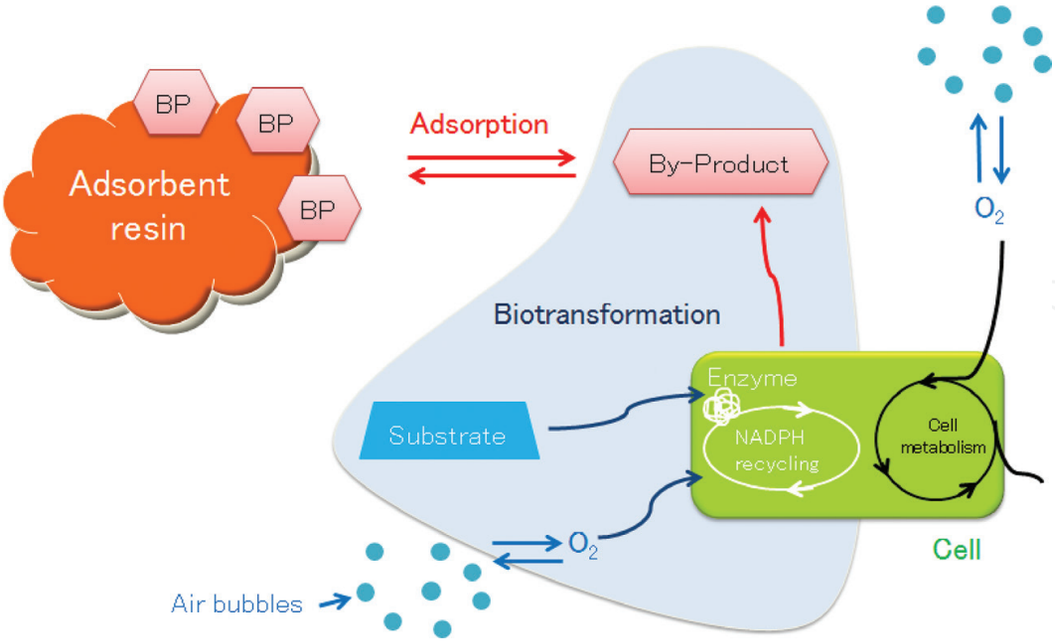


Figure 2. Schematic diagram representing the concept of extractive fermentation via *in situ* removal of by-products (BP) to enhance cell growth and production of metabolites.

The use of resin also offers an economic benefit as it is reusable. Additionally, its usage is also environmentally friendly since only a minimum consumption of organic solvent is required during preparation and elution stages. Moreover, this technique is also relatively simple to operate as resin separation following a fermentation process is relatively easy and therefore will not require the use of any high-end instrument.

In this chapter, the reports on the application of ion-exchange resin in addressing the problem of feedback inhibition from product/by-product in microbial fermentation, in different configurations: dispersed, internal, and external system are reviewed. In addition, the general methodology in developing an extractive fermentation based on the use of an ion-exchange resin is discussed. Finally, a perspective on the application of this technique is also presented.

2. Applications of ion-exchange adsorption resins in extractive fermentation

The adsorption of target product/by-product by ion-exchange resin in an extractive fermentation can be carried out either within a bioreactor (internal system) or by circulating a fermentation broth through an external column that is packed with the adsorbent (external system) (**Figure 3**). Additionally, in the former system, the resins can either be trapped in a compartment housed inside the bioreactor or dispersed freely in the culture.

2.1. Dispersed resins

The feasibility of improving the growth of attenuated gdhA derivative *Pasteurella multocida* B:2 (which is to be used as bacterial vaccine for animal) by removing the by-product

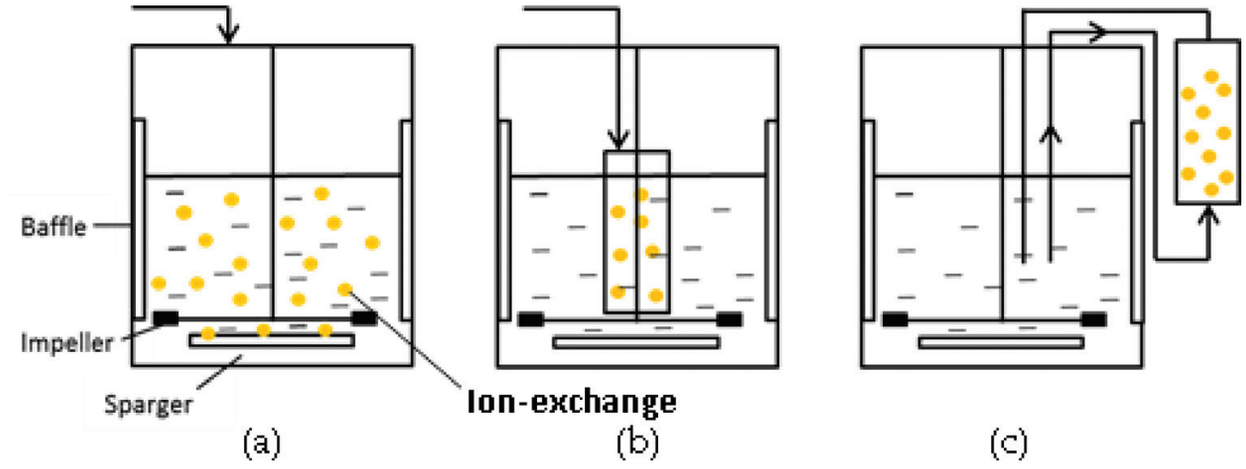


Figure 3. Schematic diagram of configuration of ion-exchange resin systems in extractive fermentation: (a) dispersed resins in culture, (b) trapped resins in internal column and (c) external column.

ammonium (NH_{4^+}) using cation-exchange resins was previously investigated [12]. The accumulation of NH_{4^+} inside cells generally results in intracellular pH change, and subsequently interrupts the activity of cytosolic enzymes [13]. Three types of resins: the weak acid cation-exchanger - Amberlite IRC86, the strong acid cation-exchangers - Amberlite IR120 H, and Dowex DRG8 H were examined. During a sorption isotherm experiment, it was determined that the highest maximum binding capacity, q_{max} (0.863 g/g) and the lowest dissociation constant, K_d (0.015 g/L) were obtained with Amberlite IRC86. Although the other two strong cation-exchangers showed high ammonium adsorption compared to IRC86, but lower cell viability was observed in their systems, which might be due to the co-removal of cation nutrients in the culture media. Additionally, when the amount of ammonium was increased from 300 to 1500 g/L, the desorption efficiency of ammonium from the resins (using 2.5 M HCl), reduced by 40 to 50%. In a shake flask culture (100 mL), the cell viability drastically reduced (5.5×10^9 times) when the concentration of IRC86 was increased from 0 to 30 g/L. The optimum resin concentration was determined to be 10 g/L, where maximum NH_4 concentration of 536.40 ± 10.85 mg/L (41% removal) and cell viability of 7.2×10^{10} (equivalent to 13-fold improvement compared to resin-free system) were obtained.

A reduction in cell yield is usually inherent in the accumulation of organic acid such as lactic acid, acetic acid, and succinic acid. Acidification of medium, followed by cytoplasm (as lactic acid diffuses into the cell), results in failure of proton motive forces, which ultimately damaging the cells [14]. However, this acidification problem may not simply be overcome by a pH control strategy, that is, addition of NaOH. It was highlighted earlier that the osmotic pressure of medium escalated due to the additional ions from NaOH, resulting in reduced cell growth [15, 16]. Therefore, Othman et al. combined the strategy of using fed-batch mode operation with an extractive fermentation of probiotic *Pediococcus acidilactici* using anion-exchange resin, Amberlite IRA 67 [16]. About 55.5 times increment in maximum viable cell concentration was achieved in the fed-batch extractive fermentation system compared to a batch mode. The maximum viable cell concentration, yield, and productivity achieved in the ion-exchange resin-added fed-batch fermentation, was 9.1, 8.5, and 8.6 times higher compared

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to a control system (without resin), respectively. Lactic acid accumulated in the former system was also lower (8.78 g/L) than system without resin (9.62 g/L). The fed-batch operation improved the fermentation performance by: (1) overcoming the substrate inhibition effect by maintaining the glucose concentration in the culture at below the inhibition level and thereby directing the glucose metabolic flux towards cell growth rather than lactic acid production and (2) enhanced the fermentation performance by removing the inhibitory by-product (i.e. lactic acid) following the *in situ* addition of ion-exchange resin.

Furthermore, an accumulation of acetate in fermentation medium also causes retardation of cell growth (due to disruption in transcription-translation machinery, stress response, and regulation in cell), and subsequently, affecting the productivity of desired metabolites or the expression of recombinant proteins (in the case of recombinant strain). *In situ* adsorption of acetate by anion-exchange resins in *E. coli* culture for producing periplasmic human interferon-alpha2b (PrIFN-α2b) was previously studied in shake flask (250 mL) and bioreactor (2 L) systems [1]. Selection of anion-exchange resin was made out of two strong base anion resins (Amberlite IRA-900 and Dowex Marathon MSA) and three weak base anion-exchange resins (Amberlite IRA-96, Diaion WA30, and Dowex M43). The selection was made based on the affinity between the resins and acetate that was determined from a sorption isotherm experiment (the affinity is indicated by the value of dissociation constant, K_d, where the lower the value, the higher the affinity). In comparison, the use of weak anion-exchange resin gave higher cell growth and PrIFN-α2b expression than the strong base anion-exchange resins. Optimisation of ion-exchange resin load for all of the resins tested was also carried out. Interestingly, for all of the resins, the cell growth and PrIFN-α2b expression were found to decrease when their concentrations were increased from 20 to 80 g/L due to higher accumulation of acetate in the batch culture.

The co-adsorption of anion nutrients such as Cl^-, SO_4^{2-} and PO_4^{2-} might be the cause of the suppressed growth and PrIFN-α2b expression. WA30 was selected and used for further experiment, as the highest cell and PrIFN-α2b concentrations were obtained, that is, 8.65 ± 0.13 g/L and 566.5 ± 4.36 μg/L, respectively. Additionally, the lowest concentration of acetic acid accumulation was also recorded (i.e. 3.49 ± 0.26 g/L) in the WA30 culture. Based on optimisation using response surface methodology (RSM), an optimum loading of 12.2 g/L was determined, where the maximum yield of 501.8 μg/L was achieved, in accordance with the predicted yield (i.e. 507 μg/L).

The effect of ion-exchange resin addition on the physiology of the cells was also investigated. Based on scanning electron microscope (SEM) analysis, the morphology of the cell grown in resin-added medium was found to be identical to the one from the control system, which is a long rod shape. However, as visualised via transmission electron microscope (TEM), a higher amount of inclusion bodies aggregates was detected in the cells grown in high resin-load culture system (i.e. 80 g/L) compared to one from low resin-load system (i.e. 20 g/L). This finding further explained the lower cell growth and PrIFN-α2b expression in the high-resin load system. Finally, it was also found that the production of PrIFN-α2b by *E. coli* in stirred tank bioreactor (STB) with *in situ* addition of WA30 resin was enhanced by 1.8-fold (578.8 μg/L) compared to system without resin (318.4 μg/L). A reduction in acetate concentration (28%) was obtained with the addition of resin after 26 h of fermentation (**Figure 4**).

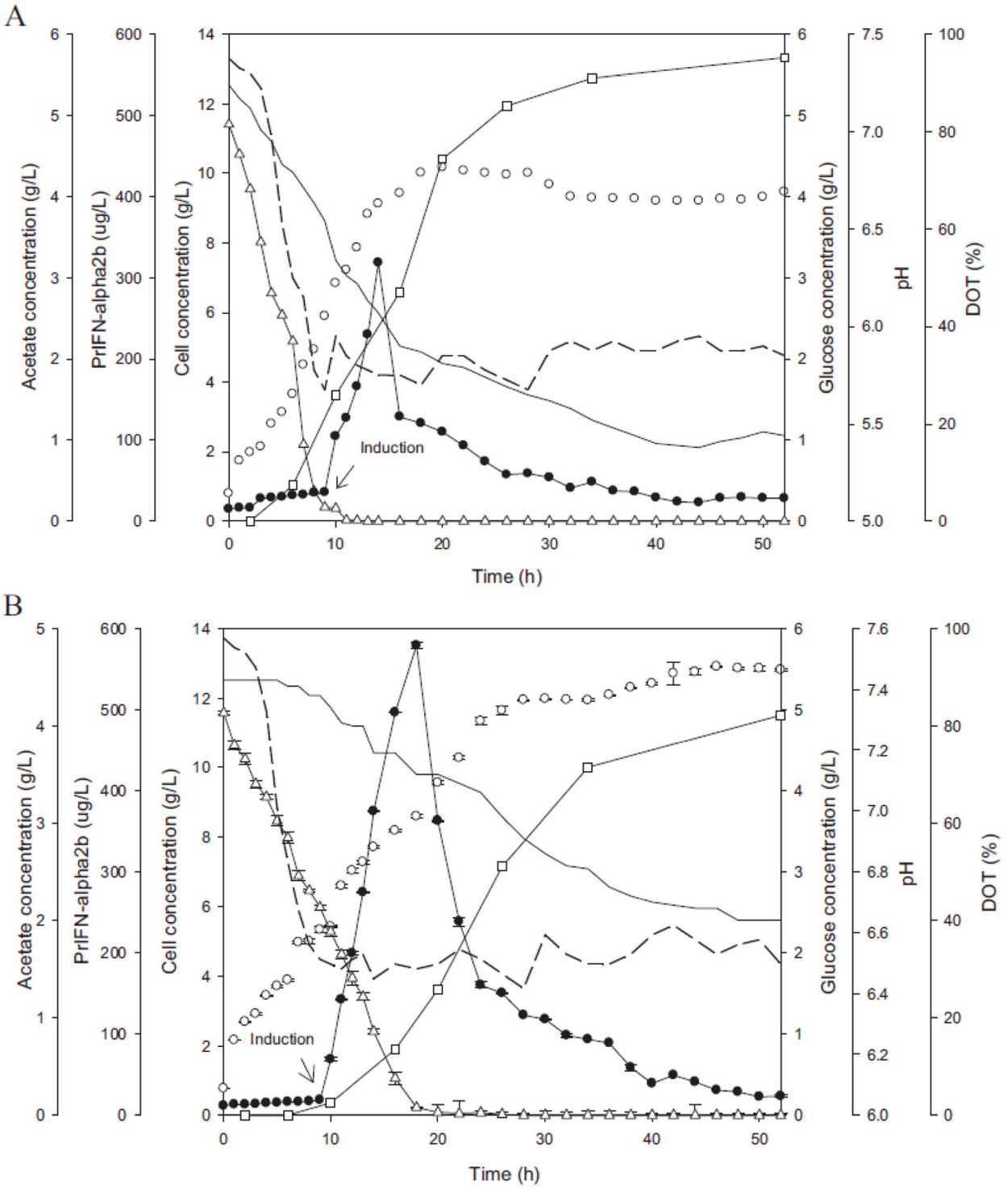


Figure 4. The time course of recombinant *E. coli* fermentation for the production of PrIFN-α2b in 2 L STB: (a) control system (resin-free), (b) with *in situ* addition of resin [1]. Symbols: (●) PrIFN-α2b concentration, (○) cell concentration, (□) acetate concentration, (Δ) glucose concentration, and (--) dissolved oxygen tension.

Another application of ion-exchange resin for removing acetic acid in a culture was reported by Chen et al. [17]. Acetic acid accumulation was also found to have a critical influence on the production of human epidermal growth factor (hEGF) by recombinant *Escherichia coli* JM101.

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Growth of *E. coli* was not occurred at 20 g/L of acetic acid concentration. The weak basic IER A-D3-1 macroporous resin was added to a shake flask culture and a batch culture in a 2.5 L bioreactor. In fermentation using bioreactor, where 33 g/L of resin was used, a 10% increment in expression level was obtained, relative to resin-free system. The resin was found to be non-toxic to the cell, albeit a minimum decrease in growth and production of hEGF was observed. This phenomenon was attributed to the co-removal of nutrient components such as CH_3COO^-, PO_4^{3-}, SO_4^{2-}, OH^-, Cl^- and other charged amino acids, as a result of their non-specific complexation with the resin.

In the production of the self-toxic antifungal agent, cercosporamide by fungal culture LV-2841, it was found that no significant improvement in yield was obtained following extensive optimisation on medium (types and concentrations of carbon (C) and nitrogen (N) sources) and fermentation conditions (aeration and temperature). Therefore, an extractive fermentation via *in situ* addition of ion-exchange resin to adsorb the product cercosporamide was done. The study was carried out in various fermentation scales, ranging from 100 μL to 10 L. Over 100-fold increase in production titre was achieved when the potato dextrose broth was supplemented with 1–3% w/v wet Diaion® HP20 resin or AmberliteTM XAD-7.

Moreover, the problem of low titre (less than 10 mg/L) was encountered in the fermentation of *Burkholderia thailandensis* E264 for producing thailandepsin A, a natural anticancer analog [6]. Low titre can deter further preclinical/clinical development of the natural drug. Thus, *in situ* addition of a polyaromatic adsorbent resin, Diaion HP-20 to the culture, coupled to a systematic optimisation of fermentation conditions was carried out in order to increase the product titre. Optimisation of Diaion HP-20 concentration was initially done by single factor experiments. The addition of resin at 4% (w/v) (after 12 h of fermentation) resulted in 91% increment in thailandepsin A production. Nevertheless, reduced production of the thailandepsin A was observed with further increase in resin concentration. It was presumed that at high resin loads, bacteria's nutrients adsorption was affected due to the resin attachment to the bacteria and/or nutrient. Additionally, there might also be larger shear forces as a result of limited space. Furthermore, at higher dissolved oxygen concentrations, which were resulted by the addition of the resin, the cell growth might as well have been inhibited.

Previously, an attempt to increase the production of teicoplanin, an antibiotic produced by *Actinoplanes teicomyceticus* in a 5 L STB using ion-exchange-based extractive fermentation has been reported [18]. In a preliminary experiment, the threshold level for the cell growth and production inhibition was determined to be from 10 to 25 mg/L. In selecting the suitable resin for subsequent experiment, resins/adsorbents: Diaion HP-20 (adsorption by ionic properties), Amberlite XAD-16 (adsorption by hydrophobicity), charcoal (adsorption by polar properties), and silica gel (adsorption by decolourisation of teicoplanin) were added to the fermentation broth at a concentration of 5% (v/v) at the inoculation stage and incubated for 120 h. The desorption step was performed using 80% methanol. Among all of the resins tested, Diaion HP-20 exhibited the highest adsorption and desorption performances followed by XAD-16. Although a high adsorption was achieved by charcoal, no recovery was achieved during desorption, indicating a strong adsorption between the teicoplanin and adsorbent. Since Diaion HP-20 is an ion-exchanger and a polyaromatic resin polymerised with polystyrene

and divinylbenzene, the capture of the teicoplanin by the resin was therefore based on ionic and hydrophobic interactions. Under a resin concentration of 5% w/v, which was supplemented to the culture at inoculation stage, a teicoplanin concentration of 134 mg/L (about 4.2-fold increment) was achieved after 120 h of fermentation.

Beside inhibitory problem, enzymatic degradation of product also commonly occurs during fermentation. One example of such product is pristinamycin, an antibiotic produced by *Streptomyces pristinaespiralis* [19]. The feasibility of increasing the productivity of pristinamycin production by *in situ* addition of resins to the culture in Erlenmeyer flask and 3 L STB has been examined. The types of resins that were being investigated include HP-20, XAD-16, JD-1, and HZ-817. Based on the outcome of batch binding experiment, JD-1 was selected to be used for the subsequent study, as high adsorption capacity (25.7 ± 1 mg/g) and desorption ratio (84.2 ± 1%) were achieved. In Erlenmeyer flask cultivation, a reduction in dry cell weight (from 16 to 13 mg/mL) was observed, when the resin load was increased from 0 to 16% w/v. The pristinamycin production was also increased by 1.83-fold when JD-1 was added at the 20 h of fermentation compared to addition at just after inoculation, because the onset of pristinamycin synthesis occurred on the 20 h. When the ion-exchange system was adapted to a 3 L bioreactor, the pristinamycin synthesis reached 0.8 g/L, which was about 1.25-fold increase over a resin-free cultivation.

2.2. Internal adsorption column

An internal adsorption column configuration (in a bioreactor) afforded the advantage of avoiding the influence of shear force from impeller that could have a detrimental effect on the resin. Although the same benefit can be attained from the use of an external column system, additional advantages can also be found with this design over the external variant, where better environmental conditions, such as pH, temperature, dissolved oxygen tension (DOT), aeration rate, and mass transfer rate can be achieved [20]. Next, product recovery can be done within one bioreactor as the fermentation (*in situ* product recovery), and this improves overall productivity, as well as providing a simpler operation. Bae et al. reported on the development of a novel internal column system to recover prodigiosin-like pigment produced from the fermentation broth of *Serratia* sp. KH-95 [20]. The bioreactor was designed with a second compartment made by internal 316 stainless steel filter and perforated support for a HP-20 adsorbent. **Table 2** summarises the performance of the system relative to external column, internal-dispersed, and without resin (control) systems. Notably, this novel system afforded 1.8-fold higher in pigment productivity than that obtained in the external column-bioreactor system.

The phenomenon of cell adsorption on the resin was reportedly encountered in dispersed and external resin systems, as the pigments were still attached to the cell wall. It was also highlighted that the conditions (e.g. pH, DOT, substrate concentration, etc.) in the external column and internal bioreactor medium were possibly varied. Thus, this might also resulted in the lower performance in the external resin system since the cell is recycled throughout the whole system.

2.3. External adsorption column

The main drawback inherent to dispersed resin system is resin attrition caused by the shear force of impeller(s) and the clogging of the valves and shaft seals in bioreactor [20]. This

No	System	Recovered product concentration (g/L)	Maximum cell concentration (g/L)	Pigment productivity (g/L/h)
1	Internal column	13.1	<1.0 (6)	0.44
2	External column	7.2	3.0 (15)	0.24
3	Dispersed adsorbent	9.8	<0.5	0.33
4	Resin-free system (control)	5.2	NA	NA

All adsorbent systems were carried out in 2.5 L bioreactor (1 L working volume), while the volume of the resin used was 100 mL. Acidified methanol was used to desorb the resin. The number in the bracket indicates the time taken to reach the maximum cell concentration (in hour). NA: data not available.

Table 2. Comparative evaluations of different resin application configurations: Internal, external, and dispersed, relative to resin-free system (control) in the fermentation *Serratia* sp. KH-95 to produce prodigiosin-like pigment [9].

effect can be pronounced in an aerobic fermentation, where an agitation is usually required. Thus, an external adsorption system is proposed in order to overcome any possible effect of shear force on cells from the collision of resins. Generally, in the concept of external resin extractive fermentation, a separation unit (resin-loaded column) is connected to a bioreactor externally, and a fermentation broth is passed through the column for capturing of product/by-product, subsequently, the broth is returned to the bioreactor for reuse. Furthermore, the broth reuse also afforded further advantages, that is, higher conversion of nutrient/substrate and the reduction in input water requirement [21].

2.3.1. Integrated bioreactor-packed bed column

In situ citric acid recovery in *A. niger* W1-2 fermentation by anion-exchange resin was previously developed [21]. The resin-loaded packed-bed column (4.0 × 50.0 cm) was connected to a 1 L bioreactor (0.7 L working volume) system. The broth was passed through the column only after 3 days of fermentation, at a frequency of once a day and a flow rate of 5 mL/min. The resin was found to be not attracted towards nutrients (i.e. residual sugar and NH_4Cl) except for KH_2PO_4. In comparison with a conventional fermentation, reduction in fermentation time (6 days), increment in productivity (1.6-fold) and sugar conversion (12.6%) were achieved by the integrated system.

A study to overcome end-product inhibition during the fermentation of *Serratia* sp. KH-95 for producing prodigiosin-like red pigment using ion-exchange resin was previously carried out [5]. Purified pigment was added to a shake-flask culture of *Serratia* to confirm the effect of product inhibition. Selection of resin (among HP-20, SP-850, and XAD-16) was made based on its adsorption performance in fixed bed column mode, where the pigment-containing culture was loaded from the top of the column. Although SP-850 showed the highest adsorption capacity (about 11.8% and 38.1% higher than HP-20 and XAD-16, respectively), it exhibited the lowest desorption ability (about 15% and 20% lower than HP-20 and XAD-16, respectively). Thus, HP-20 was selected and used for subsequent studies. The effect of resin addition time (0–15 h after inoculation) on the fermentation performance was also investigated in shake flask fermentation. Overall, it was found that cell growth was suppressed once the resin was added. Notably, the cell growth only reached 75% of the control (resin-free system) when

the resin was added during inoculation. However, the growth steadily increased to 95% at 10 h of resin addition time. This phenomenon was attributed to the reduction in casein content in the culture (about 23.5–47.1% reduction relative to control) due to undesired adsorption to the added resin that resulted in amino acids depletion in the medium. On the other hand, the production of pigment increased correspondingly with the increase of addition time. The maximum concentration of pigment (5.85 g/L) was obtained at 10 h of addition time (40% increment relative to control).

Furthermore, when resin amount was increased from 5 to 20% (v/v), cell growth and casein concentration were found to reduce. Nevertheless, 10% (v/v) was chosen as the optimum load, as the maximum production of pigment was obtained. Finally, the extractive fermentation was adapted to a bioreactor scale (5 L), which was connected to a packed-bed column. Similar to shake flask culture, the highest production was also achieved with 10% v/v resin load. Overall, 31% increment was afforded in the integrated system compared to a resin-free bioreactor system.

2.3.2. Integrated bioreactor-expanded bed adsorption

Tan et al. designed an integrated STB-EBA system for removing acetate to enhance the production of PrIFN-α2b by *E. coli* [22]. In comparison with a packed-bed column, EBA system allows for direct handling of biomass-laden fermentation broth without the need for any intermediate clarification method. Due to the bed expansion characteristic of EBA, where a large void volume is created, cell/particulate will pass through the column, while the target compound will be captured by the adsorbent in the column. The system was set-up based on a 2 L STB (1 L working volume), that was coupled to an UpFront FastLine 10 column (UpFront Chromatography A/S, Copenhagen, Denmark) (i.d 10 mm) packed with a WA30 anion-exchange resin (**Figure 5**). The effects of EBA parameters: amount of resin loaded (0–12 g/L) (and hence, the sedimented bed height, H_0 from 0 to 150 mm), column superficial linear velocities (from 240 to 900 cm/h), and culture viscosity (3.2–113.9 mPa.s) on the fermentation performance were first studied. It was found that at 150 mm sedimented bed height (12 g/L resin concentration), the highest dry cell weight (14.97 g/L) and total acetate adsorption (5.45 g/L) were attained. Further increment in sedimented bed height was not possible as the resin was expanded to the top of the column and overflowed. In addition, the optimum superficial linear velocity was determined to be 900 cm/h (degree of expansion of 2), where only a small circular movement of particles but not dispersed was observed. The resin was found to be packed tightly against the upper adaptor net following a bed expansion, when the viscosity was high (113.9 mPa.s), resulting in blockage of column. However, the viscosity of the fermentation broth was determined to be well below that point (15.75 mPas), and hence no problem was posed.

Meanwhile, for a dispersed resin system, the mixing time was found to increase at low agitation speeds (100–200 rpm). In addition, at high agitation speeds (>400 rpm), the mixing time in the integrated STB/EBA system was not significantly different compared to without resin (control) and with dispersed resin system. The performances of three systems: resin-free, dispersed system and STB-EBA integrated system in the fermentation of the PrIFN-α2b-producing

http://dx.doi.org/10.5772/intechopen.76879

recombinant *E. coli* were summarised in **Table 3**. In comparison to without resin and dispersed systems, STB-EBA system exhibited the highest PrIFN-α2b concentration, specific yield and volumetric productivity, shorter growth cycle and time to achieve the maximum PrIFN-α2b concentration. Besides, the simplified clarification of the culture broth (afforded by the use of EBA) reduced the overall length of downstream processing. Notably, with the integrated system, the PrIFN-α2b production was improved by 3-fold and 1.4-fold over that achieved in the resin-free and dispersed system, respectively.

Other reports on the implementation of such STB-EBA set-up can also be found in the literature, albeit with the use of different types of resin [23–26]. Apart from bioreactor, the external unit EBA column has also been incorporated with extraction tank for recovering active compounds from herbs [27, 28].

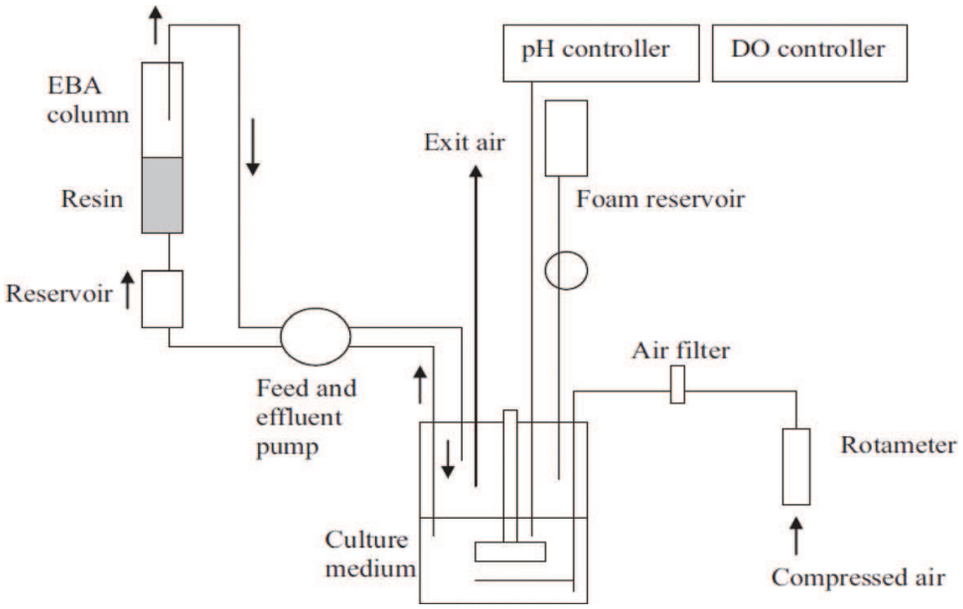


Figure 5. The STB-expanded bed adsorption column design for efficient removal of acetate during the production of PrIFN-α2b by *E. coli* [22].

System.	Maximum cell concentration (g/L)	Time to achieve maximum cell concentration (h)	Maximum PrIFN-α2b concentration (μg/L)	Time to reach maximum PrIFN-α2b concentration (h)	Time to reach threshold level of acetate concentration (≈4.0 g/L)
Resin-free (control)	9.57	16	289.5	12	>13
Dispersed system	12.76	12	638.8	12	>13
Integrated STB-EBA	14.97	24	867.4	11	>21

Table 3. Comparative fermentation performance of the three systems: Resin-free, dispersed system and STB-EBA integrated system in the production of PrIFN-α2b by *E. coli* [22].

3. General methodology in the development of ion-exchange resin-based extractive fermentation

Based on the earlier studies on the extractive fermentation using ion-exchange resin, the general methodology in the development of this technique can be systematically outlined as below:

1. Execution of batch fermentation of a particular culture under a predetermined optimum conditions

Time course fermentation profile of the specific strain will need to be constructed, where the concentrations of cell, substrate concentration, product, and by-product throughout the fermentation are included. The critical concentration of product/by-product, where the cell and product inhibition starts to occur can be identified based on the turning points of the curves.

2. Investigation on the effects of by-products/products on the growth of cell and metabolite production

In the preliminary study for determining inhibitory effect, a pure by-product/product is added to a fermentation culture that is operated in a batch mode operation. The concentration of the by-product/product is varied (which generally covers the range of the real-world concentration as determined earlier) and its effects on the cell growth and metabolite production are monitored. Determination of the threshold level, that is, the critical concentration, where the repression on cell growth and production occur is then being done.

3. Selection of suitable ion-exchange resin for *in situ* adsorption of target product/by-product

The main criteria for selecting a suitable ion-exchange resin mainly depend on the adsorption capacity and selectivity of the resin towards the target product/by-product [29, 30]. Thus, preliminary information on the properties of target molecule such as charge and stable pH range should be gained prior to the addition of resin. The general rule of thumb is that the charge of the selected resin must be the opposite to the charge of the target product/by-product. For *in situ* removal of by-product in a culture that contains extracellular target product, when possible, the charge of the by-product and product must also be different to one and another to ensure selectivity of adsorption. In cases where the charge of by-product and extracellular target product is similar, a resin with higher specificity and affinity towards by-product must be selected. Generally, weak ion-exchangers display a pH-dependent function, while strong ion-exchangers function over a wide pH range. Weak anion-exchange resins are highly ionised only at pH below 7, while weak cation-exchangers start to lose their ionisation below pH 6. Although strong ion-exchangers are generally more powerful as they can cover a wider pH, but their selectivity may not be necessarily higher than weak ion-exchangers. Essentially, when selecting a resin, it is always helpful to refer to the manufacturer's instructions for the pH range of potential resins.

The selection of suitable resin should also be made based on the physical properties of the resin, that is, size and density. The particle size of resin also influences the performance of adsorption, especially when operating in a packed-bed column mode. Smaller size resin particle generally afforded higher resolution in separation (during the elution stage, where

packed-bed mode is always preferred to minimise the use of buffer) but will not tolerate high flow rate, which otherwise could results in high backpressure. Conversely, larger size resin particle permits high flow rate but the resolution is relatively lower than the small size resin. Furthermore, in a special system like EBA system, the density of resin also has a significant effect on the performance of adsorption, as it influences the stability of bed expansion. In EBA operation, a larger diameter resin is also preferred as it enables the use of large pore column adapter screen. It is worth to note that the optimal choice of adsorption bead size and density, together with column hardware and operating conditions, ensures maintenance of bed expansion without loss of adsorbent in the column effluent [31].

For integrated packed-bed column-bioreactor system, the *in situ* recovery of product/by-product basically is not a straightforward process and can be rather challenging, considering the viscosity of cell-laden fermentation broth. Therefore, in this system, the selection of a suitable resin size is even more crucial. A bigger resin is generally more tolerable towards a highly viscous fermentation broth in mitigating pressure build-up.

With that, irrespective of any resin application (dispersed, external, or internal systems), initial screening of resin for the adsorption of a specific product/by-product is very crucial, which must be examined on a case-by-case basis, especially when the product and by-product are closely related in terms of charge properties.

Batch sorption experiment is usually being carried out at the first place to select a suitable ion-exchange resin. This is implemented by adding potential ion-exchange resin(s) (with varied concentration) to cell-laden fermentation broth (as cell could significantly influence the adsorption behaviour of adsorption). Experimental data (i.e. concentration at equilibrium and adsorption capacity) are then fitted to an adsorption isotherm such as Freundlich, Langmuir, Brunauer-Emmett-Teller (BET), etc. The Langmuir isotherm is the most commonly used model for describing adsorption isotherm, which describes a mono-layer adsorption with energetically identical sorption sites and without mutual interactions between the adsorbed molecules [32]. Two adsorption parameters, that is, maximum specific uptake capacity (q_m) and dissociated constant (K_d) can then be calculated using a graphical analysis, based on the selected adsorption isotherm model. The selected resin should generally yields high q_m (high binding capacity) and low value of K_d (exhibiting high affinity towards target molecule). In addition, selection should also be made with regard to the desorption efficiency of resin. Some resins might exhibit high binding capacity towards target product/by-product but somehow give low desorption efficiency.

Ideally, apart from the target product/by-product, the resin must show the minimum interaction with all the nutrients in the medium. Nevertheless, during a fermentation, minimum nutrient(s) removal might be tolerated provided that high degree of yield and productivity is achieved. Therefore, a preliminary experiment on *in situ* addition of resin into fermentation culture is very important. Analysis on the content of nutrient ions such as Cl^-, SO_4^{2-}, and PO_4^{2-} and so on in the cultivation medium during the preliminary fermentation (with added resin) may also be done to examine the selectivity of the resin. The information on the co-adsorption of nutrient(s) (if any) by the used resin is useful to provide a more accurate interpretation on the effects of resin on cell growth and metabolite production. Besides, the toxicity effect of the selected resin on cell growth can also be examined through the preliminary experiment. If these factors are found to significantly affecting the fermentation performance, re-selection of resin must be made.

In addition, examination on cell morphology during the fermentation following the addition of resin is also important. The probability of the unwanted cell-resin adsorption and shear effect, which could negatively affect the fermentation performance, can be scrutinised here.

4. Optimisation of adsorption parameters and operating variables

The optimum concentration of ion-exchange resin should be determined with regard to fermentation performance. The general information on the binding capacity of the potential resin (which usually can be obtained from the manufacturer's data), in conjunction with the estimated product/by-product concentration (as determined experimentally earlier in batch fermentation) will help in the rough estimation of the working concentration range. The typical trend observed is improvement in cell growth and metabolite production, when the resin concentration is raised; further, addition of resin will, however, result in suppression of cell growth and metabolite production.

Next, the time of resin addition (in optimum load as determined before), that is, during the inoculation stage or at later stage of fermentation also needs to be determined. Typically, resin addition is done during the inoculation stage for dispersed/internal system; while for external resin system, a resin-loaded column is directly connected to the bioreactor when fermentation is started. Additionally, for external resin system, optimisation of flow rate of medium through the resin column should be carried out. The ideal flow rate is the fastest flow rate that still afforded high adsorption capacity, similar to an ion-exchange chromatography operation. Effects of agitation speed on the culture, in the presence of resin in the culture or when the bioreactor is connected to external resin column, in terms of mixing, shear effect (more significant in dispersed resin system), and DOT may also need to be further optimised.

4. Conclusion

Extractive fermentation using ion-exchange resin for removal of product/by-product of fermentation is mainly employed to mitigate the problem of feedback inhibition. In addition, for *in situ* product removal, additional advantages of shortening downstream processing steps improve process economics and productivity, and minimising product lost and degradation can be achieved.

Research on the selectivity of adsorption or even desorption is very important especially for high-value biologics. However, so far, most of the earlier studies have not focused on the purity aspect of the desorbed products. In addition, it was highlighted in many earlier works that at high resin concentrations, reduction in fermentation performances (i.e. cell growth and metabolite production) was encountered. The main research question: 'what is/are the primary cause(s) of the performance decline - removal of like-charge nutrient component(s), shear effect on cell/product, or reduced mixing efficiency in the presence of high resin load?' however remains. Detailed study on the interaction of resin with medium components may be carried out to gain further insight on this.

In general, the development of new ion-exchange technologies is driven by the needs for having a higher capacity and selective adsorbent. The use of new resins, for example, resins

with alternate support matrices, that are able to operate at higher conductivities medium (dilution/diafiltration prior to adsorption will not be required), single-use/disposable resins (to overcome the hassles of cleaning-in-place (CIP), validation, and to improve overall process economic), and so on in the extractive fermentation technique should also be explored.

In addition, the feasibility of combining ion-exchange resin-based adsorptive techniques with other separation technique such as electric fields- and membrane-based technique may also be scrutinised. The use of immobilised cell to protect them from shear stress and/or unwanted interaction with the adsorbent may also be studied. Ultimately, the operation of the ion-exchange resin-based extractive fermentation in continuous mode may deserve special attention. On a separate note, the factor of cost of resin and lifetime of resins should also be taken into account during the selection especially for large-scale operation. Finally, considering that there are abundant of available experimental findings on specific products/by-products-resins adsorption, the data should be generalised to assist the selection of resin in future.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Notations

ABE	acetone-butanol-ethanol
BET	Brunauer-Emmett-Teller
BP	by-products
CIP	cleaning-in-place
DOT	dissolved oxygen tension
EBA	expanded bed adsorption
hEGF	human epidermal growth factor
K_d	dissociated constant
PrIFN-α2b	periplasmic human interferon-alpha2b
q_m	maximum specific uptake capacity
RSM	response surface methodology
SEM	scanning electron microscope
STB	stirred tank bioreactor
TEM	transmission electron microscope

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