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REVIEW ARTICLE

Recent development of unconventional aqueous biphasic system: characteristics, mechanisms and applications

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ABSTRACT

Aqueous biphasic system (ABS) is widely used in the recovery, extraction, purification and separation of proteins, enzymes, nucleic acids and antibodies. The ABS with high water content and low interfacial tension offers a biocompatible environment for the recovery of labile biomolecules. Process integration can be achieved using ABS by incorporating multiple-steps of purification, concentration and purification of biomolecules in a single-step operation which often results in high product recovery yield and purity. Conventional ABS is usually formed by aqueous solutions of two polymers or a polymer and a salt above a critical concentration. The high viscosity of polymer-based ABS causes slow phase separation and hinders the mass transfer of biomolecules, whereas polymer/salt ABS is characterized by high ionic strength resulting in the loss of bioactivity of recovered biomolecules. These limitations have encouraged the development of novel ABS which is more cost-effective for various biotechnological applications. This review discusses the characteristics and mechanisms of several types of emerging unconventional ABS using phase-forming components such as hyperbranched polymers, special salts, surfactants, magnetic fields, the addition of nanoparticles and incorporation of various solvent. Moreover, several novel applications of ABS for different separation purposes such as microfluidic-based ABS, ABS bioreactors, application of ABS as an analytical tool, and ABS micropatterning are discussed in this review. In the last section of this review, a comprehensive summary of process integration using ABS for extractive fermentations, bioconversion, crystallization and precipitation is also supplemented for the comprehensive review of various types and applications of ABS in recent years.

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Aqueous biphasic system; unconventional application; process integration; recovery; advances; separation

Introduction

Aqueous biphasic system (ABS) is a liquid–liquid separation technique widely applied in the extraction and purification of various biomolecules. The two-phase formation, which was resulted from the mixing of the aqueous solutions of starch gelatin, was first discovered in 1896 by Martinus Willem Beijerinck. The two-phase formation was later applied in the separation of biomolecules by Per-Åke Albertsson and has been widely used since the mid-1950s [1]. ABS is commonly referred to as the two-phase formation observed when aqueous solutions of two incompatible polymers or a polymer with a salt component are mixed above a critical concentration [2,3]. In the previous literature, the common polymers used for ABS formation were polyethylene glycol (PEG) and dextran [4], whereas phosphate, sulfate and citrate are among the common salt components used to construct the biphasic system [5]. The partition efficiency of the target biomolecules in an ABS is often determined by molecular weight and the concentration of phase-forming components, the hydrophobicity of the ABS formed, and the pH and temperature of the operating system [6].

ABS is an attractive approach in the recovery and separation of labile biomolecules because of the simple setup and low operating costs with high scalability and feasibility for continuous and automated operation [6–8]. The high-water content (70–90%) and low interfacial tension of the biphasic system conserves the structure and functionality of the biomolecules [2]. The single-step operated ABS with a cost-effective approach and desirable recovery yield and purity have prompted the application of ABS in industrial processes for the separation and enrichment of various biomolecules [6,9,10].

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Conventional polymer/polymer and polymer/salt ABS often resulted in the loss of bioactivity and functionality of the recovered biomolecules due to the high hydrophobicity or the presence of the high salt concentration [8,11]. Therefore, there is a surge in demand to develop unconventional ABS for the recovery of biomolecules for improved recovery efficiency, lower investment costs and alternatively more biotechnological applications.

This review discusses the characteristics, mechanisms and recent advancement of unconventional ABS including hyperbranched polymer-based ABS; special salt-based ABS; aqueous micellar biphasic systems (AMBS), magnetic ABS, the application of nanoparticles as additives in ABS and aqueous biphasic flotation (ABF). The unconventional application of ABS is inclusive of microfluidic-based ABS, an ABS bioreactor, an ABS for analytical purposes and also ABS micropatterning is also highlighted. Furthermore, process integration using ABS in extractive fermentation, bioconversion, crystallization and precipitation is also discussed.

Development of unconventional ABS

Hyperbranched polymer-based ABS

A hyperbranched polymer, commonly known as "smart polymer," was first introduced in 1991 as a replacement for conventional phase-forming polymers in ABS [12,13]. Amphiphilic polymers exhibit poor solubility in the salt-based ABS because the salt molecules tend to promote aggregation of the polymers [13]. Smart polymers with various functional groups often exhibit high biodegradability, biocompatibility, chemical and thermal stability toward different biomolecules which enhances ABS selectivity [12,14]. A hyperbranched polymer with both low melting point and low viscosity, because of the low chain entanglement structure, is an ideal phase-forming component for ABS construction [15,16]. The capability of hyperbranched polymer to covalently and noncovalently bond with a wide array of biomolecules can enhance the separation efficiency of the ABS formed toward the target biomolecules upon optimization [14]. Moreover, hyperbranched polymers display indistinguishable properties similar to ideally branched dendrimers despite a lower manufacturing cost because they can be synthesized in one single-step unlike the dendrimers. Figure 1 shows the structure of the hyperbranched polymer and the dendrimer.

Thermo-separating polymer is one the hyperbranched polymer which was widely used as the phase-forming components during the construction of ABS [17]. In year 2003, *Kluyveromyces marxianus* endo-polygalacturonase was recovered with an ABS of thermo-separating co-polymer Ucon 50-HB-5100. A 10-fold concentration of the enzyme with enzyme activity of more than 95% was recovered [18]. Moreover, ABS of thermo-separating EOPO copolymer and maltodextrin was applied to investigate the feasibility for the separation of bovine serum albumin, lysozyme and trypsin and was proven to be a cost-effective alternative for protein separation with the recovery yield obtained ranging from 60 to 98% [19]. The thermo-separating polymers can be easily recycled and reused repetitively because they are readily separated into the polymer-concentrated phase and an aqueous phase upon heating above their respective critical temperatures. In this manner, the total processing cost for the overall separation process can be substantially reduced because of the polymer's reusability.

Chicaroux et al. [15] demonstrated the application of hyperbranched polyesteramide Hybrane DEO 750 8500 (HB) as the phase-forming chemicals in an ABS

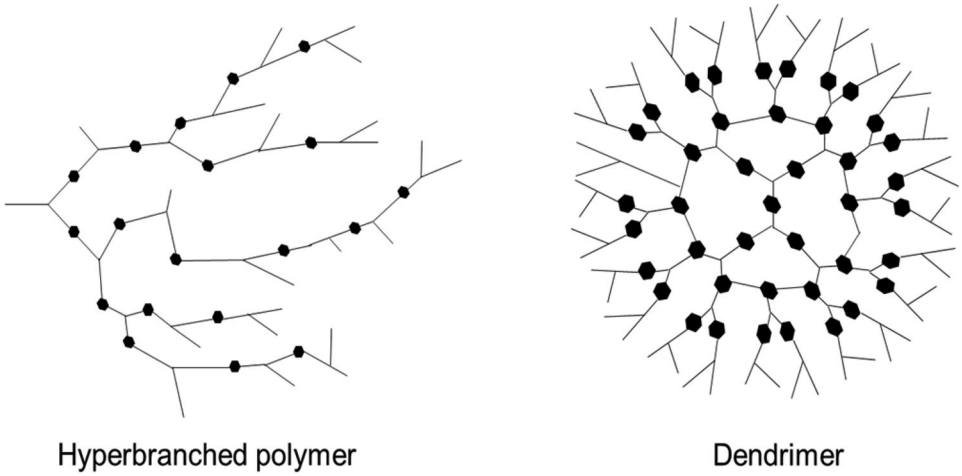

Figure 1. Structures of hyperbranched polymer and dendrimer.

composed of dextran T40 for the partitioning of L-serine. The HB polymer consists of 7 polyethylene glycol (PEG) chains which are further divided into 7 branched segments. The results showed that lower concentrations of dextran was required for phase formation in the hyperbranched polymer-based ABS when compared to the conventional PEG 8000/dextran ABS [15]. The low dextran concentration which resulted in the low system viscosity is preferable for the industrial practices of downstream processes in order to eliminate the potent complications associated with the high system viscosity along the separation processes. The L-serine showed superior partition preference to the dextran-enriched phase in the hyperbranched polymer-based ASB when compared to the conventional PEG/dextran ABS [20].

Overall, hyperbranched polymer-based ABS exhibits low system viscosity, high chemical and thermal stability. A feasibility to recycle and reuse the phase-forming polymers which would alternately minimize the total processing cost for the separation and purification of the target biomolecules when compared to the conventional polymer-based ABS.

Special salt-based ABS

Ionic liquids (IL) are emerging as extraction solvents which are generally types of salt that contain poorly coordinated ions and are present in a liquid state at a temperature below 100 °C or even room temperature. However, IL is often thermally stable over a wide range of temperatures and the electrically neutral molecules of IL made them the excellent electrolytes for the separation process of various biomolecules. The non-volatility, non-flammability and low melting points of IL provide safe and environmental-friendly phase-forming components for ABS construction [21]. The IL is regarded as a designer solvent because of the tunable physico-chemical properties that can be altered by modifying its cationic and anionic constituents [22]. The tunable physico-chemical property is ideal for the recovery of the target biomolecules with maximum extraction efficiency. Furthermore, ILs with low viscosity, negligible vapor pressure, high solvation ability, high chemical and thermal stability, high selectivity and the recyclability are favorable for extraction processes when compared to other organic solvents [21,23].

ILs are categorized into hydrophobic and hydrophilic ILs. Hydrophilic ILs are formed by high charged density salts and often introduce inorganic ions and salts that complicate the separation process and wastewater treatment procedure [24]. Hydrophobic ILs are usually more expensive when compared to the hydrophilic ILs because of the high production cost [21]. Examples of hydrophilic anions which constitute hydrophilic ILs are chloride and iodide, whereas hydrophobic ILs usually consist of hydrophobic anions such as hexafluorophosphate (PF_6^-) and bis(trifluoromethylsulfonyl)amide anion ($N(SO_2CF_3)_2^-$) [25]. Ionic liquids can also be classified into cationic and anionic ILs [26]. The most common cationic ionic salts used in the ABS applications are imidazolium, ammonium and the phosphonium salt whereas anionic ILs are carboxylic acid and amino acid [21,26]. IL-based ABS integrates the advantages of IL and ABS with low viscosity, negligible emulsion formation, short phase separation time, immediate mass transfer rate, low vapor pressure and strong solvation power for a wide range of biomolecules can significantly improve the recovery efficiency of the system [21,27].

IL-based ABS, often coupled with salts components such as citrate, phosphate and sulfate for phase formation and the extraction of target biomolecules, is usually determined by the salting-out effect. To reduce the salt application and disposal to the wastewater stream, glucose was proposed as to replace the salt components for the recovery of succinic acid *via* sugaring-out extraction in previous studies [28]. Furthermore, IL can also be applied as an additive in the ABS to strengthen the phase formation and alter the polarity of the polymer-rich phase for better extraction efficiency of biomolecules [29,30]. The addition of IL to the PEG-rich top phase changes the chemical and physical properties of the phase. The salting-out inducing IL with higher tendency in the hydration complexes formation excludes the biomolecules to the salt-rich bottom phase, whereas salting-in inducing IL improves the extraction ability of the polymer-rich phase by enhancing the solute–solvent interactions [31]. Moreover, the presence of the benzyl group or double bounds in IL cations are also responsible for enhancing the extraction efficiency of biomolecules in IL-based ABS. Table 1 shows several examples of previous studies which adopted ILs as phase-forming components and additives in ABS for recovery of biomolecules.

Aqueous micellar biphasic system (AMBS)

The aqueous micellar biphasic system (AMBS) is constructed with a thermo-induced ionic or nonionic surfactant to form micellar-rich and micellar-poor phases above the cloud point temperature [41,42]. Ionic surfactants include cationic, anionic and amphoteric surfactants which differed by the charge of surface-active

Table 1. Types of ionic liquids applied in ABS in previous studies.

Ionic liquids applied	Target bioproduct	Recovery yield	References
As phase component			
1-Ethyl-3-methylimidazolium tetrafluroborate	*Bacillus cereus* cyclodextrin glycosyltransferase	78.00%	[32]
1-Octyl-3-methylimidazolium bromide	Succinic acid	85.50%	[33]
N-Butylpyridinium chloride	Papain	95.77%	[34]
1-butyl-3-methylimidazoliumte dicyanamide	Flavonoids	93.35%	[35]
Tri(isobutyl) methylphosphonium tosylate	Bovine serum albumin	100%	[36]
Cholinium 2-[bis(2-hydroxyethyl) amino]ethanesulfonate	*Burkholderia cepacia* lipase	99.30%	[37]
Cholinium lactate and cholinium dihydrogen phosphate	Immunoglobulin G	>80%	[38]
As adjuvants			
1-Butyle-3-methyl-imidazolium chloride	Gallic acid	80–98%	[29]
1-Butyl-3-methylimidazolium tetrafluoroborate	*Bacillus subtilis* β-mannanase	89.65%	[39]
1-Butyl-3-methylimidazolium methanesulfonate	*Escherichia coli* L-asparaginase	87.94%	[40]

groups, while the nonionic surfactant is uncharged and water soluble because of the presence of oxygen-containing hydrophilic groups [43]. Surfactants molecules tend to aggregate at a certain critical concentration due to the reduced solubility of the micelles above the cloud point temperature because of the inter-micellar interaction that results in the density difference and phase formation [41].

AMBS enhances the recovery efficiency of target molecules with micellar formation to entrap the target molecule through electrostatic interactions [41]. The self-assembling characteristic of surfactant micelles determines the partition behavior of target biomolecules by altering the micellar properties, thereby facilitating the separation of target biomolecules. Furthermore, micelles formation offers an amphiphilic environment for selective partitioning of the target biomolecules based on the hydrophobicity of biomolecules [42].

The common surfactants used in the AMBS in the previous literature are Triton X-100, Triton X-114, Tween 80, sodium dodecyl sulfate (SDS), dodecyltrimethylammonium bromide (DTAB) and the detergents belong to the alkyl polyoxyethylene family (CmEOn) [39,43]. Considering the abovementioned attractive features, surfactants were applied in ABS for the separation of clavulanic acid [42], mangostins [38,44], the protease enzyme [45], phenolic compounds [46], nisin [47] and mammalian genomic DNA [48].

Magnetic ABS

The magnetic field can be incorporated into the conventional ABS to enhance the selectivity and rate of phase separation in the circumstances such as when the phase-forming components applied are too viscous. The phase density difference is extremely low when the volume ratio of the system is either very low or very high [49]. The selective distribution of target biomolecules in the ABS is determined by their electrical characteristics and the physicochemical differences [50]. Magnetic ABS was first introduced by Wikström et al. [51] in which the magnetically susceptible additives such as iron oxide particles and ferrofluids were incorporated into the two-phase system. Results showed that the ferrofluid reduces the separation time by a factor of 35 when a magnetic field was applied while iron oxide accelerates the phase separation by a factor of 70 regardless of the phase-forming components applied [51].

In view of the efficacy of magnetic ABS in accelerating the settling time of phase separation, Suzuki et al. [52] further explored the use of affinity ligand-bound fine magnetic particles in the ABS construction. The addition of IgG-bound Eudragit-Mag to a PEG/phosphate ABS has successfully increased the partition coefficient of protein A up to 35-fold. Later, magnetic ABS was adopted by Kamihira [53] to purify the staphylococcal protein A from recombinant *Escherichia coli* fermentation. The human IgG was added and immobilized into fine magnetic particles. The addition of IgG-modified magnetic particles enhances the selectivity of protein A in the ABS. The enhanced partition of protein A to the top phase of the ABS allows the elimination of cell debris and increases the rate of separation.

Figure 2 illustrates the schematic diagram of magnetic ABS. The two-phase system is prepared with an appropriate concentration of the phase-forming solution prior to the incorporation of the magnetically susceptible materials [49]. The system mixture is stirred and continuously pumped into a separator coupled with magnetic field on the wall. Phase separation was achieved when the aqueous phase containing magnetically susceptible material coalesced and drawn toward the column wall while the other aqueous phase leaves the column [49,54].

Magnetic aqueous two-phase fishing has been applied in the hybrid process technology for antibody

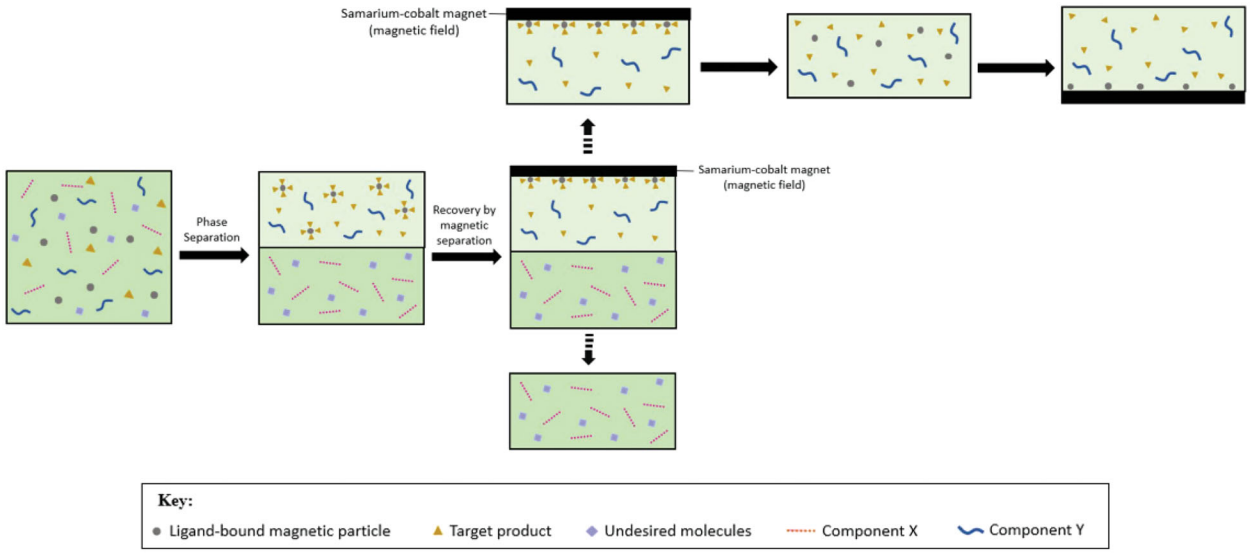

Figure 2. Schematic diagram of magnetic ABS.

purification from cell culture supernatants [55]. The addition of surface-modified magnetic particles (MPs) to the PEG/dextran ABS reduces the phase separation time from 40 min to 25 min. Ninety-two percent of antibody with a purity of 98% were recorded when excellent binding capacity was attained between the gum arabic coated particles modified with aminophenyl boronic acid (GA-APBA-MP) and the phase system.

Nanoparticles as an additive in ABS

Small size nanoparticles with a large surface area enhances the adsorption efficacy of target biomolecules to the nanoparticles and the uneven distribution of nanoparticles in the ABS generates two distinct ionic entities which alternately enhances the partition efficiency and the selectivity of target biomolecules without changing their physiochemical properties [56–58]. The incorporation of nanoparticles in the ABS also enhances the purification factor of target biomolecules by promoting the partition of unwanted materials to the other phase of the ABS.

The partition behavior of the distinct conjugate in the biphasic system is characterized by the resultant physiochemical interaction between the particles and the phase-forming molecules, which is mainly determined by the surface properties of the nanoparticles [59]. Therefore, the types and concentration of the nanoparticles incorporated in the ABS are the factors affecting the partition behavior of the target biomolecules in the ABS. For instance, graphene oxide, graphene, aluminum oxide and titanium oxide are amongst the potential nanoparticles which can be incorporated in the ABS. In previous studies, the effect of gold, silver, copper and silicon oxide nanoparticles on the partition behavior of different biomolecules have been evaluated [60].

The partition efficiency of cephalexin in the PEG 6000/citrate ABS was enhanced by 59% with a highest partition coefficient of 5.74 obtained when the graphene oxide was added [57]. Meanwhile, PEG 3350/magnesium sulfate ABS, coupled with the gold nanoparticles, has successfully recovered 92% of invertase and the increasing concentration of nanoparticles was shown to enhance the partition coefficient [60]. In later studies, Towfighi et al. [56] incorporated both the graphene oxide and carbon nanotube in the PEG 6000/salt ABS for the partitioning of Doxorubicin. The presence of these nanoparticles was reported to enhance the extraction efficiency ranged from 80% – 4000% in regard to the types of nanoparticles applied in the system.

Aqueous biphasic flotation (ABF)

Aqueous biphasic flotation (ABF) integrates the principles of ABS and solvent sublation (SS) by incorporating the use of gas bubbles, commonly nitrogen gas or compressed air in the ABS [10]. The ascending gas stream allows the surface-active compounds with both the hydrophobic and hydrophilic group to adsorb to the surface of the bubbles within the biphasic system. The adsorbed target compound then migrates toward the top phase of the ABS, overcoming the interfacial tension in the ABS [61]. Figure 3 illustrates the schematic diagram of ABF for separation of biomolecules. Compressed gas is continuously pumped into the flotation column passing through the sintered glass disk to

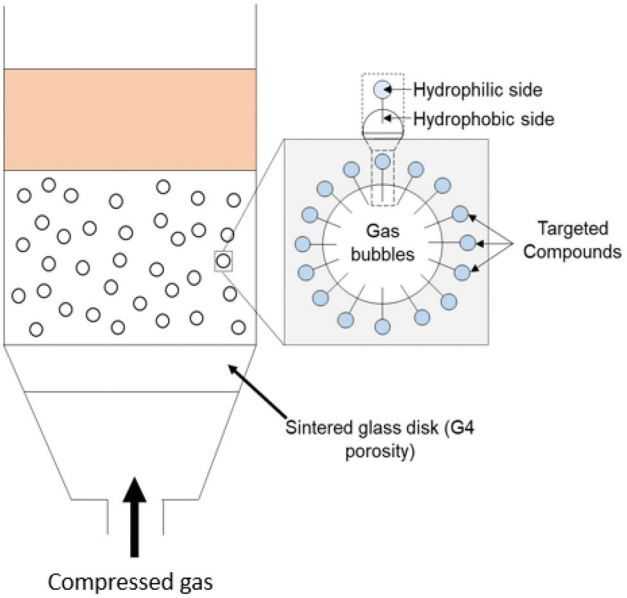

Figure 3. Schematic diagram of aqueous biphasic flotation.

generate small gas bubbles throughout the separation process [62].

The gas bubbles generated is enclosed within a thin water film which is readily ruptured and dissolved in the aqueous top phase, thereby enhancing the recovery performance of biomolecules [62]. The combination of SS and ABS is advantageous to the separation and concentration process of biomolecules with high separation efficiency, high concentration coefficient, minimal organic solvent consumption and environmentally friendly [63,64]. High concentration coefficient of target biomolecules can be achieved with ABF in which high concentration coefficients correspond to the high ratio of the concentration target of biomolecules in the top phase of the biphasic system to the initial concentration of target biomolecules in the aqueous phase. Other than the common process parameters of conventional ABS (phase composition; system pH; tie-line length (TLL); volume of crude loaded), the efficiency of ABF is also affected by the gas flow rate and the flotation time [61].

ABF has previously been used in the separation of penicillin [65], puerariae [63], lipase [66] and microalgal protein [67]. In comparison to conventional ABS, the separation of biomolecules using ABF offers a better separation efficiency and an enrichment factor. The ABF system was used to recover andrographolide from *Andrographis paniculata* with a total recovery yield of 5.85% in 30 min of flotation time and at a gas flow rate of 50 ml/min nitrogen [68]. Meanwhile, 70.3% *Pediococcus acidilactici* Kp10 bacteriocin-like inhibitory substance (BLIS) was recovered using PEG 8000/sodium citrate ABF system with 30 min of flotation time and 20 ml/min of flow rate [61]. Triton X-100/xylitol ABF system of 15 min of flotation time and 25 ml/min of flow rate has successfully extracted 87.49% of *Burkholderia cepacia* lipase with the separation efficiency of 86.46 [69].

Unconventional applications of ABS

Microfluidic-based ABS

Conventional ABS is often performed in a macroscale setup in the laboratory which requires milliliters of fluid volumes for the construction [70]. The incorporation of the microfluidic technology to the ABS allows the separation and detection of biomolecules such as cells isolation inclusive of the isolation of leukocytes and erythrocytes for haematological analysis by applying only microliters to picoliters of fluid volumes [71,72]. The microfluidic setup only requires small volumes of reactants with minimum gravitational forces for optimal separation results [72]. Both the sample and reagent volumes can be substantially minimized for rapid separation and therefore the overall setup costs are reduced. However, the application of the microfluid device ABS is more favorable for small sample size [70]. The high phase viscosity and the microscale system dimension ensures that the Reynold's number remains low ($Re < 1$) and thereby allows continuous and effective separation. The low Reynold's number also simplifies the laminar flow with stable formation. The presence of laminar flow in microfluidic channels permits the cells to position promptly near the interface which alternately enhances the cells sampling of the two-phases [71]. Partitioning of the particles only occurs when cells are attached to the interface and the cell attachment is usually dependent upon the distribution of flow rate and the inertial force of the particle movement at the confluent point of the two solutions [73]. The cells trapped at the interface of microfluidic ABS could be easily recovered, which is impracticable using the conventional macroscale ABS [71].

Microfluidic ABS is an ideal approach for the rapid separation and concentration of biological products with larger surface area, low emulsification and sample consumption [74]. The small size system is advantageous for the separation of biomolecules because of the high resolution and high sensitivity toward the separation and detection of the sample and reagents [75]. The microfluidic devices offer large surface area-to-volume ratios of streams which decreases the cells travel distance and results in a rapid separation of the biomolecules between the phases and therefore no mixing

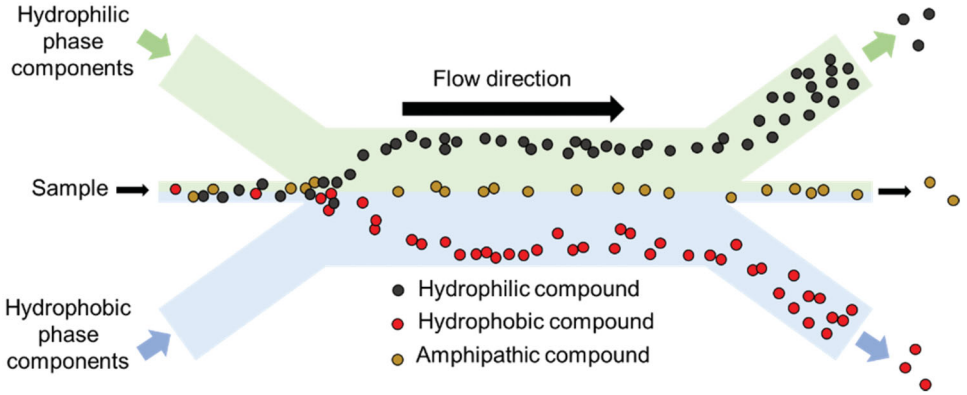

Figure 4. Schematic diagram of microfluidic ABS.

process is demanded [73]. In contrast, conventional macroscale ABS requires longer times for complete phase separation and occasionally stirring is required [70]. Figure 4 illustrates the schematic diagram of the microfluidic device design. The ABS microdevices consisted of three inputs which merged into a single main channel and then diverged into three outputs. The width of the input and output channels are 50 μm each, which merged and diverged to form a device with a width of 150 μm and a length of 20 mm. A consistent aqueous fluid environment with a stable interface is developed in the microfluid device, which allows the simple handling of the location of cells and the interface position [71]. Microfluidic ABS also greatly shortened the operation time without affecting the extraction yield in comparison to the macroscale [74].

Tagged protein from *Escherichia coli* lysate was isolated using the microfluidic ABS with a recovery yield of up to 85% by removing approximately 85% of the contaminant protein [76]. The study concluded that microfluidic ABS could be used for the purification of protein in continuous single-step operation with low-cost reagents. In 2004, Yamada et al. [73] investigated the feasibility of ABS on the microscale for the continuous partitioning of cells in a microchannel. The partition efficiency of plant cell aggregates with diameters varying from 37 μm to 96 μm has successfully enhanced the microfluidic ABS. A total of 90% of purified protein crude bacteriorhodopsin was successfully recovered from crude cell extracts of *Halobacterium salinarium* with the microfluidic ABS composed of PEG and detergent [74]. In addition, PEG/dextran microfluidic ABS was performed to concentrate the leukocytes from whole blood samples by enhancing the ratio of leukocytes to erythrocytes by a factor of 9.13 [71]. The use of a microscale PEG/phosphate buffer with sodium chloride ABS for the extraction of monoclonal antibodies (mAbs) was also successfully demonstrated and the extraction yield was proven to be similar to the macroscale ABS [75].

Abs bioreactor

The application of ABS in the recovery of biomolecules can be extended to the large-scale bioprocesses by incorporating the two-phase principle in a bioreactor operation for enhanced productivity in the integration of upstream and downstream processing. Figure 5 shows a model of an ABS bioreactor in continuous mode. In a two-phase partitioning bioreactor, cultivation product is transferred into a reaction tank consisting of two other aqueous solutions. The whole system is partitioned into two phases in which the phase with undesirable cell debris and substrate will be discarded. Meanwhile, the phase with the target molecules will be transferred to a different tank [77]. In this context, the production and extraction of target biomolecules are carried out simultaneously within the bioreactor, resulting in the effective and instant separation of target biomolecules from the unwanted materials.

The mass transfer rate of the low bioavailability of target biomolecules in the bioreactor can be enhanced by incorporating the two-phase partitioning principle. In this manner, the transfer of apolar toxic compounds can be monitored and the product degradation prevented [77]. In an ABS bioreactor, the operating temperature determines the settling rates of the biphasic system within the bioreactor because of the changing properties of phase-forming components, whereas the flow rates determine the hydrodynamic residence time and subsequently alters the overall extraction performance of ABS [74]. Moreover, the flow rates of the ABS bioreactor can result in firm dispersion and changes the phase inversion point of the biphasic system, and alternately modifies settling rates.

ABS bioreactors have been applied during the production of small aroma compounds, 2-phenylethanol (2-PE) from *Kluyveromyces marxianus via in situ* product removal mechanisms with an enhanced yield [78]. ABS bioreactors also effectively increase the resveratrol yield

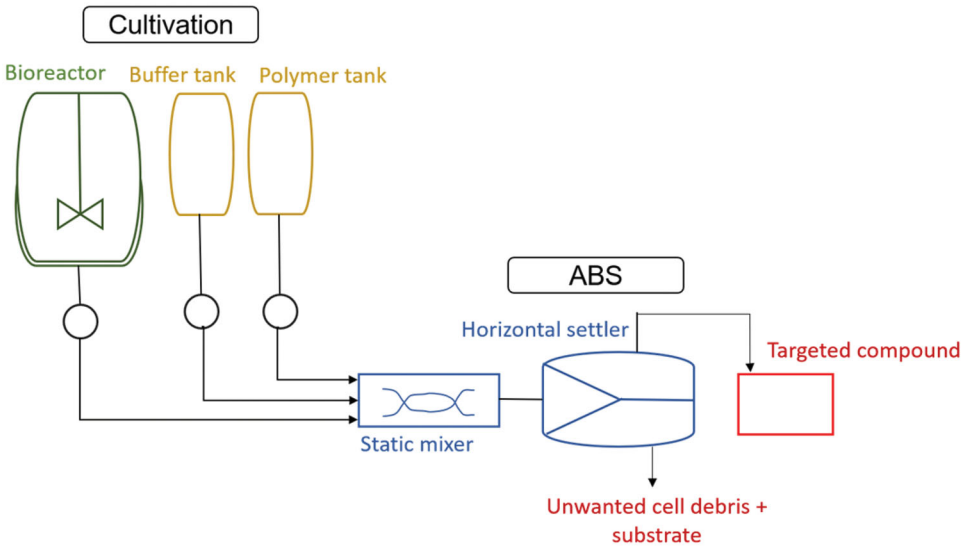

Figure 5. Schematic diagram of ABS bioreactor.

from grape seeds by 4.36-fold with a total yield of 224.61 ± 0.35 μg/g resveratrol and conversion rate of 85%. Compared to the conventional homogeneous one-phase system, the two-phase system improves the microorganism bioconversion rate and thereby enhances the yield of resveratrol [79]. An ABS bioreactor showed excellent efficacy in the continuous production of high-value low molecular weight compounds such as carotenoids from *Spirulina platensis* by enhancing the low productivity of the bio-compound from microalgae [80]. In view of this, ABS bioreactors are regarded to be a potential alternative in replacement to conventional homogeneous one-phase bioreactors with enhanced productivity.

Abs for analytical purposes

Despite the application of ABS in the downstream processes, ABS is adopted as an analytical tool to determine the structural, functional and physicochemical properties of biomolecules by providing quantitative information on the characteristics of biomolecules *via* determination of the partition coefficient in the ABS [81,82]. Partitioning of biomolecules in an ABS is highly dependent upon the characteristics and conformation of both the phase components and the target biomolecules. Differences in the physicochemical properties of different biomolecules determine the interactions between biomolecules and the phase-forming components molecules, results in selective distribution in the ABS. Therefore, the changes in the structural conformation and the differentiation of similar biomolecules can be detected using the ABS [1,82]. The hydrophobicity of various biomolecules can also be estimated by determining the partition behavior of the biomolecules in the system [83]. For instance, the biological activity, purity and homogeneity of recombinant human growth hormones (rhGH) can be determined by quantifying the partition coefficient of rhGH between the two immiscible phases [81].

The ABS can be classified as "charge-sensitive" and "charge-insensitive" based on the isotonic concentrations of phosphate salt and sodium chloride present in the system and is used for cells separation. Cells separation can be attained using "charge-sensitive" ABS due to the differential electrophoresis mobility of cells, whereas the membrane hydrophobicity of cells determines the differential partition of cells in "charge-insensitive" ABS [84]. For instance, ABS was adopted to investigate cells surface properties such as normal and cancerous cells *via* the partition behavior of cells. Previous studies revealed that patients diagnosed with malignant tumors showed a higher partition coefficient of total plasma proteins in comparison to the partition coefficient of total plasma proteins of healthy patients [50].

Abs micropatterning

In recent advances, ABS was adopted for the continuous extraction process which integrates the bacterial cultures for the patterning of multiple cell populations [85]. ABS creates a well-defined cellular microenvironment for the micropatterning technique, allowing for the partition of cells and small biomolecules into one specific phase [86]. In ABS micropatterning, bacteria cells were first homogenized into one of the phase-forming solutions and was later added as droplets into

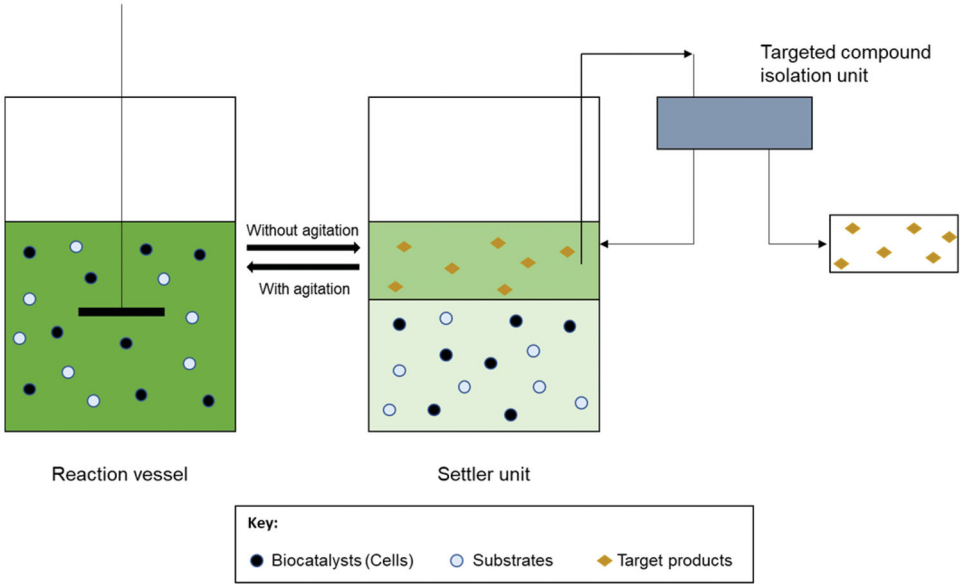

Figure 6. Schematic diagram of extractive fermentation and extractive bioconversion using ABS.

the second phase-forming solution containing the culture dish, resulting in the formation of two distinct phases [86,87]. The attachment of cells to the culture substrate leads to the removal of polymer solutions. The ABS micropatterning overcomes the limitations of conventional micropatterning approaches with the use of simple pipetting tools and easy operation. The low interfacial tension of ABS prevents the overgrowth of bacterial cells by retaining the physical confinement of bacterial cells. The transfer of biomolecules across the phases reduces the nutrient depletion and excessive waste formation [88].

In view of the abovementioned, ABS micropatterning can be used in the development of bacterial micro-colony sensors for the biochemical detection by arraying various biochemical engineered biosensor bacteria [86]. Furthermore, ABS can be applied for the formation of bacterial biofilms with high precision and specific localizations [89,90]. The idea of ABS micropatterning has been performed by Yaguchi et al. [89] in the production of ABS-derived biofilms for bacterial interaction studies. The experimental results revealed that β-lactamase-producing biofilm enhanced the survival of ampicillin-sensitive strain up to 3600-fold and showed ampicillin resistance.

Process integration using ABS

Extractive fermentation or cultivation

Extractive fermentations using ABS for the integration of cell cultivation and downstream product purification into a single step process allows the instant recovery of target compounds immediately after the synthesis of the target compounds [91]. Immediate product removal upon synthesis provides an alternative to overcome product inhibition, product reutilization and labile product degradation in conventional fermentation [92,93]. As a result, high specific activity of target biocatalysts could be produced in a cell-free stream. Additionally, extractive fermentations using ABS can serve as a continuous fermentation method for the simplification of the scaling-up process and increases the volumetric productivity of the target compounds [91]. Moreover, ABS offers a non-denaturing environment for the synthesized biomolecules as well as stabilizes the cells making it a meaningful approach as a replacement for conventional fermentations [94].

ABS extractive fermentation has been widely applied in the production of various compounds such as antibiotics [95], enzymes [91], solvents [96], toxins [97] and cells [94] over the decades [98]. ABS can integrate the fermentation and recovery of the target products in a single-step approach. Microbial cells are cultivated in one phase of the ABS, while the target product is partitioned into the other phase of the ABS eliminating the need of biomass recovery (Figure 6). In this manner, the target product is recovered in one phase once it is produced, whereas the cells and undesired substrates are accumulated in the opposite phase [99]. Cells culture can be reused for several cycles of fermentation provided if the cells viability is maintained [91]. The recycling of cell culture shortens the cultivation time and reduces substrate consumption for the preparation of a new inoculum batch [92].

Selection of suitable phase components is important for sustaining cell growth, target protein production and its purification. Various parameters including pH, agitation, fermentation temperature and time will also be evaluated to achieve optimum conditions for the fermentation process with enhanced yields [100,101]. In a previous study, PEG/dextran ABS was favorable for the recovery of alkaline phosphatase from *Bacillus licheniformis* MTCC 1483 in comparison to PEG/salts ABS because of interactions between the anionic amino acid on the protein surface and metal ions which influenced enzyme partitioning toward the PEG-rich top phase, thereby lowering the partition coefficient of alkaline phosphatase in the PEG/salts ABS [100].

ABS extractive fermentation was also used for the production and extraction of extracellular *Burkholderia pseudomallei* lipase [91]. The high recovery yield of lipase (92.1%) with optimum ABS composed of 9.6% (w/w) PEG 8000 and 1.0% (w/w) dextran T500 revealed the potential of ABS to integrate the fermentation and recovery of biomolecules in one-step operation. In addition, PEG/citrate extractive ABS was employed for the instant recovery of *Aspergillus tamarii* URM4634 protease with a high recovery yield of 98.4% and a concentration factor of 2.14 [92].

Extractive bioconversion

The high-water content and low interfacial tension features of ABS provide a gentle environment for the biocatalyst reaction. The low interfacial tension of ABS facilitates small phase droplet formation during the homogenizing of phase-forming components. This reduces the migration distances and promotes mass transfer of desired compounds in the biphasic system. Whereas, the minimization of product inhibition and degradation due to instant removal of products is achieved by incorporating ABS into the extractive bioconversion [4,7,102]. Extractive bioconversion using ABS enables the retention and recycling of the biocatalysts-containing phase in the subsequent downstream processing due to the continuous partitioning of target products to the product-containing phase [4].

Figure 6 shows the schematic representation of extractive bioconversion using ABS. Biocatalysts are added to the reaction vessel containing the well-mixed phase components and substrates to initiate the reaction. Phase separation is achieved by pumping the phase mixture into a settler unit. The product that settled on the top phase will be extracted to an isolation unit for product recovery [103]. Extractive bioconversion can be performed either in a single step or a multiple step unit operation. It can also be merged with other separation techniques such as adsorption and ultrafiltration for target product isolation in order to enhance the recovery yields of the target products [103].

Extractive bioconversion with ABS was performed for the synthesis and recovery of cyclodextrins (CDs) using *Bacillus cereus* cyclodextrin glycosyltransferase [4]. A total of 13.7 mg/mL of CDs recovered with the optimum condition was composed of 7.7% (w/w) polyethylene glycol (PEG) 20,000 and 10.3% (w/w) dextran T500. Chew et al. [104] also produced a total of 79.8% of hydrolyzed poly-caprolactone (PCL) from *Burkholderia cepacia* lipase extractive bioconversion using ABS composed of 19% (w/w) PEG 3000 and 8.1% (w/w) potassium phosphate at 40 °C and pH 7.0. These studies have highlighted another milestone for the applications of ABS in process integration using ABS.

Extractive crystallization

In an extractive crystallization using ABS, the aqueous phase solution was transformed into a solid crystal. Crystallization is often used in the separation of components which are difficult to be recovered by distillation and heat-labile materials [105,106]. In the general extractive crystallization process, the soluble target protein was first partitioned to one specific phase of the ABS, followed by the crystallization process which transforms the target protein from liquid to a solid state [107]. Extractive crystallization is proven as an effective alternative for the purification of salts components in which the concentrated salt solution homogenizes with a selected solvent and results in salt crystallization because of the mutual insolubility of the water and solvent [108]. The crystal structural components formed in the salt-induced crystallization process can be enhanced if PEG is applied as the phase-forming solution [107].

Extractive crystallization using PEG-based ABS was performed by Taboada et al. [105] in the isolation of pure anhydrous sodium sulfate crystals showed a notable increase in the yield of sodium sulfate crystals from 58% to 100%. The ABS extractive crystallization was employed for the recovery and reuse of phosphate salts as a fertilizer in agriculture with the addition of phosphoric acid into the methanol/phosphate ABS [109]. In the later study, Huettmann et al. [107] developed a PEG 2000/sulfate ABS for the simultaneous crystallization and extraction of a single-chain antibody which increased the PEG concentration from 2% (w/v) to 4% (w/v) which has shown a significant enhancement in the crystallization yield of the single-chain antibody from 63% to 87%.

Extractive precipitation

Affinity precipitation in ABS is mainly dependent on the interaction between target molecules and the affinity ligands coupled to a reversibly soluble-insoluble polymer. This has resulted in the formation of an insoluble biomolecule-affinity ligand complex which is subsequently transferred to one of the phases in the biphasic system [110,111]. The combination of ABS and the protein-polyelectrolyte is commonly applied to concentrate or purify enzyme and macromolecules using the poly-charged macromolecules of opposite electric charge to the target biomolecules [112]. The high selectivity of biomolecules could be attained with low polyelectrolyte concentrations in ABS and the resultant insoluble complex can be easily re-dissolve through a pH change or salt addition, making it an ideal approach for industrial scale production.

The incorporation of an affinity ligand into ABS was first studied by Tjerneld et al. [113] in 1987. The extraction of lactate dehydrogenase (LDH) into the PEG-rich top phase was enhanced by the addition of dye Procion yellow HE-3G. In 1994, the integration method of ABS and affinity precipitation was also studied for the purification of LDH from porcine muscle extract. In the addition of a ligand carrier, Eudragit S 100 [114] enhanced the partitioning of LDH to the PEG-rich top phase of ABS comprising PEG 8000 and dextran T250, leading to the precipitation of a Eudragit-dye-LDH affinity complex. A yield of 54% of LDH was recovered which proved to be an excellent strategy for the purification of components [114].

In the later study, Malpeidi et al. [115] integrated ABS and its precipitation with polyethyleneimine (PEI) and a positively charged flexible chain polymer for the purification of trypsinogen from bovine pancreas. Under optimized conditions with 0.25% (w/w) PEI addition, fivefold of purification and 84% of yield of trypsinogen were achieved in the citrate-rich bottom phase whereby nucleic acid contaminants were successfully precipitated. In addition, the combination of ABS and polyelectrolyte precipitation was employed for the purification of pepsin from bovine abomasum homogenate. A recovery yield of 48.4% of pepsin with a purification factor of 9.0 was attained in the PEG-rich top phase of optimized PEG 1450/phosphate ABS. The insoluble complex was formed in the biphasic system due to the addition of chitosan, a cationic polyelectrolyte for the precipitation of acidic enzyme pepsin [112].

Challenges and future prospects

In the development of various unconventional ABSs both *via* chemical and physical approaches have resulted in an enhanced recovery efficiency during a reduced operation time. However, the overall operational costs are often associated with phase-forming components applied and additional additives required for the ABSs in large-scale operation. Therefore, the efforts of recycling and reuse of the phase-forming components could contribute to reduction of the overall operational costs in industrial scales and thereby enhance the feasibility in practically apply the ABS on the industrial scales. The potential of the of ABS in process integration have significantly expanded its application in separation, purification, concentration and the clarification of numerous bioproducts by reducing the processing steps in overall upstream and downstream processes. Nonetheless, bench-scale experiments are required for the optimal operation of ABS either as primary separation methods or integrated with other downstream processes. There are unavoidable differences present in the bench-scale and industrial-scale operations and therefore further researches on reducing the separation time and product harvesting time are often demanded for optimal operational efficiency and productivity in industrial-scale implementation of ABS.

Conclusion

Unconventional ABS can improve the efficiency and performance of the separation process in terms of the purification and recovery yield by adopting novel phase-forming components or incorporation with certain small physical components. More studies on the development of ABS should be performed to provide a more cost-efficient approach for the recovery of biomolecules with a higher purification and yield. Process integration in ABS has remarkably simplified the operation of bioprocess which has attracted many industries to incorporate ABS into the process operation. Considering the advantages of these combinations, the development of unconventional ABS is worth deeper research to achieve desired goals that are not effectively met by conventional discrete upstream and downstream processes.

Disclosure statement

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