Development of a continuous aqueous two-phase flotation process for the downstream processing of biotechnological products

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ABSTRACT

Aqueous two-phase flotation (ATPF) is an alternative downstream process for direct recovery of biotechnological products from crude biosuspensions. ATPF is a combination of two phase extraction and flotation, resulting in high recoveries and product concentrations. In addition to selecting a suitable two-phase system, the process design must be optimized to realize an efficient ATPF process. In this study, the focus was on the process strategy. As an example of a biotechnological product, the technical enzyme phospholipase was purified. The influence of different initial enzyme concentrations on the separation process was investigated and limitations of the separation efficiency were identified. Based on these findings, batch ATPF was transferred first to semicontinuous ATPF and later to continuous ATPF. Thus, this work describes for the first time a continuous ATPF, which showed 2.5 times the purification capacity of batch ATPF in the same flotation time.

1. Introduction

Biotechnological products range from smaller molecules such as penicillin with functional properties to macromolecules such as enzymes that catalyze biochemical reactions. They are often synthesized by microorganisms during a fermentation process and are either accumulated in the cell (intracellular products) or expressed into the biosuspension (extracellular products). Due to the complexity of the fermentation medium and the sensitivity of biological substances to external influences (e.g. pH, temperature, mechanical stress), the separation and purification of biotechnological products is difficult. In order to achieve the desired product concentration and purity, several process steps (e.g. centrifugation, (ultra-)filtration, or ion exchanger) usually have to be effectively combined. Product losses, equipment and energy costs add up over the process chain and reduce the proceeds. Since the production of so called secondary metabolites is independent of cell growth, more and more upstream processes are carried out continuously. If the downstream can also be carried out continuously, the effort in production can be minimized by saving tanks for intermediate storage (space and investment costs) as well as the associated energy costs (mainly caused by pumps) [1].

Aqueous two-phase flotation (ATPF) is a modification of solvent sublation and was first described in 2009 as an alternative purification method for penicilin G [2]. Like solvent sublation, ATPF involves flotation within a two-phase system. Hence, ATPF is a combination of aqueous two-phase extraction (ATPE) and flotation. While in solvent sublation, mostly organic compounds are flotated into a solvent phase, in ATPF, water forms the main component of both phases, resulting in high biocompatibility. The mild conditions make ATPF particularly suitable for the separation and purification of biotechnological products [3]. The two phases form when phase-forming components (polymers, salts, or alcohols) are present in sufficiently high concentrations as aqueous solutions. In polymer-salt systems, the heavier bottom phase usually has a high salt concentration, while the lighter top phase consists mainly of polymers in addition to water. In ATPF, the biosuspension is transferred to the bottom phase or mixed with the phase-forming components. The top phase is then added on top and gassing is started at the bottom of the flotation cell. Porous media such as sintered glass membranes generate gas bubbles, to the surface of which substances with hydrophobic areas attach during their ascent through the bottom phase and thus float up into the top phase. These substances are dissolved there during ascent, or at least after bursting of the bubbles at the surface. The top phase serves as a product-collecting extraction phase, which requires the target molecules to have an affinity for the top phase. The affinity of a substance to the top phase can be described by the distribution coefficient K_P, which is calculated from the ratio of the concentrations of the substance in the top phase to the bottom phase [4]. While in ATPE a mass transfer occurs only by diffusion, in ATPF the transport into the top

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phase additionally takes place by flotation. This allows comparable recoveries to be achieved with a significantly lower volume ratio V_R of top phase to bottom phase [3]. Bi, Li, and Dong (2009) were able to achieve a separation efficiency comparable to that of ATPE for the ATPF of penicillin G although they used less than one-fifth volume of the top phase. Similar results were obtained for the ATPF of baicalin, where the top phase was reduced tenfold compared to ATPE [5]. In addition to the reduction of polymers, which both reduces costs and makes ATPF environmentally friendly, high concentrations can be achieved due to the low V_R. Tan *et al.* (2014) achieved a concentration factor (ratio of enzyme concentrations in top to bottom phase) of 92 by ATPF of a lipase compared to 1.5 for ATPE at the same yield. Other advantages of ATPF besides the high concentration and high separation efficiency are environmental compatibility and economic efficiency by saving phase-forming components [3]. Since its introduction in 2009, ATPF has been intensively researched for the direct purification of various, biotechnological products from biosuspensions. In addition to pharmaceutical low-molecular-weight compounds such as penicillin G [2,7], baicalin [5], or lincomycin [8], high-molecular-weight compounds such as enzymes have also been successfully separated [6,9–17]. ATPF can also be promisingly used in the extraction of proteins from microalgae [18,19] or in the extraction of ingredients from plants [20–22]. Despite these numerous investigations of ATPF for various applications, the focus is mostly on the optimization of the two-phase system. Especially the selection of suitable phase components recycling of phases are the focus of several studies [2,3,10–13,15,17–20,23,24]. Most studies on ATPF take place at laboratory scale (smaller than 300 mL) using glass filters for gas input and do not describe a scalable process or process integration. The largest scale of ATPF described so far is about 2.5 L, however, a non-scalable converted water dispenser is used as a flotation cylinder [12]. In order to be able to use ATPF on an industry level, process and apparatus optimization are absolutely necessary. Previous work has shown that process parameters, such as bubble size and gas flow rate during aeration, have a decisive influence on the stability of the aqueous two-phase system and on the kinetics of the ATPF [25]. During this work, it also became apparent that separation efficiency might be limited by diffusive equilibrium. It was suggested that back-diffusion of enzymes from the top phase to the bottom phase counteracts flotation. This finding indicates that an alternative process strategy, such as a stepwise or continuous exchange of the top phase, could increase the efficiency of ATPF.

The studies presented in this paper include the characterization of the influence of the initial enzyme concentration on the ATPF as well as the establishment of a (semi-)continuous and continuous phase exchange. To the best of the authors' knowledge, this is the first time a continuous ATPF has been presented.

2. Material and methods

The materials and methods used in the experiments are described below.

2.1. Aqueous two-phase system (ATPS)

The ATPF experiments were performed in an aqueous two-phase system (ATPS) consisting of salt and polymer. The phase-forming components tri-sodium citrate dihydrate and polyethylenegylcol (PEG) 1000 were used. In the bottom phase, the citrate concentration was 25.8 % (w/w) and the PEG 1000 concentration was 0.7 % (w/w) in all experiments. The initial enzyme concentration ($x_{BOT,0}$) in the bottom phase varied between 0.6 and 2.7 % (w/w) and was 1.5 % (w/w) in the semicontinuous and continuous ATPF experiments, respectively. In the top phase, PEG 1000 formed the largest mass fraction with a concentration of 39.4 % (w/w). The citrate concentration in the top phase was 3.0 % (w/w) and no enzyme was in the top phase at the beginning of the flotation. The density difference between the phases was 101 kg/m^3 and the volume ratio V_R of top phase to bottom phase was 0.1.

2.2. Model enzyme and measurement method

In order to determine the influence of various factors such as the initial enzyme concentration, a model suspension was prepared using the spray-dried food enzyme phospholipase A_2. The powder was suspended in deionized water and added to the bottom phase to achieve the desired concentration. The enzyme concentrations in the respective phases were measured by absorbance measurement at 280 nm using a UV/Vis-spectrophotometer. Calibrations allowed the absorbance values to be converted to a mass concentration.

2.3. Aqueous two-phase flotation (ATPF)

All experiments were performed in a laboratory ATPF apparatus with a capacity of 300 mL. The exact dimensions are described in previous studies [25]. Gas bubbles were introduced via a sintered glass membrane (porosity G4). The air flow rate, adjusted via a mass flow controller, was 10 ccm for all experiments. For the continuous ATPF, an additional flotation with 20 ccm was performed. All experiments were carried out at 20 (±2) °C.

In batch ATPF, the enzyme-enriched bottom phase was first added to the flotation cell and then the top phase was carefully added on top of the bottom phase without mixing the phases. With the start of the gas introduction, the flotation time was counted and samples were taken at different time points using syringes for concentration determination. The separation efficiency E is calculated from the initial measured volumetric enzyme concentration in the bottom phase $c_{BOT,0}$ and the volumetric enzyme concentration at flotation time t ($c_{BOT,t}$), according to Equation (1).

$$E \quad \left(1 \quad \frac{c_{BOT,t}}{c_{BOT,0}}\right) \cdot 100\% \tag{1}$$

For semicontinuous ATPF, the initial procedure was analogous to batch ATPF. However, after a flotation time of 30 min, the gassing was stopped and then half of the top and bottom phase volumes were removed and replaced by fresh phases. The enzyme-enriched bottom phase was refilled, and unloaded top phase was carefully added again from above. Subsequently, the gassing was started again. The semicontinuous phase exchange was repeated analogously every 30 min.

For continuous ATPF, the first 30 min were analogous to batch and semicontinuous ATPF. After 30 min, continuous phase exchange was performed using peristaltic pumps (experimental setup shown in Fig. 1). For this purpose, pump 1 was used to feed enriched bottom phase (BOT) at the bottom of the flotation cell near the gas bubble introduction and, to the same extent, purified bottom phase was withdrawn about 2 cm

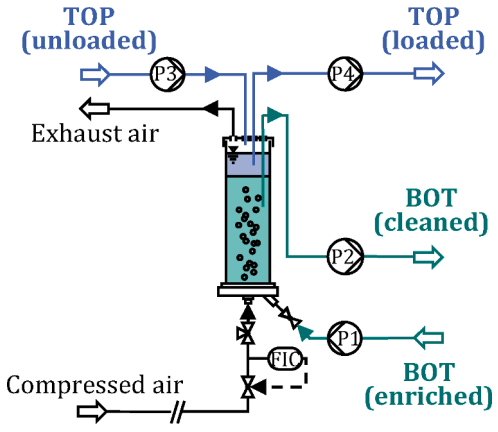

Fig. 1. Schematic illustration of a vertical ATPF apparatus for continuous flotation.

below the aqueous phase boundary between top and bottom phase using pump 2. The top phase (TOP) was exchanged using both synchronized pumps 3 and 4, with the unloaded top phase being fed at the surface and the loaded top phase being removed in the middle of the top phase. The exchange rates were set so that the complete flotation volume was exchanged once within 60 min of flotation.

3. Results and discussion

To evaluate the ATPF as a separation process, it is important to determine kinetics of enzyme concentrations in the top or bottom phase by sampling. It is known that the separation efficiency increases steeply at the beginning of the ATPF and flattens out in the further course of time [3]. It has also been observed that the kinetics of separation efficiency can be represented by an exponential function and approaches a threshold value asymptotically as flotation time progresses [25]. The separation efficiency thus appears to be limited depending on the two-phase system and the substance to be flotated. Considering findings from two-phase extraction, two possible reasons are most likely for this. First, a concentration equilibrium of enzymes between top phase and bottom phase due to thermodynamics could counteract mass transfer through the gas bubbles with increasing flotation time. On the other hand, the uptake capacity for macromolecules such as enzymes could be limited, since a high amount of polymer already occupies the free places in the water lattice. This behavior is known as the volume exclusion effect. [4]

To obtain information about the limiting factors during ATPF of the model enzyme phospholipase A2, experiments were performed with different initial enzyme concentrations. The kinetic of the ATPF was determined to identify an efficient range and then perform a stepwise exchange of the phases (semi-continuous ATPF). By defining the exchange rate, a continuous phase exchange could thus be realized (continuous ATPF).

In order to characterize the influence of the initial enzyme concentration in the bottom phase, flotations with different initial concentrations ($x_{BOT,0}$ 0.6–2.7 % (w/w)) were performed on model enzyme PLP at a constant aeration of 10 ccm with glass membrane G4. The changes in enzyme concentrations during the flotation time for top or bottom phase are shown in Fig. 2 A and B, respectively. The enzyme concentrations at the end of ATPF after 120 min ($x_{BOT,120}$ and $x_{TOP,120}$) as a function of the initial enzyme concentration for the top or bottom phase are shown in Fig. 2 C and D, respectively.

In all experiments, steep increases in enzyme concentration in the top phase and steep decreases in the bottom phase were observed during the first 30 min of flotation time. Thereafter, the curves asymptotically approach a final value. It is noticeable that the more enzyme was present at the beginning, the steeper the increase or decrease at the beginning and the higher the enzyme concentration at the end in the top phase and the lower in the bottom phase.

Different increases in the first 30 min reflect different amounts of mass transfer of the enzyme from the bottom to the top phase. From the principle of ATPF, it is clear that mass transfer arises from diffusion and flotation [3]. For the different progressions of the different experiments two reasons are conceivable. One possible explanation is a predominant influence of diffusion at the beginning of ATPF. The more enzyme present in the bottom phase at the beginning, the larger the concentration difference with the initially unloaded top phase. Since diffusion is driven by concentration gradients, the greater diffusive mass transfer takes place where the difference is highest. The second possibility is based on different collision probabilities between the enzyme molecule and the air bubbles. For successful flotation, collisions between the enzymes and the gas bubbles are necessary for the enzymes to attach to the bubbles.

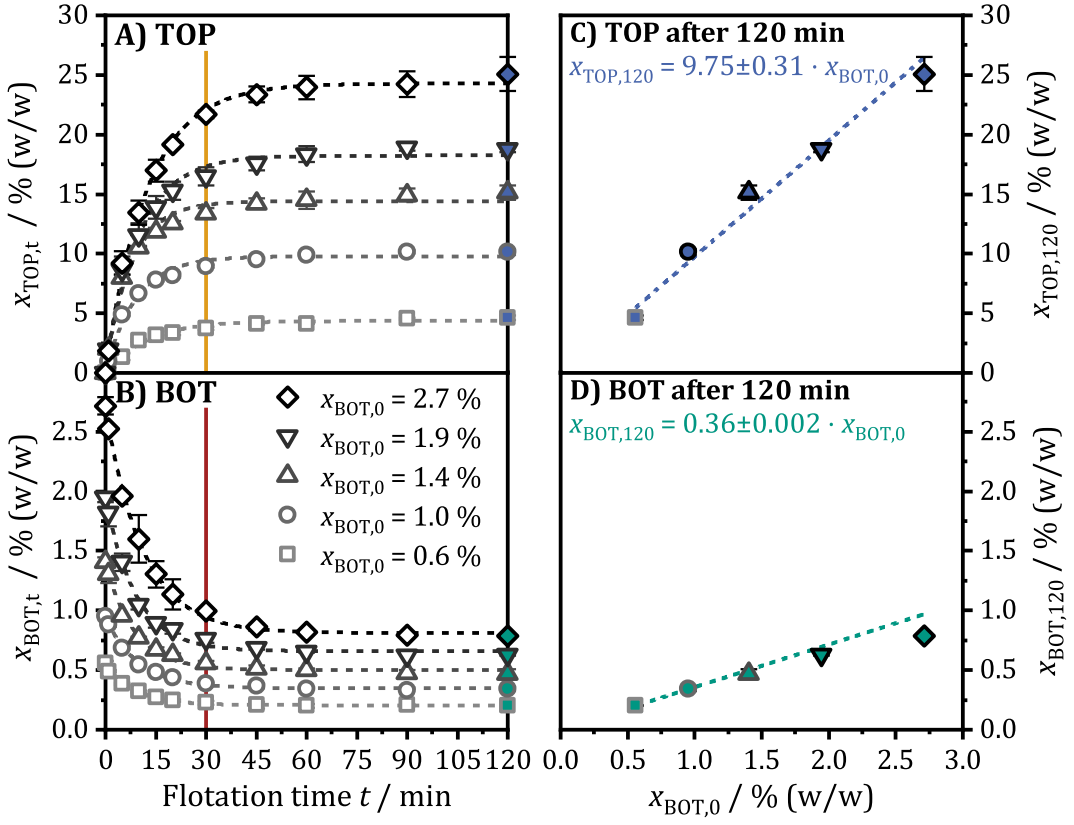

Fig. 2. Time course of emzyme concentrations in the top (A) and bottom phase (B) during ATPF of a phospholipase for different initial enzyme concentrations $x_{BOT,0}$. Linear dependency of enzyme concentrations after 120 min of flotation ($x_{BOT,120}$ and $x_{TOP,120}$) in the top (C) and bottom phase (D) as a function of $x_{BOT,0}$ (R^2 for both fits higher than 0.99).

At constant gas input, the collision probability between bubble and enzyme increases with the number of enzyme molecules in the bottom phase. The flotative mass transport is therefore highest where the most enzyme is present.

If the collision probability alone, i.e. the different numbers of enzyme molecules that attach to a bubble during its ascent in the bottom phase, was responsible for the different concentration changes, the enzyme concentrations in the bottom and top phases would all have to aim for the same final level. By decreasing the number of enzyme molecules in the bottom phase, the flotation rate and $x_{BOT,t}$ would steadily decrease. This is not the case for either the bottom phase or the top phase. Rather, there is a linear relationship between initial enzyme concentration and final concentrations after 120 min ATPF in both bottom phase (Fig. 2 D) and top phase (Fig. 2 C). A volume exclusion effect, which causes a maximum enzyme concentration in the top phase, can therefore be excluded in the range investigated here. However, the stagnation of the enzyme concentrations in the course of the flotation time (from approx. 60 min) suggests that a state of equilibrium is reached. The equilibrium between the enzyme concentration in the top phase and the enzyme concentration in the bottom phase can be described by the partition coefficient K_P, which is 31.3 ± 2.7 for all ATPF-experiments after 120 min. This concentration equilibrium, which is reached independently of the initial enzyme concentration, suggests that diffusion effects play a crucial role during ATPF. At the beginning of ATPF, the top phase is still unloaded, so the floated enzyme molecules can freely dissolve after the gas bubbles have reached the top phase or burst at its surface. With increasing flotation time, the enzyme concentrations approach the equilibrium state and the enzymes attached to the bubbles do not pass into the top phase, since diffusion can no longer take place without concentration gradient between bottom and top phase.

As can be seen from Fig. 2 A and B, the ATPF seems to be particularly efficient in batch operation, especially at the beginning, since the mass transfer is strongest there. With a look at the changes in the enzyme concentrations in the top (Δx_{TOP}) or bottom phase (Δx_{BOT}) between the start of ATPF and after 30 min flotation time, a linear dependence between the initial enzyme concentration and the changes in the concentrations in the respective phases can be seen. These relationships are shown in Fig. 3. The fit equation for the bottom phase shows that 60 % of the enzymes pass into the top phase within the first 30 min, regardless of the initial enzyme concentration in the bottom phase. This corresponds to a separation efficiency of 62 %, which was reached after 30 min in earlier experiments [25] (also shown in Fig. 4 A).

The results suggest that diffusion plays a crucial role during ATPF. Although an increase in bubble surface area flux causes an increased flotation rate [25], diffusion determines whether the enzymes enter solution in the top phase. At the beginning of ATPF, a large concentration gradient between the phases leads to an easier transition of enzymes from the bubble to the top phase. The subsequent flattening of the concentration profiles by nearing the equilibrium state can be attributed to reduced diffusion. Although the enzymes still float through the bubble ascent into the top phase, they cannot dissolve there due to the lack of a concentration gradient. They remain in the liquid film consisting of bottom phase, which formed when the bubble was generated. After the bubbles burst at the surface, this film forms a liquid droplet that sediments back into the bottom phase, causing the enzyme molecules to be transported back. The concentration equilibrium that forms between the top or bottom phase thus limits the separation efficiency of the ATPF. Consequently, ATPF is most effective when there is a high enzyme concentration in the bottom phase and a comparatively low one in the top phase. In batch operation, this is only possible at the beginning of the ATPF. A stepwise exchange of the phases by unloaded top phase and loaded bottom phase or a continuous phase exchange is therefore promising, as this prevents the combination of low concentrated bottom phase and simultaneously high enzyme concentrations in the top phase.

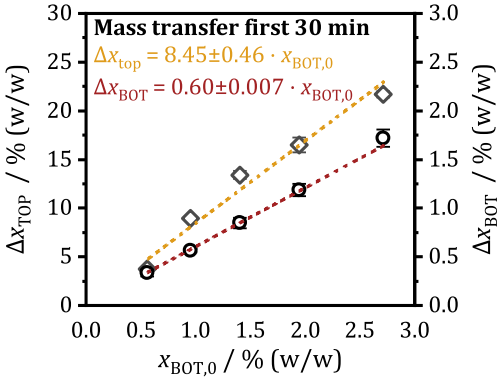

Fig. 3. Mass transfer during first 30 min of ATPF for different initial enzyme concentrations $x_{BOT,0}$. Linear fits show linear dependencies between $x_{BOT,0}$ and enzyme concentrations in the top (Δx_{TOP}) and bottom phase (Δx_{BOT}) (both times R^2 higher than 0.99).

Fig. 4 A shows the time course of separation efficiency during batch ATPF of the model enzyme phospholipase ($x_{BOT,0}$ 1.5 % (w/w)). The measured values are from Jakob, Singer, and Nirschl (2021) and represent the starting point for the development of a semicontinuous and a continuous ATPF.

In batch ATPF, as in the previously shown experiments with different enzyme concentrations, most of the enzyme transport into the top phase takes place within the first 30 min. At this point, the separation efficiency was already 62 %; in the subsequent 90 min, there was only an 8 % change in separation efficiency to 70 % after 120 min of batch ATPF. In order to make the ATPF process as efficient as possible, the aim was to run it semicontinuously by stepwise exchange of the phases every 30 min. The resulting separation efficiency curve is shown in Fig. 4 B. Initially, a similar increase in separation efficiency can be observed within the first 30 min. After the exchange of half of the respective phase volumes, the separation efficiency drops and then rises again steeply within the next 30 min to a value above 60 % before the next phase exchange takes place. In total, 2.5 times more enzyme-loaded bottom phase was purified than in batch ATPF during 120 min of flotation. The removed top phase showed only a slightly lower enzyme concentration than at the end of the batch ATPF. Thus, a significant increase in throughput can be achieved by semi-continuous exchange with almost constant concentration compared to batch ATPF.

The semicontinuous exchange of phases was used as a basis for the continuous exchange. After 30 min of flotation without phase exchange, fresh enzyme-enriched bottom phase was added at the bottom of the flotation cell or removed below the phase interface via the synchronized pumps P1 and P2 (see Fig. 1). The flow rates of pumps P3 and P4 were adjusted so that the V_R of the two phases did not change. A total of 1.5 times the batch volume was pumped between t 30 min and t 120 min, so that here, too, a total of 2.5 times the volume was purified compared to batch ATPF. With the same gassing as for the batch ATPF (10ccm), the separation efficiency, after a steep increase in the first 30 min, remains constant during the continuous phase exchange (see black triangles in Fig. 4 C). Consequently, the enzymes freshly added near the gas inlet adsorb to the bubble surfaces and are transported directly into the top phase where they are concentrated. Due to the selected time of the beginning phase exchange after 30 min, a stagnation of the mass transfer is prevented. In the following 90 min during the continuous ATPF, loaded top phase is thus continuously discharged. Increasing the gas input to 20 ccm led to a steeper increase in separation efficiency within the first 30 min and to a higher level during the continuous phase exchange in the following (see gray diamonds in Fig. 4 C). Due to the increased gas input, an increased flotative mass transfer of the enzymes into the top phase takes place. This confirms the crucial role of gas input, which has already been investigated in previous studies [25] and reveals a simple way to compensate for fluctuations during continuous ATPF

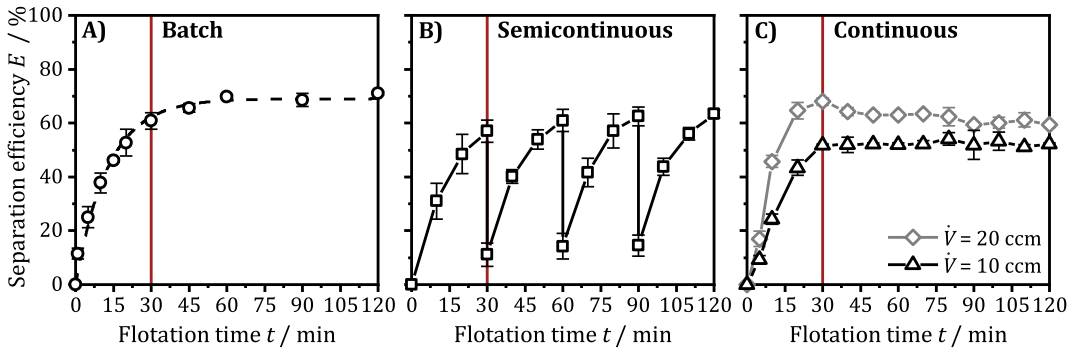

Fig. 4. Time course of separation efficiency for batch ATPF (A) (Data from [25]), semi-continuous ATPF (B) with 50% phase exchange every 15 min after 30 min flotation time, and continuous phase exchange (C) after 30 min flotation time.

operation to ensure consistent purification rates and high enzyme concentrations in the discharged top phase.

Both the stepwise and continuous exchange of the top or bottom phase increased the throughput (volume of purified bottom phase) by a factor of 2.5 compared to batch ATPF. These two modes of operation are preferable to batch operation for the purification of biosuspensions. The increased throughput can reduce either process times or plant volume. In the case of continuous production of biomolecules, continuous ATPF in particular is a promising purification method. Efficient concentration and purification of the target substance can be achieved with low equipment requirements. If further subsequent purification or formulation steps are necessary, these can be carried out simultaneously with lower volumes, which greatly reduces energy and equipment costs.

4. Conclusion

Batch ATPF has proved to be a dynamic process where mass transfer of the enzyme into the collection top phase follows an exponential progression during flotation time. The initial enzyme concentration in the bottom phase determines the final enzyme concentration in the bottom and top phases, which are caused by the distribution equilibria known from two-phase extraction. There are linear dependencies of initial enzyme concentration and final concentrations in the bottom and top phases. Thinking of an industrial process, these correlations can be used to achieve a wanted product concentration in the top phase, which can be crucial for following process steps like further purification, mechanical dewatering, or thermal drying. The main mass transfer was recognized at the beginning of the ATPF during the first 30 min of flotation. During the further progress of ATPF, mass transfer into the top phase decreases until final separation efficiency and die distribution equilibria are reached. To overcome the limitation caused by the decreasing concentration gradient during advancing flotation time, a step wise exchange of both phases, enriched bottom phase and unloaded top phase, was realized. Consequently, a 2.5-fold phase volume could be processed in 120 min of ATPF with the same separation efficiency. The transfer rate of this semicontinuous ATPF was used to define the continuous exchange of the phases using peristaltic pumps and hence realizing a successful continuous ATPF, which was described for the first time. The results demonstrate that continuous ATPF is more efficient than batch ATPF. Thinking of industrial downstream processes of biotechnological products, a continuous ATPF has even more advantages compared to batch ATPF. In the case of a continuous upstream process, continuous purification will lead to reduction of apparatus equipment (e.g. storage tanks) smaller ATPF cells, and allows direct further processing of the product-loaded top phase.

Further process development of ATPF should include an optimization of the ATPF apparatus which enables direct flow of the continuous exchange of bottom and top phases to guarantee high concentration gradients and hence faster separation. In combination with a suitable gas input and implementation of the online measurements of product concentration and phase stability, this will allow an ideal process control. To create an efficient industrial ATPF process, the findings presented should be used to design a scalable continuous pilot ATPF plant.

CRediT authorship contribution statement

Lucas Jakob: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization. **Marcel Heinzmann:** Methodology, Investigation, Validation, Formal analysis. **Hermann Nirschl:** Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] T. Li, X.-B. Chen, J.-C. Chen, Q. Wu, G.-Q. Chen, Open and continuous fermentation: Products, conditions and bioprocess economy, Biotechnol. J. 9 (12) (2014) 1503–1511, https://doi.org/10.1002/biot.201400084.
[2] P.-y. Bi, D.-Q. Li, H.-r. Dong, A novel technique for the separation and concentration of penicillin G from fermentation broth: Aqueous two-phase flotation, Sep. Purif. Technol. 69 (2) (2009) 205–209, https://doi.org/10.1016/j.seppur.2009.07.019.
[3] S.Y. Lee, I. Khoiroh, T.C. Ling, P.L. Show, Aqueous Two-Phase Flotation for the Recovery of Biomolecules, Sep. Purif. Rev. 45 (1) (2016) 81–92, https://doi.org/10.1080/15422119.2015.1007147.
[4] M. Rito-palomares, Aqueous Two-Phase Systems for Bioprocess Development for the Recovery of Biological Products, Springer International Publishing, Cham (2017), https://doi.org/10.1007/978-3-319-59309-8.
[5] P.Y. Bi, L. Chang, Y.L. Mu, J.Y. Liu, Y. Wu, X. Geng, Y. Wei, Separation and concentration of baicalin from Scutellaria Baicalensis Georgi extract by aqueous two-phase flotation, Sep. Purif. Technol. 116 (2013) 454–457, https://doi.org/10.1016/j.seppur.2013.06.024.
[6] J.S. Tan, S. Abbasiliasi, Y.K. Lin, M.S. Mohamed, M.R. Kapri, S. Kadkhodaei, Y. J. Tam, R.N.Z.R.A. Rahman, A.B. Ariff, Primary recovery of thermostable lipase 42 derived from recombinant Escherichia coli BL21 in aqueous two-phase flotation, Sep. Purif. Technol. 133 (2014) 328–334, https://doi.org/10.1016/j.seppur.2014.06.048.
[7] P.-Y. BI, L. CHANG, H.-R. DONG, Separation behavior of penicillin in aqueous two-phase flotation, Fenxi Huaxue/ Chinese J. Anal. Chem. 39 (3) (2011) 425–428, https://doi.org/10.1016/S1872-2040(10)60428-8.
[8] M. Li, H.-r. Dong, The investigation on the aqueous two-phase floatation of lincomycin, Sep. Purif. Technol. 73 (2) (2010) 208–212, https://doi.org/10.1016/j.seppur.2010.04.002.

[9] P.E. Kee, L.S. Cheah, P.K. Wan, P.L. Show, J.-W. Lan, Y.H. Chow, H.S. Ng, Primary capture of Bacillus subtilis xylanase from crude feedstock using alcohol/salt liquid biphasic flotation, Biochem. Eng. J. 165 (2021) 107835, https://doi.org/10.1016/j.bej.2020.107835.
[10] B. Jiang, L. Wang, J. Na, X. Zhang, Y. Yuan, C. Liu, Z. Feng, Environmentally-friendly strategy for separation of α-lactalbumin from whey by aqueous two phase flotation, Arab. J. Chem. 13 (1) (2020) 3391–3402, https://doi.org/10.1016/j.arabjc.2018.11.013.
[11] R. Sankaran, P.L. Show, Y.J. Yap, Y. Tao, T.C. Ling, K. Tomohisa, Green technology of liquid biphasic flotation for enzyme recovery utilizing recycling surfactant and sorbitol, Clean Technol. Environ. Policy. 20 (9) (2018) 2001–2012, https://doi. org/10.1007/s10098-018-1523-5.
[12] P. Mathiazakan, S.Y. Shing, S.S. Ying, H.K. Kek, M.S.Y. Tang, P.L. Show, C.W. Ooi, T.C. Ling, Pilot-scale aqueous two-phase floatation for direct recovery of lipase derived from Burkholderia cepacia strain ST8, Sep. Purif. Technol. 171 (2016) 206–213, https://doi.org/10.1016/j.seppur.2016.07.017.
[13] Y.K. Lin, P.L. Show, Y.J. Yap, C.P. Tan, E.-P. Ng, A.B. Ariff, M.S.B. Mohamad Annuar, T.C. Ling, Direct recovery of cyclodextringlycosyltransferase from Bacillus cereus using aqueous two-phase flotation, J. Biosci. Bioeng. 120 (6) (2015) 684–689, https://doi.org/10.1016/j.jbiosc.2015.04.013.
[14] C.H. Tan, P.L. Show, C.W. Ooi, E.-P. Ng, J.-W. Lan, T.C. Ling, Novel lipase purification methods - a review of the latest developments, Biotechnol. J. 10 (1) (2015) 31–44, https://doi.org/10.1002/biot.v10.110.1002/biot.201400301.
[15] P.L. Show, C.W. Ooi, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, M.S.M. Annuar, T. C. Ling, Recovery of lipase derived from Burkholderia cenocepacia ST8 using sustainable aqueous two-phase flotation composed of recycling hydrophilic organic solvent and inorganic salt, Sep. Purif. Technol. 110 (2013) 112–118, https://doi. org/10.1016/j.seppur.2013.03.018.
[16] S.V. Pakhale, M.D. Vetal, V.K. Rathod, Separation of Bromelain by Aqueous Two Phase Flotation, Sep. Sci. Technol. 48 (6) (2013) 984–989, https://doi.org/10.1080/01496395.2012.712596.
[17] P.L. Show, C.P. Tan, M.S. Anuar, A. Ariff, Y.A. Yusof, S.K. Chen, T.C. Ling, Direct recovery of lipase derived from Burkholderia cepacia in recycling aqueous two-phase flotation, Sep. Purif. Technol. 80 (3) (2011) 577–584, https://doi.org/10.1016/j.seppur.2011.06.013.
[18] W.N. Phong, P.L. Show, W.H. Teh, T.X. Teh, H.M.Y. Lim, N.S.B. Nazri, C.H. Tan, J.-S. Chang, T.C. Ling, Proteins recovery from wet microalgae using liquid biphasic flotation (LBF), Bioresour. Technol. 244 (2017) 1329–1336, https://doi.org/10.1016/j.biortech.2017.05.165.
[19] R. Sankaran, P.L. Show, Y.-S. Cheng, Y. Tao, X. Ao, T.D.P. Nguyen, D. Van Quyen, Integration Process for Protein Extraction from Microalgae Using Liquid Biphasic Electric Flotation (LBEF) System, Mol. Biotechnol. 60 (10) (2018) 749–761, https://doi.org/10.1007/s12033-018-0111-6.
[20] C.E. de Araújo Padilha, P.V.F. Dantas, C.d.C. Nogueira, A.L.d.S. Leitao, H. N. Almeida, D.F. de Santana Souza, J.A.d. Oliveira, G.R. de Macedo, E.S. dos Santos, Enhancing the recovery and concentration of polyphenols from camu-camu (Myrciaria dubia H.B.K. McVaugh) by aqueous two-phase flotation and scale-up process, Sep. Sci. Technol. 53 (13) (2018) 2126–2135, https://doi.org/10.1080/01496395.2018.1442865.
[21] H.Y. Leong, C.W. Ooi, C.L. Law, A.L. Julkifle, T.C. Ling, P.L. Show, Application of liquid biphasic flotation for betacyanins extraction from peel and flesh of Hylocereus polyrhizus and antioxidant activity evaluation, Sep. Purif. Technol. 201 (2018) 156–166, https://doi.org/10.1016/j.seppur.2018.03.008.
[22] K.S. Khoo, K.W. Chew, G.Y. Yew, S. Manickam, C.W. Ooi, P.L. Show, Integrated ultrasound-assisted liquid biphasic flotation for efficient extraction of astaxanthin from Haematococcus pluvialis, Ultrason. Sonochem. 67 (2020) 105052, https://doi.org/10.1016/j.ultsonch.2020.105052.
[23] R. Sankaran, P.L. Show, Y.J. Yap, H.L. Lam, T.C. Ling, G.T. Pan, T.C.K. Yang, Sustainable approach in recycling of phase components of large scale aqueous two-phase flotation for lipase recovery, J. Clean. Prod. 184 (2018) 938–948, https://doi.org/10.1016/j.jclepro.2018.02.301.
[24] C.E.d.A. Padilha, P.V.F. Dantas, F.C. Sousa Júnior, S.D. Oliveira Júnior, C.d. C. Nogueira, D.F.d.S. Souza, J.A. de Oliveira, G.R. de Macedo, E.S. dos Santos, Recovery and concentration of ortho-phenylphenol from biodesulfurization of 4-methyl dibenzothiophene by aqueous two-phase flotation, Sep. Purif. Technol. 176 (2017) 306–312.
[25] L. Jakob, J. Singer, H. Nirschl, Importance of gas input in aqueous two-phase flotation (ATPF), Chem. Eng. Sci. 233 (2021) 116391, https://doi.org/10.1016/j.ces.2020.116391.