Timo van Beek[1]
Michael Budde[2]
Jan van Esch[1,*]

Membrane-Freeze Concentration Hybrid for Temperature-Sensitive Biomolecules

Investigation, Application, and Techno-Economic Benefits

In order to close the technology gap between membrane technologies and spray/freeze-drying ideally with a technology that avoids thermal stress to sensitive enzyme solutions, the limits of freeze concentration for this application have been investigated. On laboratory scale it was found that average crystal sizes are > 300 μm despite high viscosity and ice separation is possible up to 42 % solids and > 1000 mm^{2}s^{-1} viscosity. No activity loss was observed during concentration. A combination of two-stage freeze concentration with a filter and wash column for ice liquid separation in an integrated setup with ultrafiltration has the greatest potential and was shown to be economically feasible in three out of four cases studied.

Supporting Information available online

1 Industrial Enzymes: Markets and Applications

Modern enzyme technology started in the beginning of the 20th century with first attempts of Otto Röhm who used trypsin from cattle pancreas extract for leather processing and who filed the first patent for application of tryptic enzymes in detergents in 1915 [1]. Over 100 years and many technological milestones later, enzymes are used in large quantities in applications ranging from detergents, animal feed, dairy, baking, textiles, pulp and paper, food processing or manufacturing of antibiotics, adding up to an overall market size of approximately 5 billion US $.

Main contributors to this success story from a technological point of view certainly are the development of large-scale sterile fermentation technology for antibiotics production in the 1940s which paved the way for efficient production of enzymes from microbial systems and of course the introduction of genetic tools in the 1970s and 80s that allowed construction of highly efficient expression systems and the design of tailor-made enzymes with ideal properties for the desired application.

The biggest share of the technical enzyme market is held by detergent enzymes (proteases, lipases, cellulases) with about 1 billion US $, followed by enzymes for animal feed like phytases, xylanases or proteases with a market of about 800 million US $ a^{-1}. Starch and sugar processing enzymes like cellulases, amylases or glucose isomerases are suspected to be in the range of 500–600 million US $. Markets for baking and leather processing are expected to be in a range of 500 million US $, respectively.

Commodity enzymes as described here require robust und economic manufacturing processes. Expression systems that allow extracellular excretion of the enzymes are preferred, as cell disruption with concomitant release of cell proteins, DNA, and other undesired substances can be avoided. Typically, the biomass is removed by simple unit operations like filtration or centrifugation, yielding the crude enzyme product which is then further processed via concentration and/or purification steps depending on the later use, e.g., decoloring of enzyme solutions for the application in detergents, or sterile filtration to comply with microbiological specifications of the product.

Additionally, technical enzymes must perform under extreme (nonphysiological) conditions like high pH values and high temperatures (detergent applications) or must survive harsh production steps like the hygienic treatment of forage. Nowadays, the scientist can choose from a large tool box of protein engineering methods to tackle these challenges [2, 3].

A consequence of the above-mentioned stability of technical enzymes is that they are relatively stable throughout their production process, which usually takes place in well-controlled equipment at 5–10 °C during downstream processing to limit enzyme losses and to control bioburden. Therefore, the loss of

[1]Timo van Beek, Jan van Esch
jan.van.esch@GEA.com
GEA Technology Center for Freeze Concentration and Melt Crystallization, de Beverspijken 7B, 5221 's Hertogenbosch, The Netherlands.

[2]Dr. Michael Budde
BASF SE, G-ENB/OP – O819, Phytase factory, Carl-Bosch-Strasse 38, 67056 Ludwigshafen, Germany.

enzyme activity during the production process is usually an inferior problem for producers of technical enzymes, but the picture changes, if the liquid enzyme concentrates need to be dried, which is usually done by spray-drying for technical enzymes. Of course, activity losses can be reduced if spray-drying parameters are optimized and suitable stabilizers have been identified, but typical losses for feed enzymes such as phytase, xylanase or glucanase still remain in the range of 10 % [4], which holds true also for many other commercial enzymes [5]. Therefore, technologies that enable dewatering of enzyme solutions without affecting their activity beyond the technical limitations of membrane processes could significantly contribute to reduction of production costs.

2   Freeze Concentration Introduction and State-of-the-Art in Ice Separation

The most common methods for removing water from dilute aqueous solution are membrane filtrations with limitations on viscosity, evaporation with limitations on temperature stress, foaming and fouling or drying with limitations on temperature stress and energy costs. An alternative way of taking water out of a solution is by freezing the water to ice crystals and then removing this water in the form of ice crystals. This principle is illustrated in Fig. 1. The positive aspects of freeze concentration are by nature the low temperature and absence of thermal stress, a closed system without vapor space which avoids losses of volatile components and absence of microbial activity which allows for weeks of continuous operation without intermediate cleaning. The low temperature also imposes limitations because it leads to high viscosity and to conditions with poor crystal growth with as result difficult ice separation.

Various crystallization systems are on the market. An overview can be found in [6, 7]. GEA uses in this study its IceCon® design for the crystallizers. This is a typical configuration for small- and medium-sized plants and features a wall-cooled drum crystallizer in which an anchor mixer is fitted equipped with scrapers to keep the walls ice-free and with internals for enhanced bulk mixing attached to the arms of the anchors.

Effective ice removal and separation can be done in wash columns specifically developed for this purpose. Based on the mechanical ice transport there are two types of wash columns that are distinguished, namely, the hydraulic and the piston-type wash column [8]. These have a typical limit for trouble-free operation with pure water production around 30–50 mm^{2}s^{-1} viscosity (GEA experience, [9]). To overcome this limitation, a multiple-stage setup can be selected in which ice flows to the most dilute stage and the concentrated liquor countercurrently to the most concentrated stage. The ice separation with a wash column is done in the stage with lowest viscosity and concentrate separation in a filter integrated in a production vessel or crystallizer with integrated filter at the point of the highest concentration in the system. There are also more open systems like overflow tanks with ice scrapers or centrifuges, but they are not considered for this study where aseptic production is a final goal.

The wash columns separate the ice by making a compressed bed of ice which is forced from bottom to top through a column. The bed is continuously fed from the bottom and scraped of at the top. As it moves at relatively slow speed from bottom to top, wash water is pressed into the bed from top to bottom to provide an intensive washing of the ice. The ice bed transport is generated by hydraulic forces of a slurry pump (HWC) or the direct mechanical action of a piston (PWC). The liquid leaves the column via a filter which is integrated in filter tubes in the column or in an annual ring around the column (HWC) or via a filter plate which is mounted on top of the piston (PWC). The typical filter width is 150 μm.

Unlike wash columns, the filters do not really separate the ice but they separate the final product and make a more concentrated ice slurry. The filters as presently applied are typically used for final product recovery in multiple-stage freeze concentration plants. They are characterized by a large flow in a draft tube vessel along a scraped surface filter of typically 100–150 μm in sieve width. Disadvantages are the high power input and a relatively large vessel volume. The ice separation systems are illustrated in the Supporting Information.

The limitations of the ice separation/freeze concentration are seen as a function of viscosity, residence time, crystal size, and filter mesh, presence of solids other than ice, number of stages, and requirement on the quality of produced water. Already 30 years ago GEA tried to reach higher final concentrations using decanters added to multiple-stage orange juice plants. [10].

This initiative was abandoned due to a too high addition of costs and complexity in operation compared to the benefit of extra concentration. Tab. 1 gives an overview of GEA experiences for maximum concentration that can be economically achieved in various food applications.

3   Development Target

As mentioned in the introduction, there is an interest from producers of technical enzymes to close the technology gap between membrane technologies and spray/freeze-drying ideally with a technology that avoids thermal stress to sensitive enzyme
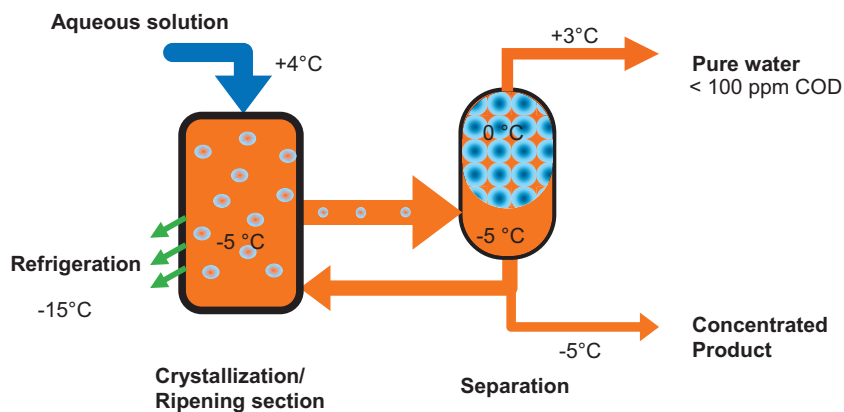

Figure 1. Schematic diagram of freeze concentration.

Table 1. General trends of freeze concentration for various products.

Product	Crystal size[a]	Max. solids content concentrate[b] [wt %]
Alcoholic beverages	Small	38
Fruit juices	Average to small	45
Coffee	Average	40
Dairy, whole milk	Large	44

[a]Small 200–500 µm, large 700–1000 µm averages; [b]alcohol also counted as solid, more correct is 1–water %.

solutions. An extra step always leads to extra complexity, so the revenues must make up for this disadvantage. This means that there must be a clear increase in solids content of the feed to the drying step.

Enzyme solutions and in general protein solutions, which have been handled by GEA in earlier applications, have a higher viscosity then encountered in typical freeze concentration application like juice concentration. High viscosities do not only have a direct impact on the actual functioning of the separation equipment but also on the crystal growth. For instance, it has been observed in multiple-stage orange juice concentration plants that in the most dilute stage at a viscosity of $8 \, \mathrm{mm^2 s^{-1}}$ the crystals grow to around 500 µm while in the most concentrated stage at a viscosity of $150 \, \mathrm{mm^2 s^{-1}}$ the achievable crystal size is limited to around 150 µm. As a consequence it was expected that one may have crystals that are well below 50 µm on average, leading to different filter designs or even rendering the development unfeasible.

The solution must also be economically viable and the sum of opex (lower energy) and capex must outperform the conventional systems. Absence of losses in activity or product is an important precondition for every new technology considered a technically feasible alternative.

In summary, the goals for a meaningful development of a concentration system for enzymes are:

– Total solids content high enough to make a step compared to membranes, target > 40 % solids.
– Separation that can handle the high viscosities and accompanying crystal sizes that are expected and well beyond what is feasible in present systems, target viscosities between 500 and $1000 \, \mathrm{mm^2 s^{-1}}$.
– No reduction in activity of the enzymes.
– Based on lower energy costs and saving in dryers investment, a payback time of less than three years.

4 Exemplary System

From our point of view, phytase was an ideal test system for the freeze concentration studies. It is available at big quantities, making freeze concentration experiments at pilot-scale possible. As described in the introduction, ultrafiltration also

here has its limits at about 27–30 % solids content, as transmembrane fluxes will drop dramatically at higher concentrations and therefore higher viscosities. Higher phytase concentrations would on the one side open the possibility for new liquid formulations with higher enzyme activities and on the other side reduce the water load for spray-drying, which is done for solid formulations.

For more than two decades, phytase is used as feed additive in animal nutrition. Here, BASF was the first to introduce phytase into the market in the early 1990s [11]. In diets for poultry and pigs, phytase dramatically minimizes the amount of phosphate excreted by the animal. The phosphorus load of manure is reduced by approx. 30 %, leading to a significant decrease of the environmental burden in animal husbandry. In addition to the beneficial environmental impact, phytase also helps the farmer to be economically more successful: Natuphos® cleaves phytate, i.e., the plant-bound phosphorus storage form, which is indigestible for poultry and pigs, and thereby minimizes the need of supplementation of inorganic phosphorus. In addition, it releases other phytate-bound nutrients such as calcium, zinc, copper, and magnesium but also amino acids. Finally, it also allows animals to metabolize more energy out of the same amount of feed.

Feed enzymes like phytase are produced by fermenting the filamentous fungus *Aspergillus*. Today, Natuphos® is one of the most important feed additives and is applied all over the world. Recently, BASF launched its new phytase Natuphos E®, which has been designed to meet the customers' needs for more efficient phosphorus release from phytic acid and higher thermostability in the feed manufacturing process.

To check if the phytase is not an exception in its behavior, tests were repeated on a smaller scale with hydrolyzed whey proteins. The proteins were WPC 60 from Serumitalia. First testing was done with powder and final tests were performed on fresh cooled solution.
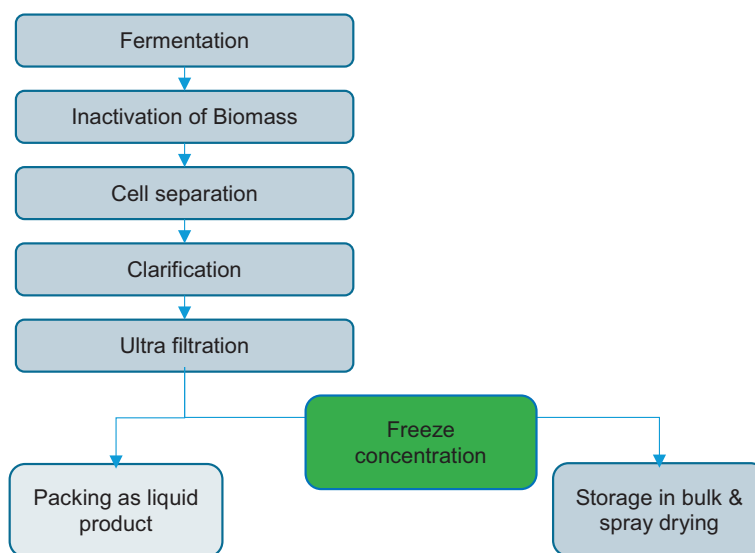

Figure 2. Exemplary system phytase, production scheme.

5   Methods

To investigate the freezing point curve, samples of the feed, dilutions of the feed, and concentrates from the lab-scale plant were put in individual test tubes and cooled down until crystallization started. A cooling bath was used to cool the samples down and a glass thermometer served as a stirrer, this way the freezing point could be measured during stirring.

When the crystallization had started, the sample would be removed from the cooling bath so the crystals would melt. The temperature of the sample during melting was relatively stable and this temperature reading was noted as the freezing point.

The viscosities of the samples were measured using an Ostwald tube viscosimeter making Tamson close to the freezing point of the samples. For determination of the concentration a Brix meter Atago pocket refractometer type PAL 1 was employed. The precision of the Brix meter was 0.1 % Brix. The relation between Brix and total dissolved solids (TS) was determined on a number of samples and is defined as: TS = Brix×0.7687 + 3.8035 for phytase and TS = Brix×0.91 for WPC 60.

Temperatures were measured with thermometers with an accuracy of 0.1 °C and in the crystallizer with temperature probes with an accuracy of ± 0.5 °C.

The crystal size distribution was determined by analyzing crystal photos. The setup for taking the photos is displayed in Fig. 3 below (different crystallizer). Ice slurry was pumped out of the crystallizer and passed through a microscope with a modified object glass. This glass was in a thermostated block and consisted of a chamber that was squeezed to the object glass to put the crystals in focus every time a picture was taken. The pump which was placed after the microscope to prevent melting in the pump before taking pictures, returned the slurry to the crystallizer. The pumping was approximately 6 L per hour. In the final setup the tubing was insulated with foam.

To analyze the crystal size, the photos were loaded into Autocad. In the lens of the microscope a measure line of 10 mm is integrated. This line is used to scale the photo back to the actual size. In this way, it is possible to measure the actual crystal size with the 'dimension' function of Autocad. For determination of the size, the biggest and smallest diameter of a crystal were measured and averaged.

The test setup for the continuous crystallization and separation experiments for the wash column separations and for the filter separation is schematically illustrated in the Supporting Information.

For the continuous test on freeze concentration of the phytase solution, a crystallizer was designed to fit the size of the separation equipment. This stainless-steel crystallizer was equipped with a scraped filter section attached to the crystallizer. The total content of the crystallizer was mixed and the filtrate was withdrawn with a hose pump.

The wash columns used for the phytase testing were scaled down 3-cm diameter versions of existing 6-cm diameter wash
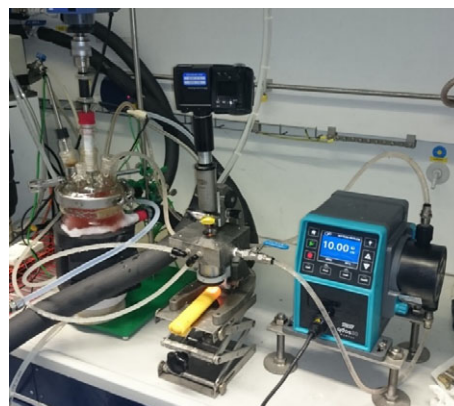

Figure 3. Crystal size measurement setup (note different crystallizer).

columns. The standard design for a 6-cm column is with a scraper at the top of the ice bed that only removes ice when the ice bed is compressed with the upward stroke of the piston. This ensures the bed is kept compact and homogeneous. Due to size limitations, the test column was not equipped with a scraper, but with a small melter that is working continuous. In the PWC this leads to some melting of the bed between the strokes and, as a result, the bed is not always as compacted and the washing is not as good as in a larger-size commercial wash column.

6   Results

The viscosity and freezing point curve for the phytase tests are presented in Fig. 4. As expected, the viscosity of the solutions is increasing to large values. The target to reach a final concentration of 41–44 % solids could be achieved despite the high viscosity.

The crystal size was analyzed in detail and at two different residence times of 2.5 and 1 h, respectively. Some typical crystal pictures are displayed in Fig. 5. The results are summarized in Fig. 6, which represents the average crystal size D50 (diameter
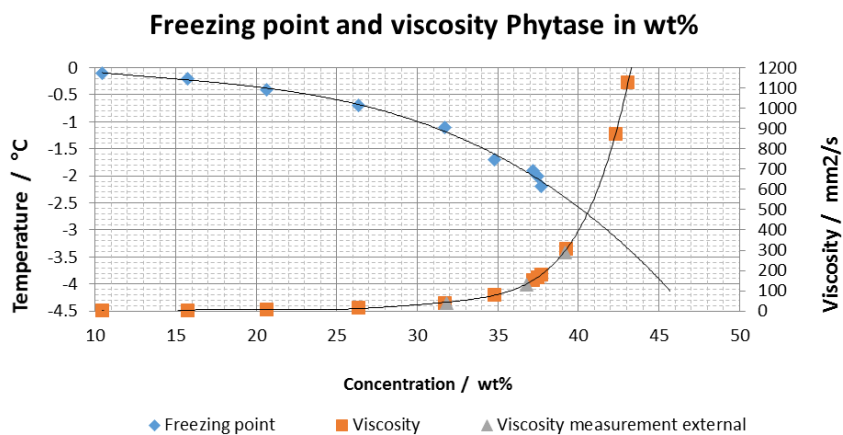

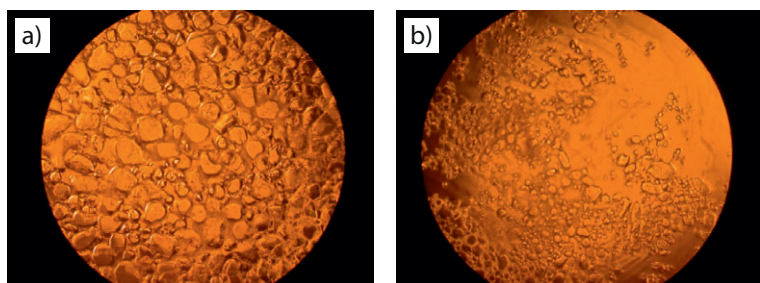**Figure 4.** Freezing point and viscosity phytase solutions.

Figure 5. Typical pictures of ice crystals in phytase solutions; (a) 2.5 h residence time, 37 °Brix; (b) 1 h residence time, 51 °Brix.
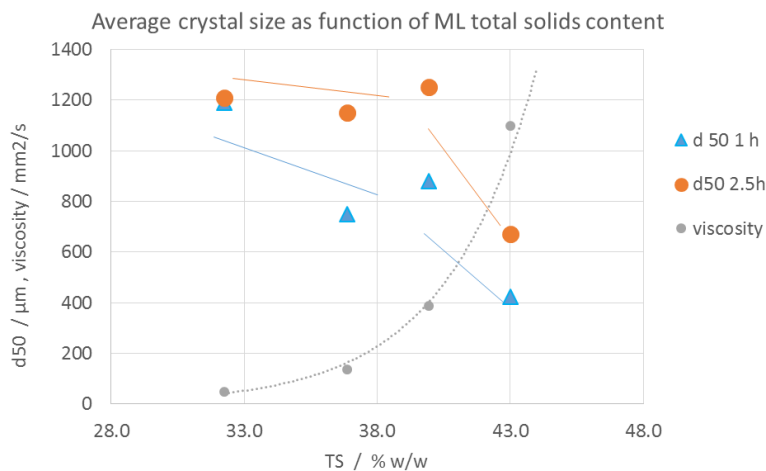

Figure 6. Average crystal size and viscosity as function of TS content.

at which half of the crystals by mass are bigger and half smaller). The crystal size distributions are indicated in the Supporting Information. As can be seen from the pictures, the crystals are fairly rounded, no needles or plates were observed.

From the crystal size and growth analysis it is apparent that the crystals on average grow very well and form large crystals. Average values of above 1000 µm are exceptionally high for freeze concentration plants. Only at the highest solids content and highest viscosity one can observe that the average crystal size is clearly reducing. This effect is most pronounced in the case of the shorter residence time of 1 h. From the population balance it is also visible that the nucleation rate for this condition is an order of magnitude higher than at lower concentration. This increase in nucleation rate will result in clearly smaller crystals if going to viscosities higher than tested in this research.

The crystal size analysis has a fairly high statistical component in it because of the strong influence of the few very large crystals and the relatively low number of crystals analyzed (around 300–400 per data point) which is the consequence of the non-automated method to be used. However, it is a valid method for analyzing the trends. The lines indicated in Fig. 6 are indications of trends by the author.

In summary, it can be said that the size of the ice crystals will not make special separation designs necessary and that conventional designs with filters of 75–150 µm will lead to losses of ice

in the final product of only a few percent. Residence times of 1 h for all but the most concentrated situations are sufficient and, when confirmed in pilot-plant trials, can serve as a basis for crystallizer design.

The activity was measured in four samples that have been collected at the beginning, in the middle, and at the end of a run. The fourth sample is from the crystallizer on the morning after the end of the test when the ice has melted down and diluted the sample again. The results are demonstrated in Fig. 7, where the activity per gram of solids is normalized with the first sample as 100 % of the activity. Although even a trend is obvious which indicates an increase in activity, we like to interpret this more conservative and conclude that the freeze concentration does not have a negative effect on the activity.

The operation of the wash column becomes more difficult with higher concentration/viscosities. This is expressed in a pressure drop in the filter with ice bed. When this pressure drop came above 5–6 bar, the operation was stopped. It was observed that more than doubling the filter open area by changing the sieve width does not have much influence on the pressure drop. The pressure drop is mainly determined by the characteristics of the ice bed along with the viscosity of the concentrate.

For the filter, the maximum pressure that could be applied on the crystallizer plus feed tank of 3 bar defined the limit of the operation. When this pres-
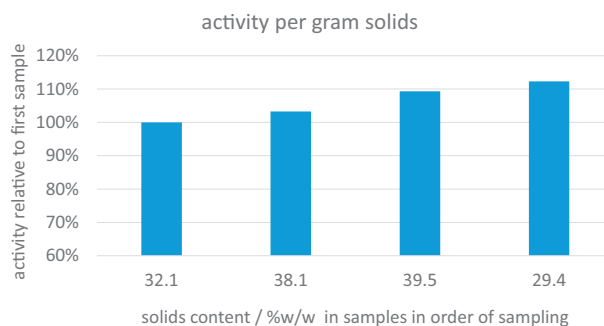

Figure 7. Development of the specific activity during freeze concentration.

sure was reached and higher viscosities were tried, the hose pump created a vacuum for which the discharge system was not designed. In a system with higher feed pressure to the filter higher viscosities may be handled. The limits of application that can be extracted from these experiments are summarized in Tab. 2. The tests were repeated with whey protein concentrate 60 with identical results.

Table 2. Achievable concentrations for the investigated separation technologies in freeze concentration of phytase.

	Max. Concentration [°Brix]	Max. concentration [wt %]	Viscosity [mm²s⁻¹]	Viscosity [mPa s]
Hydraulic wash column	45.6	38.9	270	320
Piston wash column	46.5	39.5	340	400
Filter (no washing)	51.1	43.1	1126	1340

spray-drying and freeze-drying were considered. As a reference plant, a membrane preconcentration till 27 % solids with following drying was considered. Cost estimations were based on available information inside GEA for the different technologies and must be considered as rough budget indications only.

7   Translation of Laboratory Results to a Plant Concept

As described in the previous paragraphs, the maximum concentration with the freeze concentration technology is with a filter as separation system. A single-stage system with freeze concentration and filtration will produce a concentrate and ice slurry. After the ice gets melted, this gives, compared to the feed, a dilute solution. The ice slurry needs to be pumpable, so one cannot go to extremely high ice concentrations and the final concentration would not meet the target of > 40 % TS. For that reason a two-stage setup is required. In the low-concentration stage, more favorable conditions exist for ice separation in a wash column and in the high-concentration stage a filter is used for concentrate production at maximum concentration.

The ice slurry from the filter stage goes to the low-concentration stage without melting. Because the wash column is working under conditions where no pure water is produced, the water with some product is sent to the membrane filtration system which precedes the freeze concentration, creating an integrated system with membrane filtration and freeze concentration. This setup is illustrated in Fig. 8. For special purposes, this setup can be extended to a salting-out or antisolvent crystallization of the produced enzymes or proteins. This leads to a complex and costly plant and is only interesting for very valuable crystals.

8   Economic Evaluation

The economy of a freeze concentration plant depends on the size of the installation and the drying system applied. If there is a very small system where for a special product only 10 kg h⁻¹ are dewatered, it normally makes no sense to add an extra processing step. Exceptions may be if this leads to extra product recovery of a very special product or, e.g., if an existing plant can be doubled in capacity without changes to the dryer.

The more interesting case is where there is a middle- to large-sized dryer. Consequently, a plant size was selected with 400 and 3500 kg h⁻¹ of dewatering in the freeze concentration system for a preliminary economic analysis. For drying, both

9   Conclusions and Outlook

Freeze concentration of enzyme and protein solutions was found to be in principle technically feasible and meaningful based on laboratory-scale test work and general experience with freeze concentration and membrane filtration.

From the economic evaluation the following points can be concluded:

– In combination with a spray dryer, there are no savings on capex for the small to medium capacity and low savings for the large capacity. What is saved in the dryer investment is spent in the freeze concentration. This makes the energy saving/opex the driver for introduction of the freeze concentration technology in combination with spray-drying. In the current example, this is feasible for the larger plant with a

Table 3. Preliminary economic evaluation.

| FC net dewatering [kg h⁻¹]	400	3.500
Plant solids production capacity [10³ kg a⁻¹]	1.800	15.900
Net savings spray-drying (SD) [€ a⁻¹]	276.849	1.791.722
Net savings freeze-drying (FD) [€ a⁻¹]	995.385	5.578.722
Investment FC [€]	1 000 000	2 700 000
ROI [a] (spray drying benchmark)	3.6	1.5
ROI [a] (freeze drying benchmark)	1.0	0.5
Assumptions		
Steam cost [€ t⁻¹]	25	
Electricity cost [€ kWh⁻¹]	0.08	
Steam consumption SD [kg_steam kg⁻¹W⁻¹]	3	
Electricity consumption SD [kW kg⁻¹W⁻¹]	0.2	
Electricity consumption FD [kW kg⁻¹W⁻¹]	2	
Steam consumption FD [kg_steam kg⁻¹W⁻¹]	2	
Electricity consumption FC [kW kg⁻¹W⁻¹]	0.09	
Electricity consumption MF [kWh t⁻¹]	10	
Value reduction hardware [% a⁻¹]	10	
Operation hours [h]	6000	
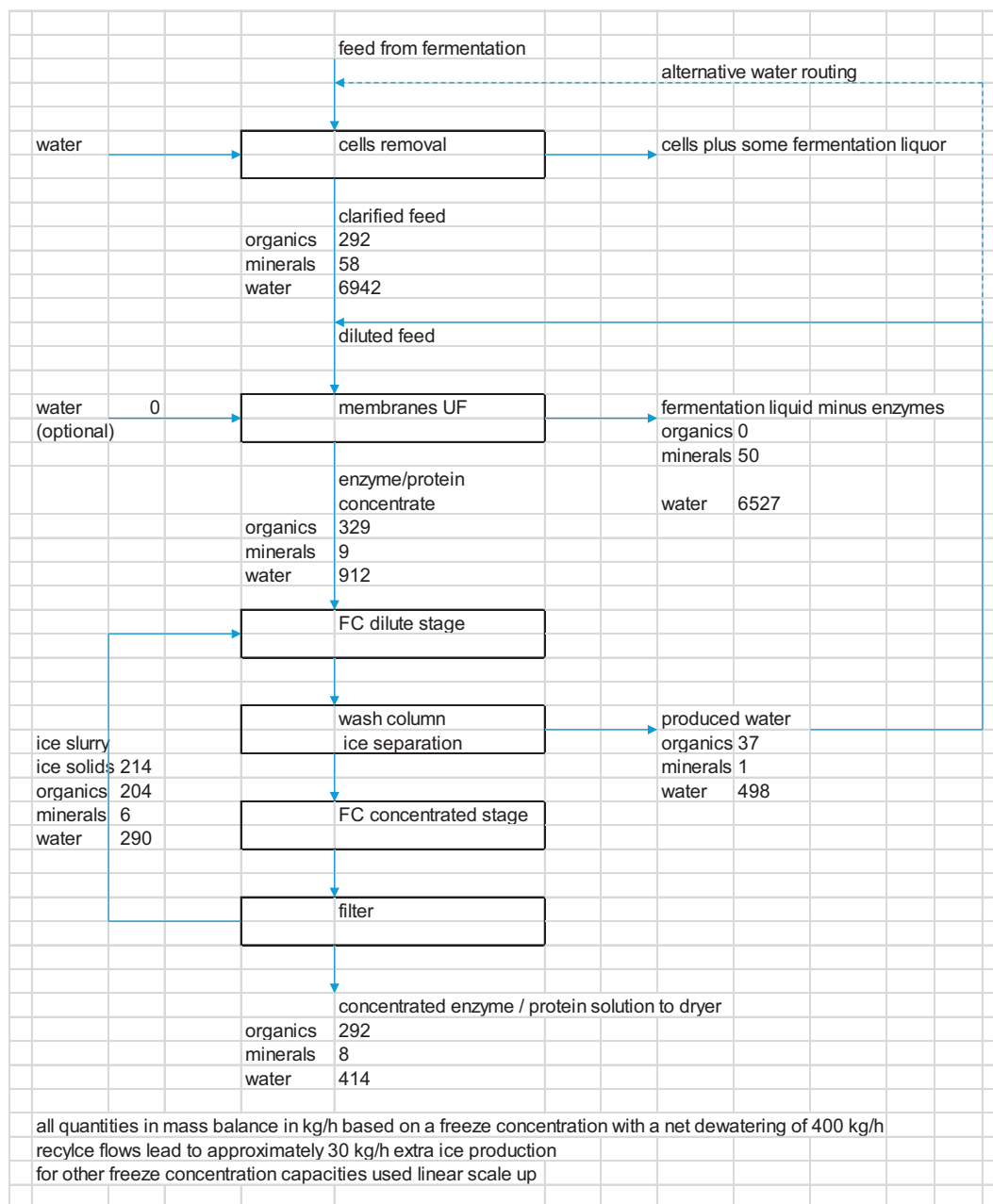
Figure 8. Schematic diagram of the hybrid system involving membranes and freeze concentration.

payback of 1.5 years but not for the smaller plant with a payback of around 4 years.

- In combination with freeze-drying the freeze concentration is always economically feasible both from a perspective of capex and opex.
- A further contribution to the economic feasibility of a freeze concentration between ultrafiltration and spray-drying might arise from a higher overall product recovery, as a large proportion of water can be removed without loss of activity (Fig. 7 and current drying losses [5]). Further experiments that investigate the activity loss of highly concentrated enzyme solutions during spray-drying are necessary to eval-

uate if these benefits really materialize. For freeze-drying these profits can be reasonably expected.

The laboratory results are currently confirmed in a pilot plant. The behavior of the filter construction at low viscosities is proven and at higher viscosities the test program is ongoing. For low viscosities, the new filter is ready to go to market for freeze concentration applications but also as a thickener in melt crystallization at the end of 2018. For high viscosities, the go to market is planned for the first quarter of 2019.

Acknowledgment

References

[1]   K. Buchholz, J. Collins, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 3747–3762.

[2]   J. M. Woodley, *Curr. Opin. Chem. Biol.* **2013**, *17*, 310–316.

[3]   U. T. Bornscheuer, *Nature* **2012**, *485*, 185–194.

[4]   R. Barendse, H. P. Harz, R. J. Betz, (Gist Brocades), *US005827709A*, **1998**.

[5]   A. Pilosof, V. Sánchez, in *Handbook of Industrial Drying*, 4th ed. (Ed: A. S. Mujumdar), Taylor & Francis, London **2015**.

[6]   J. Machado, Y. Ruiz, J. Auleda, E. Hernandez, M. Raventos, *Food Sci. Technol. Int.* **2009**, *15*, 303–315. DOI: https://doi.org/10.1177/1082013209344267

[7]   J. Sanchez, E. Hernandez, J. Auleda, M. Raventos, *Food Sci. Technol. Int.* **2011**, *17*, 5–13. DOI: https://doi.org/10.1177/1082013210382479

[8]   F. Qin, M. Yang, X. Yang, X. Chen, A. Abeynake, *AIChE J.* **2009**, *55 (11)*, 2835–2847.

[9]   D. Verdoes, M. Nienoord, in *Melt Crystallization: Fundamentals, Equipment, Applications* (Eds: J. Ulrich, H. Glaade), Shaker Verlag, Aachen **2003**.

[10] R. Ruemekorf, M. Koeleman, W. Swinkels, *8th World Congress of Food Science and Technology*, Toronto, September **1991**.

[11] S. Haefner, A. Knietsch, E. Scholten, J. Braun, M. Lohscheidt, O. Zelder, *Appl. Microbiol. Biotechnol.* **2005**, *68*, 588–597.