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Optimization of Removal of D-Trehalose from Commercial Food Colorant by Diafiltration with Ultrafiltration Membranes

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A dia-ultrafiltration process for purification of phycocyanin-based solutions is presented. The aim of the process is to remove additives present in a commercial food colorant, mainly D-trehalose. Optimization of parameters such as molecular weight cut-off, initial concentration of feed, process temperature and transmembrane pressure has been conducted. The focus has been on obtaining a fast and effective removal of additives, while at the same time keeping operational conditions mild enough to avoid degradation and hereby loss of colour.

1. Introduction

The increased focus on a healthy lifestyle among the population has led to a trend away from synthetic additives towards natural ones in the food industry. A common natural source of blue pigments is phycobiliproteins, which can be extracted from different cyanobacterial species such as *Spirulina* (Herrera et al. 1989).,Recently Patel et al. (2005) further extracted phycobiliproteins from *Phormidium* and *Lyngbya* species. The blue colour of these pigments is caused by tetrapyrrole chromophores, which are covalently bound to the proteins. Phycobiliproteins are typically commercialized as colorant mixtures with sugar and citrate salt, with the aim of increasing their stability during product shelf life (Martelli et al. 2014). Nevertheless, the available natural blue colorants show sometimes poor stability and changes of hue and intensity when exposed to different conditions such as pH, temperature, presence of additives (Chaiklahan et al. 2012) or processing conditions (Sarada et al. 1999). Cleavage of the chromophore from the proteins is a necessary step for further chemical stabilization of the tetrapyrrole chromophore. In order to avoid undesired side reactions during the cleavage process, a solution of phycobiliproteins free of sugars and citrate salt is necessary.
The difference in size between the proteins and additives is compatible with a size exclusion separation process based on membrane technology. Clarification and concentration of *Spirulina* extracts by the means of micro- and ultrafiltration (Jaouen et al. 1999) and nanofiltration and reverse osmosis (Chaiklahan et al. 2011) has been reported in literature. Diafiltration of sugars with ultrafiltration membranes is a very well explored field (Lipnizcki et al. 2002). During this pretreatment step preservation of proteins is important, since denaturation could release the chromophore allowing it to permeate through the ultrafiltration membrane.

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2. Materials and methods

2.1 Materials

The raw material selected was the *Spirulina*-based colorant Linablue G1 (DIC Europe GmbH). It was purchased in powder form and was stored in a dark, dry place at room temperature until processing. D-trehalose used for the calibration standards was analytic grade (Sigma-Aaldrich).
Optimization of the diafiltration process was carried out in a Minimem Lab Scale Membrane Filtration Unit (PS Prozesstechnick GmbH) combined with a Control Unit for Automatic Diafiltration and Continuous Operation (PS Prozesstechnick GmbH). The membranes used were Alfa Laval-GR51PP (Batch no.: 113351-03) and Alfa Laval-GR40PP (Batch no.: 110241-01) with molecular weight cut-offs (MWCOs) of 50 and 100 KDa respectively. All experiments were carried out with deionised water (Purelab Chorus, Krüger Aquacare)

2.2 Experimental set-up for diafiltration

The diafiltration process setup is shown in Figure 1. The feed vessel (T02) containing the colorant feed and a magnet was placed on top of a magnetic stirrer (MS01). Water contained in the diafiltration solvent vessel (T01) was added to the feed vessel through a diaphragm pump (P01) at a flow rate determined by the control unit through an optical level sensor (LS) which ensured the level in T02 was constant throughout the process. An HPLC pump (P02) pumped the feed at a constant flow rate to the membrane module (MM). The pressure at the entrance of the module was monitored by a manometer (PS) coupled to a venting valve (V01) to protect the HPLC pump from possible overpressure. The temperature in the membrane module was regulated by a heat exchanger (HE) connected to a cooling bath (not in the Figure). The membrane module contains a magnet aimed to create turbulence at the membrane surface, and hence was placed on top of a magnetic stirrer (MS02). The temperature and rotation speed in the module were monitored by two sensors (TS and RS, respectively). A spring valve (V02) was used to regulate manually the pressure in the module. The retentate stream was recycled to the feed vessel, and the permeate stream was collected in the permeate vessel (T03). A scale (S) was used to monitor the permeate flux.
All experiments were performed at a constant feed flow rate of 10 ml min^{-1}. The stirring in the feed vessel was 200 rpm and 500 rpm in the membrane module. The total membrane area was 25 cm^2. Cleaning of the set up between experiments consisted of a flush with water (20 min), recycle of 0.08 % NaOH at room temperature (20 min) and a final flush with water (20 min).
Water flux was monitored before use, after water rinse and after the combination of basic cleaning and water flush, to quantify the recovery of the flux and the cake formation. All experiments were carried out in triplicate.

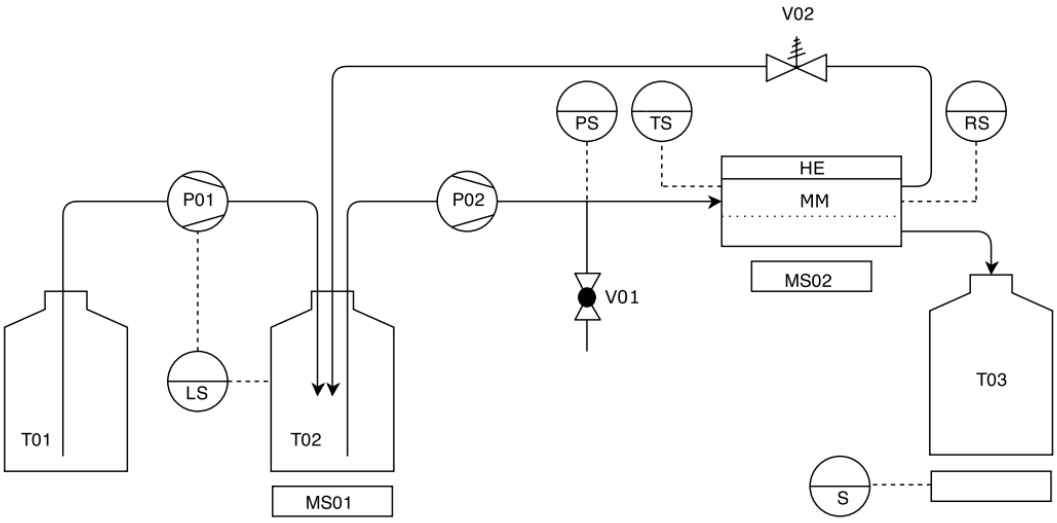

Figure 1. flow diagram of the process for purification of phycobiliproteins by dia-ultrafiltration

2.3 Analysis

D-trehalose content in permeate samples taken at intervals of 30 or 60 minutes, feeds and products was determined by HPLC equipped with a refractive index detector. Separations were performed on a Rezex™

HPLC column (300 x 7.8 mm). The mobile phase was deionized H_2O at a flow rate of 0.6 mL·min^{-1}. Analysis of the proteins in the process streams was done by HPLC equipped with a diode array detector (DAD). Separations were performed using a C18 reverse phase column (150 x 4.6 mm). Absorbance was measured at 280, 365, 616 and 660 nm. The mobile phase was a gradient mixture of H_2O and acetonitrile, both containing 0.05 % v/v of formic acid. Absorbance of feeds, final products and permeates was measured by a UV-Vis Spectrophotometer (UV-1650PC, Shimazdu).

3. Results and discussion

The intense blue colour of Linablue G1 is caused by two phycobiliproteins present in *Spirulina* genus algae: C-phycocyanin (C-PC) and allophycocyanin (APC). These proteins have been extracted from the algal cells and characterized in the literature by HPLC (Bermejo et al. 1997). Linablue G1 contains 55 % w/w of D-trehalose, 5 % trisodium citrate, and the rest is algal material. Quantification of C-PC and APC in Linablue G1 was done according to the procedure of Yoshikawa and Belay (2008) obtaining a concentration in the powder of 21.5 ±0.3 and 6.2 ±0.1 % in weight of C-PC and APC, respectively.

3.1 Molecular weight cut off

The molecular weight of the proteins was estimated from the molecular weights of their α and β subunits as reported by Moreno et al. (1997), and assuming they are found in solution as trimers $(\alpha\beta)_3$ as the lowest aggregation state. Therefore the molecular weights are expected to be 121.5 and 111.9 KDa for C-PC and APC trimmers, respectively. The molecular weights of D-trehalose and trisodium citrate are 378 and 256 Da, respectively.

Two membranes with different MWCOs were tested for filtration of Linablue G1 at a trans-membrane pressure (TMP) of 8 bar, temperature of 24 °C, and feed flow of 10 ml min^{-1}. The membrane Alfa Laval GR81PP with MWCO of 100 KDa showed a rejection of proteins R_{obs} of 0.97 ± 0.01, based on Equation 1, where C is the concentration of the species studied. For phycobiliproteins, the ratio of concentrations was substituted by the ratio of the absorbance of the streams at 616 nm.

$$R_{obs} = 1 - \frac{C_{permeate}}{C_{feed}} \tag{1}$$

Although the observed rejection was high, the protein's colour is so intense that even a small amount of permeation causes a light blue colour in the permeate stream easily noticeable by the naked eye. This fact agrees with our hypothesis that the proteins are found in trimers of molecular weight around 100 KDa, and hence can permeate through the membrane.

The membrane AlfaLaval GR51PP with MWCO of 50 KDa showed complete rejection of proteins as found by the absorbance method. Only at high concentration of Linablue G1 did some traces of protein appear in the HPLC-DAD chromatogram. The rejection of D-trehalose was 0 for both membranes. Trisodium citrate was washed out very early during diafiltration and was not quantified.

3.2 Process temperature

It is well known that permeate fluxes in membrane filtration are influenced by the process temperature due to the inverse relation between the flux and the viscosity of the permeate, which is temperature dependent. This relation is shown in Eq(2), where J is the permeate flux, ΔP is the difference in pressure across the membrane, $\Delta\pi$ is the difference in osmotic pressure across the membrane, μ is the viscosity of the permeate, and R_T is the resistance to the permeation caused by the membrane itself and other phenomena opposing resistance to the flux (if any).

$$J = \frac{\Delta P - \Delta\pi}{\mu \cdot R_T} \tag{2}$$

While a high temperature increases the flux it also promotes protein degradation. Thus the choice of operation temperature is a balance between having a short operation time at room temperature, and minimizing protein loss by working at a moderately low temperature.

Two sets of experiments were performed with constant parameters of initial concentration of Linablue G1 of 2 mg mL^{-1}, 50 mL feed, TMP of 8 bar and a feed flow of 10 mL min^{-1}. The temperatures studied were 24 and 5 °C. Because of the inherent variation in the three small membrane cut-outs, the flux is stated relative to the pure water flux at 24 °C and 8 bar for a more representative comparison. The absorbance decay was quantified from the absorbance at 616 nm of the final product and the feed. The differences observed between the two experiments are shown in Figure 2. Operation at low temperature resulted in a slightly lower flux, but compared to the reduction in protein decay, the small flux decay is not significant.

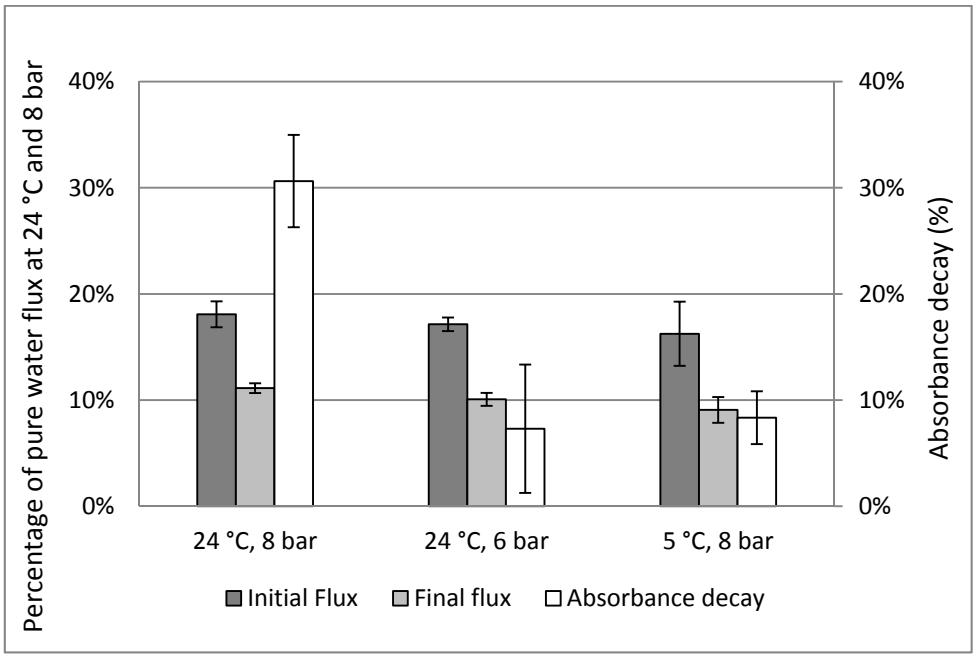

Figure 2. permeate flux and absorbance decay along filtration at two different temperatures and pressures

3.3 Trans membrane pressure

As shown in Eq(2), the flux is directly proportional to the trans-membrane pressure. However, it is known that at a certain point the increase in pressure does not cause the flux to increase any further. Comparison of two different TMPs was intended to check whether a moderately lower pressure would actually cause a flux decrease and to which extent. Two sets of experiments were performed with constant parameters of initial concentration of Linablue G1 of 2 mg mL^{-1}, 50 mL feed, temperature of 24 °C and a feed flow of 10 mL min^{-1}. The pressures studied were 8 and 6 bar.
The differences observed between the two experiments are shown in Figure 2. Operation at 6 bars does not decrease the flux significantly. It does though increase the stability of the proteins, as the absorbance decay at 616 nm before and after filtration is reduced. This can be due to the fact that at lower pressure the proteins are not subjected to the same mechanical stress and shear, hence the process is milder.

3.4 Concentration and cleaning

Four different concentrations of colorant were tested for dia-ultrafiltration to quantify the amount of D-trehalose removed per unit time and solvent volume. The concentrations tested were 0.2, 3, 6, and 10 % w/w of Linablue G1 in aqueous solution. The feed volumes were 50 mL and 1000 mL of diafiltration solvent available. The experiments were conducted until no more D-trehalose was detected in the permeate. The main difference observed was in the flux tendency, as it is shown in Figure 3.

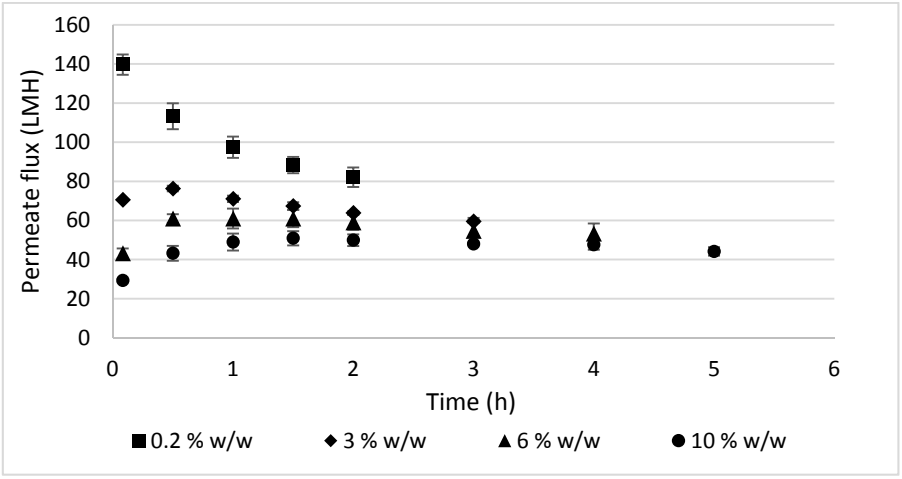

Figure 3. permeate flux at different initial concentrations of Linablue G1 in aqueous solution

The most diluted solution shows the typical flux decay trend with time, while the rest of the solutions show a low initial flux that first increases and tends to decrease after some time to converge into a constant flux when all the D-trehalose is removed. The initial increase is partially attributed to the high viscosity of the feed at the initial conditions, partly to the high solute concentration. A high concentration of proteins and D-trehalose causes build-up of concentration near the membrane that increases the resistance to the flux. As the diafiltration process takes place and the feed becomes more diluted, this influence becomes less significant. It is to be noted that since no proteins are being removed in the permeate; the initial tendency cannot only be attributed to the high concentration of proteins, because in that case that resistance would be constant throughout the process. The concentration-polarization phenomena must be caused by D-trehalose itself or a combination of D-trehalose and proteins.

The rate at which D-trehalose was removed increased with concentration, as is shown in Figure 4. The increase in filtration times is compensated by a higher degree of overall D-trehalose removal per hour and per ml of solvent used.

No significant deviation was observed in the pure water flux before operation and after performing the cleaning procedure detailed in section 2.2.

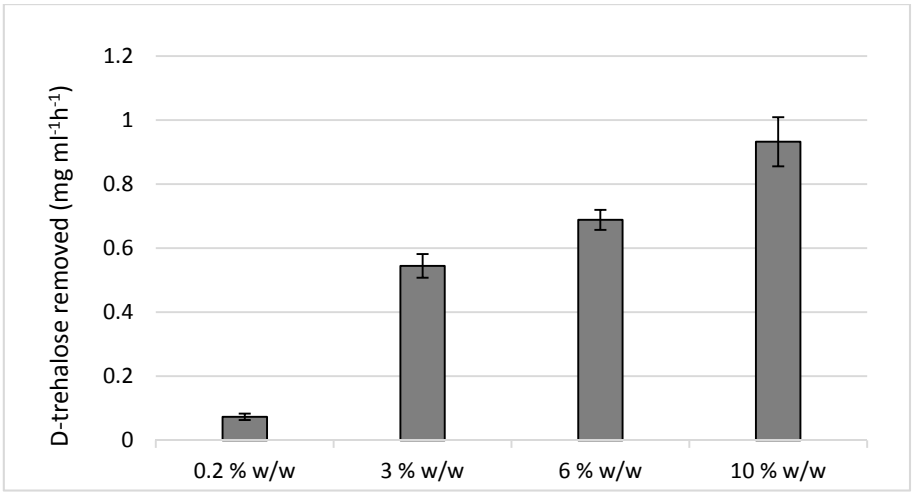

Figure 4. overall D-trehalose removal at four different concentrations of Linablue G1

4. Conclusions

The diafiltration process for removal of additives from commercial Linablue G1 by using 50 KDa polypropylene ultrafiltration membranes and water as diafiltration solvent has been proven effective, maintaining an acceptable degree of protein preservation at a process temperature of 5 °C. A trans-membrane pressure of 6 bar is preferred compared to 8 bar since it resulted in a lower degree of degradation and only a relatively small flux decrease. The optimal initial concentration tested was 10 % w/w of Linablue G1 in aqueous solution despite of its high viscosity and hence low initial flux. This concentration gave the fastest overall removal of D-trehalose. Cleaning of the membranes after use with a 0.08 % w/w solution of NaOH resulted in complete flux recovery which makes this process suitable as an industrial application.
The process suggested forms a necessary part of a method for production of a novel natural colorant based on phycobiliproteins from Linablue ®.

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