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Synthetic polymers are more effective than natural flocculants for the clarification of tobacco leaf extracts

Johannes F. Buyel[a,*], Rainer Fischer[a,b,1]

[a] Institute for Molecular Biotechnology, Worringerweg 1, RWTH Aachen University, 52074 Aachen, Germany
[b] Fraunhofer Institute for Molecular Biology and Applied Ecology, Forckenbeckstraße 6, 52074 Aachen, Germany

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ABSTRACT

The use of synthetic polymers as flocculants can increase filter capacity and thus reduce the costs of downstream processing during the production of plant-derived biopharmaceutical proteins, but this may also attract regulatory scrutiny due to the potential toxicity of such compounds. Therefore, we investigated the efficacy of three non-toxic natural flocculants (chitosan, kaolin and polyphosphate) alone and in combination with each other or with a synthetic polymer (Polymin P) during the clarification of tobacco leaf extracts. We used a design-of-experiments approach to determine the impact of each combination on filter capacity. We found that Polymin P was most effective when used on its own but the natural flocculants were more effective when used in combination. The combination of chitosan and polyphosphate was the most effective natural flocculant, and this was identified as a potential replacement for Polymin P under neutral and acidic extraction conditions independent of the conductivity, even though the efficiency of flocculation was lower than for Polymin P. None of the tested flocculants reduced the concentration of total soluble protein in the feed stream or the recovery of the model fluorescent protein DsRed.

1. Introduction

The advantages of plants for the production of biopharmaceutical proteins include their scalability and low pathogen burden (Fischer et al., 2013), but these are often outweighed by the high costs associated with downstream processing (DSP) (Wilken and Nikolov, 2012). We have previously shown that flocculation, the aggregation of dispersed particles induced by synthetic polymers, can reduce the consumables costs associated with clarification by 50% and thus improve the competitiveness of plant-based protein expression systems (Buyel and Fischer, 2014b,c). However, synthetic polymers may be toxic (Shirzad-Semsar et al., 2007), difficult to source, or incompatible with subsequent DSP steps (Buyel and Fischer, 2014b), so more sustainable alternatives are desirable. Naturally occurring flocculants include inorganic polymers (e.g. polyphosphates), biopolymers (e.g. chitosan from shrimp or fungi (Pochanavanich and Suntornsuk, 2002; Sini et al., 2007)), plant extracts (Sellami et al., 2014) or mineral clays (e.g. kaolin), and these have been used for the processing of manure (Garcia et al., 2009), lignocellulosic materials (Duarte et al., 2010) and waste water (Ji et al., 2010). Although natural polymers can also be toxic, the toxicity may be reduced by chemical modification (Khaira et al., 2014). The efficacy of flocculation can be improved if different types of agents are combined (Duarte et al., 2010; Gregory and Barany, 2011; Rytwo et al., 2013) but flocculation may also increase the rate of membrane fouling (Wang et al., 2013). Here we used a design-of-experiments (DoE) approach to compare the performance of natural flocculants, alone or combined with each other or with the synthetic cationic polymer Polymin P (BASF, Germany), for the processing of tobacco leaf extracts. The resulting predictive models identified conditions under which natural flocculants can replace synthetic polymers, their impact on dissolved protein concentrations and thus the overall product yield.

2. Materials and methods

Transgenic tobacco plants expressing the fluorescent protein DsRed were cultivated in the greenhouse as previously described

Abbreviations: DoE, design of experiments; DSP, downstream processing; LDS-PAGE, lithium dodecylsulfate polyacrylamide gel electrophoresis; NTU, nephelometric turbidity unit; pDADMAC, poly-diallyldimethylammonium chloride; TSP, total soluble protein.

* Corresponding author. Tel.: +49 241 6085 13162; fax: +49 241 6085 10000.
E-mail addresses: johannes.buyel@rwth-aachen.de (J.F. Buyel), fischer@molbiotech.rwth-aachen.de (R. Fischer).
[1] Tel.: +49 241 6085 11020.

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(Buyel and Fischer, 2014e). We homogenized 100–150 g of leaf tissue in a blender for 3 × 30 s (with 30-s breaks) in three volumes (3 mL g^{-1}) of extraction buffer (50 mM phosphate, pH 8.0, 10 mM sodium bisulfite, and either 75 or 600 mM NaCl according to DoE specifications, resulting in conductivities of ∼15 or ∼45 mS cm^{-1}). The pH was adjusted after extraction and flocculant stock solutions (60 g L^{-1}) were added to 20-mL extract aliquots, as required by the DoE setup. After topping up with buffer to 22 mL, the mixture was agitated for 20 s and then incubated without agitation for 10 min. If two flocculants were used, the above procedure was repeated for the second flocculant. The samples were then filtered through two layers of Miracloth (Merck, Germany) which behaves like 1.0–1.5 μm bag filters (Buyel and Fischer, 2014c) and the turbidity of the filtrate in nephelometric turbidity units (NTUs) was measured using a 2100P turbidimeter (Hach, CO, USA). An initial 60-run IV-optimal DoE was used to identify effective flocculant concentrations in the range 0.1–10 g L^{-1} at pH 6.0–8.0 and conductivities of 15 or 45 mS cm^{-1}. The models were refined in an IV-optimal design with 172 runs using flocculant concentrations of 0.5–3.0 g L^{-1} at pH 4.0–8.0 and conductivities of 15 and 45 mS cm^{-1} also taking interactions between two different flocculants into account. The DoE procedure is described in detail elsewhere (Buyel and Fischer, 2014a). The concentrations of total soluble protein (TSP) and DsRed were determined by the Bradford method (Simonian and Smith, 2006) and fluorescence spectrometry, respectively (Buyel and Fischer, 2014c).

3. Results and discussion

3.1. Chitosan alone induces visible flocculation in plant extracts

In an initial 60-run DoE, we measured the turbidity reduction achieved after bag filtration when chitosan (poly(D)glucosamine), kaolin (kaolinite, $Al_2Si_2O_5(OH)_4$) or polyphosphate (sodium metaphosphate, $Na_{n+2}P_nO_{3n+1}$, $n \approx 16$) were added to the extract, compared to a flocculant-free control (Fig. 1A–D). Polyphosphates can act as de-flocculants (Dobias, 1993) but also increase the hydrodynamic radius of dispersed complexes, especially when interacting with molecules of opposite charge (Cini and Ball, 2014). Anionic polymers are also effective flocculants with negatively charged particles (Menkhaus et al., 2010) and may therefore prove useful as co-flocculants if combined with highly charged cationic polymers such as Polymin P. We used flocculant concentrations in the range 0.1–10 g L^{-1}, which is ideal for chitosan (Rizzo et al., 2010; Sarika et al., 2005), overlaps with the values reported for kaolin (Duarte et al., 2010) and matches our previous results with synthetic polymers (Buyel and Fischer, 2014c). Chitosan was the only individual natural flocculant that reduced the turbidity of tobacco leaf extracts after bag filtration, achieving 40% reduction but only at pH 6.0 (Fig. 1B). This reflects the increasing solubility and positive charge of the polymer with decreasing pH due to protonation of the amine groups with a pK_a of ∼6.2–7.0 (Eierdanz, 2008; Santiago de Alvarenga, 2011). Our previous data also showed that cationic polymers are the most effective flocculants in tobacco leaf extracts (Buyel and Fischer, 2014c). When kaolin was added, the turbidity increased ∼2-fold compared to flocculant-free or polyphosphate-treated extracts (∼5000 NTUs, independent of the pH and flocculant concentration) probably reflecting the presence of >50% (w/w) sub-μm particles in the clay (Benea and Gorea, 2004; Mackinnon et al., 1993) which pass through the Miracloth filter. Because the flocculant concentration did not have a significant impact on turbidity within the investigated range, we used 0.5–3.0 g L^{-1} for all subsequent experiments, depending on the DoE requirements.

3.2. Polymin P is a more effective flocculant for plant extracts than non-synthetic counterparts

Based on an IV-optimal DoE with 172 runs, we built models describing the effect of sequential addition of the same or different flocculants (including the buffer control) on the bag filtrate turbidity as well as the concentration of TSP and DsRed. The quality of the predictive models was fair (turbidity) to good (TSP, DsRed) as shown in Table 1. The conductivity (factor D), concentration of the second flocculant (factor F) and the combination of flocculant concentrations (factor AB) had the greatest impact on bag filtrate turbidity based on the corresponding *F*-values (the mean square of the factor divided by the mean square of the residuals (Anderson and Whitcomb, 2000)). Chitosan alone reduced the bag filtrate turbidity by ∼15% at pH 6.0 and a conductivity of 15 mS cm^{-1}. In contrast, the addition of polyphosphate had no impact on turbidity and kaolin increased it 2-fold. When Polymin P was added to the extract, the bag filtrate turbidity was reduced by at least 50% across all pH, conductivity and concentration ranges. The best results (∼95% turbidity reduction) were achieved at pH ∼8.0 and a conductivity of ∼15 mS cm^{-1}, consistent with our previous results where at least 10 synthetic polymers reduced the turbidity of a tobacco extract more effectively than chitosan in this study (Buyel and Fischer, 2014c). Our results also agree with another study reporting that synthetic cationic polymers such as polyethylenimine can reduce the turbidity of lignocellulosic hydrolysates by >90% within less than 20 min, surpassing the performance of inorganic flocculants such as alum (Yasarla and Ramarao, 2012). Interestingly, we found that the turbidity was reduced more (∼80% instead of 50%) when Polymin P was the second flocculant, i.e. no other agent or buffer control was added, not even a second injection of Polymin P. A possible explanation is that when Polymin P was added as the first agent, the flocs that formed within the initial 10-min incubation period were disrupted during agitation following the addition of the second agent and did not re-form in the subsequent 10-min incubation period. This is supported by our previous observations that prolonged mixing times of more than 15–30 s reduce the effectiveness of flocculation by Polymin P (Buyel and Fischer, 2014c) and the proposed effect of shear stress on the size of flocs during bridging flocculation (Gregory, 1988). However, the repetitive or continuous addition of flocculants can also prove beneficial, if this prevents local overdosing of the polymer (Pearson et al., 2004). Temperature may also affect flocculation (Fitzpatrick et al., 2004). However, our previous experiments with Polymin P and tobacco extracts indicated that temperature does not have a significant impact on the current experimental setup (Buyel and Fischer, 2014c) and it was therefore excluded as a parameter in this investigation.

3.3. A combination of chitosan and polyphosphate is the most effective non-synthetic flocculant combination

Based on the predictive models, we used the numerical optimization tool in Design Expert to identify combinations of two sequentially added flocculants that achieved the strongest reduction in bag filtrate turbidity at pH 4.0, 6.0 and 8.0, with buffer conductivities of 15 or 45 mS cm^{-1}. The results of 100 numeric solutions for this minimization problem are shown in Fig. 2A (selection of first flocculant) and Fig. 2B (selection of second flocculant), indicating a trend toward none (no flocculant, only buffer) or Polymin P as the first flocculant and a clear preference for Polymin P as the second flocculant. This bias toward Polymin P as the only flocculant (added after an initial buffer injection) was even more obvious when only the 25 best results (achieving the lowest bag filtrate turbidities) were taken into account (Fig. 2C and D for first and second flocculant, respectively). This result correlates with the data discussed in Section 3.2: even if Polymin P is not the preferred