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Identification of Upstream Culture Conditions and Harvest Time Parameters That Affect Host Cell Protein Clearance

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ABSTRACT

During early stage bioprocess development, characterizing interactions between unit operations is a key challenge. Such interactions include the release of host cell enzymes early in the process causing losses in product quality downstream.

Using a CHO-expressed IgG1 system, the impact of cell culture duration was investigated using a 50 L bioreactor and performing scale-down protein A purification. While antibody titer doubled during the last week of culture, the post-protein A HCP levels increased from 243 to 740 ppm. Effects of pH and temperature were then explored using fed-batch ambr250 bioreactors, and parameters enabling higher titers were linked to a decrease in post-protein A product purity. These trade-offs between titer and product quality were visualised using a window of operation.

The downstream space was explored further by exposing shake flask material to shear representative of disc stack centrifugation, prior to purification, and by adding polishing chromatography. While product quality decreased with progressing cultivation, cells became more shear resistant. Polishing chromatography resulted in product fragmentation which increased 4-fold from day 10 to 24, adding constraint to achieving both efficient HCP clearance as well as high monomer purities.

These examples highlight the importance of adopting integrated approaches to upstream and downstream development strategies to enable whole process optimisation.

KEYWORDS

monoclonal antibodies, upstream processing, downstream processing, product quality, host cell proteins

INTRODUCTION

Recombinant therapeutic antibodies are a major part of today's pharmaceutical industry. In 2016, monoclonal antibodies (mAbs) represented almost 24% of all the biopharmaceuticals approved in the EU and US, and mAb sales reached $ 82 billion [1]. These market forces have lead biopharmaceutical companies to explore strategies for intensifying manufacturing processes to maximize productivity while following best practice to meet regulatory expectations regarding product quality. During attempts to intensify these processes, one class of process related impurity in particular has proven to be challenging to monitor and remove from the final drug substance and has thus come under increasing scrutiny – host cell proteins. The host cells that are used for the expression of mAbs, produce not only the desired product, but also co-express the indigenous proteins that enable the cells to grow. These so called host cell proteins (HCPs) are present in the harvested cell culture fluid (HCCF) and require separation from the mAb product during downstream processing.

HCPs are a complex mixture of a variety of proteins with significantly diverse physicochemical properties [2, 3] necessitating the use of multiple techniques for their efficient clearance. HCP removal is essential as their presence can influence drug efficacy as well as cause immunogenic responses in patients, including cross-reactivity and autoimmunity [3-7]. A recently published case concerned a mAb in phase III clinical trials, in which the drug product was found to contain high amounts of the HCP PLBL2, which caused a notable immunogenic response in ~90% of subjects, although no adverse safety effects were observed [8]. For these reasons, the FDA recommends HCPs be reduced to acceptably low

levels (<100 ppm) [9], although in reality HCP limits are case-by-case dependent and are defined from (pre-) clinical studies and manufacturing consistency lots [10, 11].

Several research groups have demonstrated that much of the HCPs associated with mAbs following protein A affinity chromatography are co-eluting with the product by association with the bound antibodies rather than by non-specifically binding to the resin [3, 12-16]. Based on this understanding, considerable research has been done to identify the specific HCP species that are being retained during protein A affinity chromatography with certain antibodies.

Work has also been done to investigate whether upstream process conditions affect the composition of HCPs present in cell culture material prior to purification. For instance, Jin et al. [2] studied how media, temperature, feeding strategy, agitation speed, process duration and cell viability impact the composition of HCPs and found viability to have the most significant effect. Tait et al. [17] have also reported that the changes in environment, metabolism and declining viability at the end of the cultivation period result in a different HCP profile at the end of the process compared to earlier days. Both the work from Jin et al. and Tait et al. suggest that the time of harvest is a crucial parameter with regards to HCP composition and that cell culture duration and cell viability should be controlled for process consistency.

To date, there has been little research to investigate the interaction between upstream effects on HCPs and the robustness of the subsequent downstream operations to remove these impurities. However, recently published data, including that by regulatory bodies, has linked upstream and downstream studies, highlighting the importance of such an approach during

process development [18]. It was investigated how changes to upstream process parameters can influence capture chromatography performance and discovered that higher dissolved oxygen levels (DO%) and higher sparge rates were associated with fewer HCPs, increased antibody monomer levels and improved protein stability with properly folded native structures. The authors suggest this is possibly because well-aerated and oxygenated cultures might be healthier and have intact machinery for protein assembly and processing which should result in fewer misfolded/sticky HCPs [18].

However, upstream and downstream process development has traditionally been performed in isolation with development activities focused on the specific function of each. Cell culture processes are developed to maximize titer and productivity, whereas HCP clearance is an objective for downstream purification and is not commonly monitored during upstream processing. There are certain disadvantages to this approach: The upstream selection criteria for the best cell lines and cultivation conditions are based primarily on titer considerations, but optimisation based on this criteria will also have an effect on the amount and composition of HCPs [2]. It is therefore possible that certain process conditions may unintentionally cause the HCCF to contain high amounts of HCPs and/or HCPs that are difficult to remove with the downstream process.

In this paper, we describe the impact of upstream processing on HCP removal in downstream operations in the context of mAb process development, and thus investigate the importance of approaching the biopharmaceutical manufacturing process holistically.

MATERIALS AND METHODS

Case Study I – Impact of harvest time on titer and product quality

Upstream Processing

A summary of the methods associated with the three case studies can be seen in Figure 1. The first case study was performed with a Chinese hamster ovary (CHO) cell line expressing an IgG1 monoclonal antibody (mAb 1). Cells were grown in a 50 L Single Use Bioreactor (Sartorius, Göttingen, Germany) using chemically defined media under defined pH, temperature and DO setpoints. On days 1, 3, 7, 10, 14 and 17 supernatant samples were taken and centrifuged in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 5 mins at 4 °C. Antibody titer was measured using a CEDEX BioHT (Roche Custom Biotech, Mannheim, Germany), while culture viability was determined by the trypan blue exclusion method using a benchtop Vi-Cell XR (Beckman Coulter, Indianapolis, IN). All titer results have been normalised to 1 as exact titers are confidential.

Downstream Processing

Supernatant samples were 0.2 µm filtered (Mini Kleenpak™ 25 mm syringe filters with 0.2 µm Supor® EKV membrane (KM2EKVS), Pall Corporation, Portsmouth, UK) and affinity purified on prepacked 0.2 mL MabSelect SuRe™ (GE Healthcare, Uppsala, Sweden) RoboColumns (Atoll GmbH, Weingarten, Germany) using a liquid handling robot (Freedom Evo 200 Robot with EVOware standard software; Tecan, Mannedorf, Switzerland). The purification protocol was a scale-down mimic of the typical downstream processing method for mAbs. Columns were equilibrated with a Tris acetate buffer (pH 7.5) before loading. This

was then followed by a column wash step with a Tris acetate buffer containing caprylate (pH 7.5) and a re-equilibration step before product elution using a sodium acetate buffer (pH 3.6). The eluate from each column was collected across 10 fractions using a 96 well collection plate, lined with 10 µL of 1 M tris base to neutralise the eluate. The UV at 280 nm was read by a Magellan Infinite 200 plate reader (Tecan, Mannedorf, Switzerland) and fractions containing high levels of protein were pooled.

HCP Quantitation

ELISAs were used to quantify total immunoreactive HCP content in processed samples using GSK's in-house anti-CHO HCP antibodies raised against media from null CHO cells. The assay has a coverage of 80%. The polyclonal mix of antibodies was immobilized on 96-well plates and diluted samples were added to the wells. Serial dilution was performed for the samples to ensure results were within the quantification range of the standard curve. After sample incubation at 24 °C and 400 rpm for 1 hour, goat anti-HCP biotinylated antibody was incubated under the same conditions, followed by a final incubation with streptavidin–horseradish peroxidase (HRP). The HRP enzymatic activities were quantified using substrate and stop solution by SeraCare Life Sciences (Milford, MA, United States), and measuring UV absorbance with the SpectraMax190 Microplate Reader (Molecular Devices, San Jose, CA, United States). Results were analysed using SoftMax Pro version 5.4.4.

Case Study II – Impact of culture conditions on titer and product quality

Upstream and Downstream Processing

A Design-of-Experiment (DoE) with mAb 2 (IgG1) was carried out using an ambr250 system (Sartorius, Göttingen, Germany) to explore the effects of cell culture pH and temperature on titer and HCP levels. For this fed-batch process, all vessels were inoculated to a defined seed density, performed with defined DO and temperature setpoints, and maintained at a defined pH for the first three days, before the pH was reduced to the DoE setpoints. A pH range from 6.8 to 7.1 and a temperature range from 32 °C to 38 °C were explored (pH and temperature setpoints are illustrated in Figure 3A). Samples were harvested on day 16 by centrifugation at 4,000 rpm for 5 min, and the supernatant material was analysed on the CEDEX and Vi-Cell as described in the 2nd section.

Downstream purification of the resultant eluates was performed in the same manner as previously described for Case Study I.

HCP Quantitation

HCP quantification was performed as described in the 2nd section using Cygnus reagents: Anti-CHO HCP antibodies (3G-0016-AF and 3G-0016-AFB, Cygnus Technologies, Southport, NC), and antigen standard (F018H; Cygnus Technologies, Southport, NC). Cygnus reagents were used as these had been raised against cell lysate and were thus expected to achieve better coverage of HCP species that might be released from cells as cultures in this case study had fairly low viabilities.

Case Study III – Harvest time interactions with subsequent DSP

Upstream Processing

CHO-expressed IgG1 monoclonal antibody (mAb 3) was cultivated in shake flasks under fixed culture parameters (batch process with defined temperature, DO and pH setpoints, and with glucose addition on day 7) using chemically defined media. Cell broth samples were taken at four time points (days 10, 17, 20 and 24 to generate samples with a wide range of viability percentages) and antibody titer and cell viability were determined as described in the 2nd section. At each time point, samples were processed as expanded upon below.

Downstream Processing

Using a rotating cell shearing device developed at UCL to mimic harsh mechanical conditions during cultivation and/or harvesting that might cause cell breakage, half of the material was sheared at 12,000 rpm for 20 seconds (previously determined to be sufficient time for full cellular break-down [19, 20]), while the other half of the material was not exposed to this particular shearing technique. Both sheared and non-sheared material was subsequently centrifuged in a Sorvall Legend RT (Thermo Scientific, Waltham, MA) at 4000 rpm for 20 mins at 4 °C, and then 0.2 µm filtered to remove all cell debris and prepare samples for affinity purification. A 4.7 mL MabSelect SuRe protein A HiScreen column (GE Healthcare, Uppsala, Sweden) was used for the purification, using the same steps as previously described for the Robocolumns, and loading to 85% of the manufacturer's suggested dynamic binding capacity. All protein A eluate samples were further processed by anion exchange chromatography in flow-through mode using 0.2 mL pre-packed ATOLL

Robocolumns. The flow-through material was collected and polished with a final cation exchange chromatography step, using 1 mL MiniChrom columns (Repligen, Waltham, MA) and a buffer with a pH below the pI of the protein.

HCP and Monomer Purity Quantitation

HCP quantitation was performed in the same manner as described for Case Study I. Product monomer, aggregation and fragmentation percentage was determined by size exclusion chromatography (HPLC-SEC) using an Agilent HPLC system (Agilent 1100 series) and a 7.8 mm x 300 mm TSKgel G3000SWXI column (Tosoh Bioscience).

Data Processing

Results were plotted in OriginPro 2016 software (OriginLab Corporation, Northampton, MA). The contour plots were created with JMP 13.2.1 (SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Case Study I – Impact of harvest time on titer and product quality

CHO-expressed mAbs are commonly produced in fed-batch processes. During cultivation, the CHO cells grow in number and produce increasing amounts of protein – both the desired antibody as well as proteins required by the host cells to survive. In the first study, samples were taken throughout the production bioreactor run of mAb 1 (including on the harvest day when culture viability was at the lowest point of 93%) in order to investigate the effect that culture duration has on not only product titer but also post-protein A HCP levels.

The purpose of this study was to investigate whether there is a trade-off between titer and HCP clearance with cell cultivation time. Figure 2 illustrates that as product titer increased throughout the cultivation, so did the amount of post-protein A HCP impurities, meaning that product amount and purity are negatively correlated, especially after 2 weeks of cultivation at which point the relative production of HCP impurities outweighs the one of antibody titer – the amount of product increased by 28% from day 14 to day 17, however post-protein A HCP levels increased by 75% during this time frame (Figure 2), demonstrating that material which is harvested later potentially requires a more extensive HCP clearance strategy. Previous literature [2, 17] has suggested that the time of harvest is a crucial parameter with regards to the HCP composition in HCCF, and our data shows that harvest time further also affects the efficiency of HCP removal during protein A chromatography, with a later harvest time resulting in decreased post-protein A product purity.

Case Study II – Impact of culture conditions on titer and product quality

As the first case study has illustrated, longer cultivation results in increasing levels of HCP impurities following protein A purification. The aim of the second case study was to explore the impact of culture conditions on the trade-offs between titer and product quality, using a Design-of-Experiment (DoE) approach which is a common industry standard to optimise process parameters during process development. An example of the DoE approach is described extensively in "A-Mab: a Case Study in Bioprocess Development" [21]. The second case study was carried out with a different molecule (mAb 2) compared to the first case study. Figure 3A shows the two cell culture parameters pH and temperature that were investigated using a centre point DoE with six replicate centre points. All cultures were

harvested on day 16 and displayed a wide range of titer and HCP levels (Figure 3B). pH and temperature conditions that promoted the production of more antibody also resulted in higher post-protein A HCP levels which shows that the correlation of increasing product amount and decreasing purity is not just caused by increasing cell culture duration, as observed in the first case study, but also by culture conditions. Figure 3B also shows cultures that are associated with low antibody titer as well as low post-protein A HCP levels. These cultures are less active, either due to low cell densities or low specific productivity rates (SPR) as a result of poor growth conditions (cf. Figure 3A). These cultures are highlighted by the grey oval in Figure 3C. Cultures 10, 11 and 12 only reached peak cell densities of 11-12 x 10^6 viable cells/mL, and culture 14 only had a cell-specific productivity rate of 11.4 pg/cell/day. Based on the observation that culture 14 (with a lower SPR yet higher cell density compared to cultures 11 and 12) produces more antibody and HCPs, the cell density seems to be the primary factor influencing the production of antibody and HCPs, followed by the cell-specific productivity rate as a secondary factor.

Based on this data, a contour plot was generated in JMP by fitting least squares regression models for the titer (R^2 = 0.95) and HCP (R^2 = 0.92) data to show the range of pH and temperature that would give rise to a window of operation, trading off both upstream and downstream responses in terms of high titer and low HCP levels respectively. Such windows of operation are commonly used to illustrate the operating conditions that are feasible for one or multiple desired responses [22]. Figure 4A shows such a feasible operating window based purely on an upstream requirement (chosen here to be a titer of at least 3 g/L) which suggests operating at the midpoint to be optimal. However, Figure 4B displays a smaller feasible

operating window generated based on both an upstream and a downstream requirement (a titer of at least 3 g/L, as well as no more than 900 ppm post-protein A HCPs, which was deemed by the authors to be a suitable early development target based on industry trends). This second plot shows that the operating range needs to be shifted to a new optimum based on a trade-off between product titer and product purity / quality (shift from red point to green point).

Case Study III – Harvest time interactions with subsequent DSP

The feasible window of operation presented in the second case study is based on HCP clearance after the critical protein A purification. The feasible operating space may widen when the whole downstream process is considered and HCP clearance during subsequent chromatography polishing steps is demonstrated. Alternatively the operating space may become smaller when adding further desired responses to the contour plot, such as high product monomer percentage, but in the second case study, all cultures were associated with low levels of aggregates and fragments post-protein A purification (data not shown) and therefore did not impact the window of operation.

These considerations were addressed in the third case study in which another molecule (mAb 3) was used to explore the whole downstream process, beyond protein A capture, and to consider how process development decisions might be affected by a mAb that is susceptible to fragmentation unlike mAb 1 and 2. The third study also explored the effect of shear damage, representative of that which might typically be found in disc stack centrifugation upon scale-up (12,000 rpm for 20 sec), at different harvest points. Cells were cultured for over three weeks to enable the analysis of material with a wide range of

viabilities. Cell broth samples were taken on days 10, 17, 20 and 24 when culture viability was 97, 76, 68 and 43% respectively, and at each time point half of the collected material was exposed to shear to see if the viability of the cells impacted upon behavior during disc stack centrifugation.

Harvest time vs. product quality

Firstly, monomer purity and HCP levels were measured following protein A purification of HCCF which had not undergone any shear treatment. The results show that throughout the culture the product quality decreases due to increased levels of post-protein A HCPs (as already observed in the first and second study) as well as increased levels of product aggregates and fragments measured after affinity purification (Figure 5). Interesting to note is that in case study I, protein A columns were loaded according to volume rather than total amount of protein, and the observed HCP increase could be explained by the increasing load levels of antibody molecules to which the HCPs could bind. However, in case study III, the same mass of antibody was loaded to each column, and shows the increase in post-protein A HCP levels with progressing culture duration as well. Regarding the increase in aggregates and fragments, previous research has linked aggregation to the low pH conditions required for protein A elution [23] as well as to a low pH hold following protein A purification as commonly used for viral inactivation [24], and while material in this case study was eluted from the protein A resin with a pH 3.6 buffer, all samples were treated in the same way and should be affected in a similar manner if the observed aggregation were purely pH dependent. Increasing product aggregation and/or fragmentation due to a concentration effect is also unlikely given that the same mass was loaded onto the column for all samples. A more likely

cause for the increase in aggregates and fragments could be the presence of proteases – it has previously been reported [2, 17] that proteases are produced during late stage cell culture and can cause product fragmentation. Cathepsin D in particular is known to co-purify and cleave the antibody product [25-27]. Accumulation of fragments could also lead to the formation of aggregates.

Shear due to disc stack clarification

Secondly, the effect of mechanical damage to cells during culture and harvest (mimicked here using an ultra-scale down shear device) on post-protein A HCP levels was characterized. Interestingly, it was determined that shearing did not cause a significant increase in HCPs in the investigated samples (results not shown). However, shearing did have a significant effect on cell breakage on samples taken on day 10 of culture. While cell viability remained unaffected, total cell number was reduced by 60% (determined through the Vi-Cell cell counter; images displayed in Figure 6A-D), at which point the levels of total HCPs produced by the cells is expected to be low (cf. Figure 2 and 5), whereas the samples from day 17 onward were more shear-resistant and did not suffer significant morphological damage. This is consistent with previous literature [28] which states that the more permeable outer membrane structure of non-viable cells can potentially absorb the impacts of stress better and thus these cells are less susceptible to it.

In addition to HCP levels, the extent of product aggregation and the effect of shear at harvest was studied. As stated, shearing was only observed to affect material samples from day 10 of culture, at which point it resulted in increased aggregation (Figure 7), potentially due to the release of antibody chains that had not been fully assembled. This raises an

interesting counter-argument to the question of whether product should be harvested earlier to avoid the presence of high HCP levels, as suggested in the first case study. Such a decision would now need to weigh not only the advantage of purer product versus the disadvantage of less product but also take into account the additional risk of harvesting shear-sensitive cells that might break and release intracellular impurities. In order to make an informed decision, this window needs to be explored further to determine if there is an optimal harvest point.

Interaction with intermediate and polishing chromatography

Lastly, monomer purity and HCP clearance were explored during intermediate and polishing chromatography steps (Figure 8). Cation exchange chromatography (CEX) was found to dramatically reduce HCP levels to below the guidance limit of 100 ppm (Figure 8A), however this step also resulted in increased product fragmentation in the case of this particular mAb 3 (Figure 8B). Figure 8B shows three interesting observations: Firstly, fragmentation increases up to 4-fold from day 10 to day 24 which could be caused by the release of proteases as discussed in the 3rd section. Secondly, fragmentation is increased even further by the cation exchange chromatography step, suggesting either that a component of the CEX process itself causes the unstable mAb 3 to fragment, or that protease-dependent fragmentation is activated on the CEX column. Thirdly, on day 10 the post-CEX fragmentation in non-sheared material is similarly low to fragment levels prior to CEX (0.5-0.8%) whereas the fragment level in the sheared material has increased by 1%. Comparing this observation to day 24, the fragmentation levels post-CEX are similarly high (3-3.2%) in non-sheared and sheared material. This supports the theory that fragmentation is caused by proteases which on day 10 are only released into the cell culture fluid upon shear-induced cell

breakage whereas on day 24 they are thought to be present in the HCCF regardless of shear exposure.

While CEX is essential for HCP clearance, the fact that it can cause fragmentation of unstable mAb product further increases the challenges for downstream purification. Previous literature [29, 30] has also shown that the use of the CEX polishing step can result in lower product quality due to on-column aggregation of mAbs. If the upstream process were amended to create material with fewer HCPs, the CEX polishing step might not be required, which would avoid a trade-off decision between two downstream responses (HCP clearance vs high monomer purity), as well as save the costs associated with including another column step in the clearance strategy.

CONCLUSIONS

As an increasing number of biopharmaceuticals are being brought to market, creating an ever more competitive market environment, biopharmaceutical companies need to speed up their drug development timelines. One important aspect of this is to improve understanding between the upstream and downstream processing groups in order to develop and produce safe and efficacious drugs of high quality in an efficient and cost-effective manner.

In this work we have shown the purity of multiple mAb products to be dependent on harvest time and culture conditions. Harvesting material at a later date for the sake of higher product quantity results in worse product quality due to the higher presence of process- and product-related impurities. However harvesting the material too early, when the cells are still

sensitive to shear damage, will cause difficulties during the harvest process as cell breakage can result in the release of intracellular impurities such as HCPs, DNA and lipids, and may cause aggregation due to release of unfolded antibody chains that have not been assembled into mature product. In addition to harvest time, culture conditions such as pH and temperature affect the purity of mAbs as setpoints that promote higher product quantity also result in worse product purity due to the higher presence of HCPs. Choosing a harvest time and culture conditions that are optimized for high antibody titer will therefore result in material that is challenging to purify, as the material being loaded onto the column contains a worse product:impurity ratio with a high presence of HCPs, that might prove difficult to clear, as well as potentially host cell DNA and lipids from membrane fragments that might enhance the co-purification of HCPs as they are being dragged through the protein A column.

More insight could be gained by using mass spectrometry to compare the HCP profiles at different time-points as well as at different upstream operating conditions, and to identify when more high-risk HCP species are being produced such as more "sticky" HCP species that prove more difficult to clear during downstream purification [31] or proteases that might cause product fragmentation [2, 17].

In addition to harvest time and culture conditions, further constraints are added when dealing with mAbs that are unstable during certain process steps such as mAb 3 which fragments during cation exchange chromatography and thus makes it more challenging to achieve desirable outcomes for multiple downstream responses – in this case HCP clearance as well as high monomer purity. Table 1 shows a summary of all the positive and negative effects discussed.

Given our emerging understanding of how upstream process conditions can affect downstream purification, it is advisable to take a more holistic approach to process development, and to consider the requirements for both upstream and downstream responses in order to select a feasible window of operation. Especially with the recent developments in perfusion or continuous biomanufacturing and the ensuing increases in antibody titers, it is becoming progressively inevitable to link upstream and downstream process development.

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FIGURE LEGENDS

Figure 1. Outline of experimental set-up. In the first case study, mAb 1 samples from a 50 L bioreactor run were taken at six points during the cultivation. Samples were centrifuged, filtered and then protein A purified, before being quantified for HCPs. In the second study, a DoE with mAb 2 was carried out to explore two cell culture parameters in order to determine the optimal upstream process conditions for high titer and low HCP levels. In the third study, mAb 3 was grown in shake flasks under fixed upstream parameters and cell broth samples were taken at four time points. At each time point, half of the material was sheared (2) while the other half of the material was left non-sheared (1). Both materials were subsequently centrifuged, filtered, and protein A purified. Next, all protein A eluate samples were further processed by anion exchange chromatography in flow-through mode and by cation exchange chromatography. All samples were analysed for product quality by measuring monomer percentage and HCP levels.

Figure 2. Effects of harvest time on product titer and HCP levels (Case Study I). Increasing product amount over the course of a cultivation is accompanied by decreasing product purity. Supernatant samples were taken throughout the 50 L STR production bioreactor run of mAb 1 (including on harvest day) and antibody concentration was measured and normalised to 1 due to confidentiality. Supernatant samples were filtered, protein A purified, and analysed for HCP quantities by ELISA. Note that the HCP data points for days 1, 3 and 7 are estimated on titer as HCP levels were below detection (< 2 ng/mL). Descriptive error bars are based on assay serial dilutions and show one standard deviation.

Figure 3. Effects of culture conditions (Case Study II). **(A)** Experimental DoE points. 14 fed-batch cultures were grown in 250 mL small scale bioreactor vessels (TAP ambr250) under a range of different pHs and temperatures. The empty, half-filled and filled symbols represent low, medium and high temperatures respectively, whereas the squares, triangles and circles represent low, medium and high pH points. All cultures were harvested on day 16. **(B)** Harvested samples were centrifuged, filtered and protein A purified, in order to determine HCP quantities, and correlate HCP data to titer results. Descriptive error bars are based on HCP assay serial dilutions and show one standard deviation. **(C)** Peak cell densities and cell-specific productivity rates were determined for each sample. Highlighted in the grey oval are cultures associated with low titers.

Figure 4. Window of operation for the second case study. Using JMP software, a contour plot was created to illustrate how pH and temperature affect the titer and post-protein A HCP results, and how the feasible operating window (white area) gets smaller when considering not just the upstream requirement of high titer (A), but also the downstream requirement of low HCP levels (B). The contour plot was generated by fitting least squares regression models for the titer (R^2 = 0.95) and HCP (R^2 = 0.92) results, and are based on the black experimental data points.

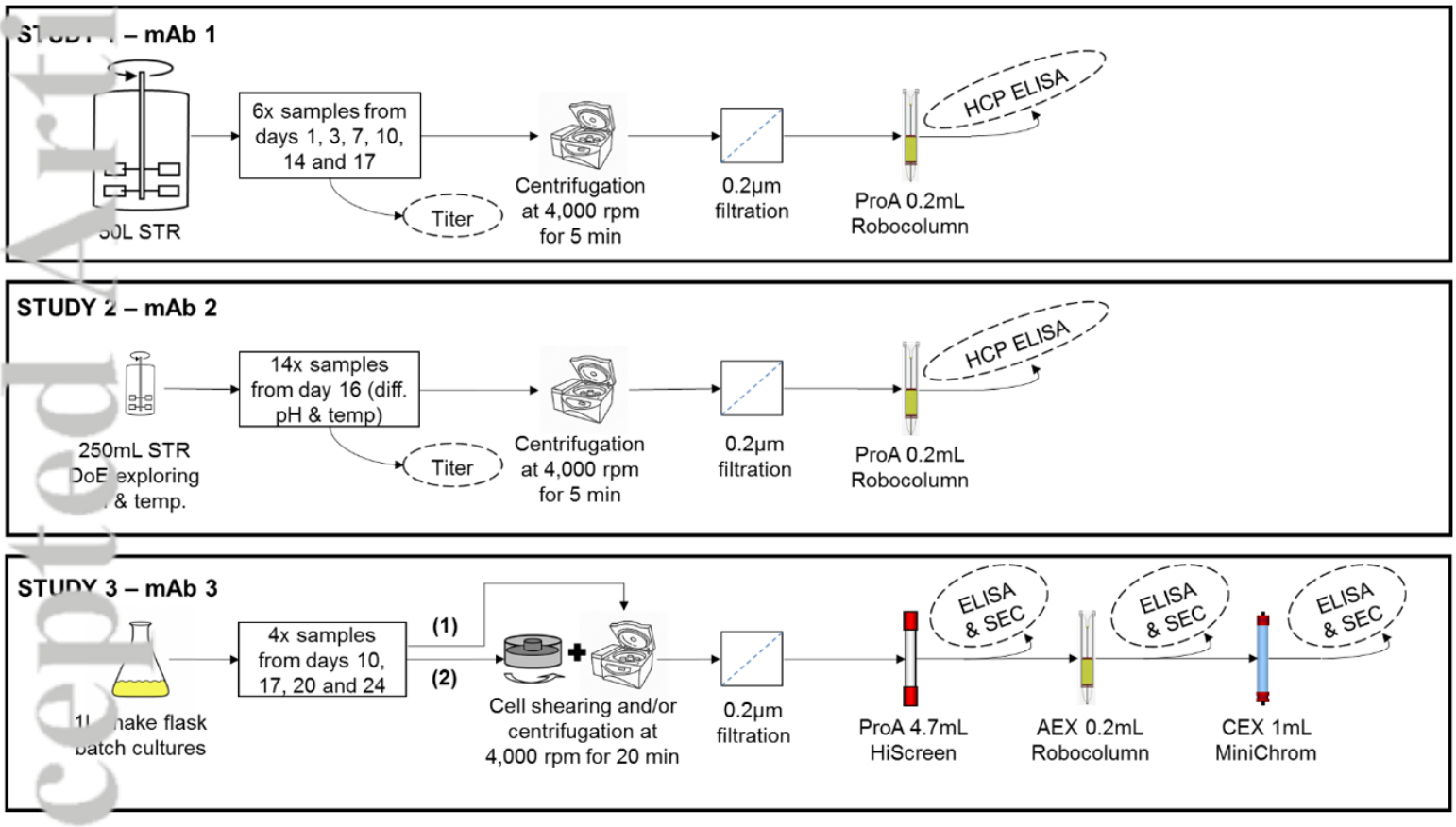
Figure 5. Effects of harvest time on product quality in the unmodified process, i.e. non-sheared samples only (Case Study III). Aggregate and fragment percentages as well as HCP levels were measured after affinity purification and were determined by analytical SEC or CHO-HCP ELISA respectively. Descriptive error bars are based on HCP assay serial dilutions and show one standard deviation.

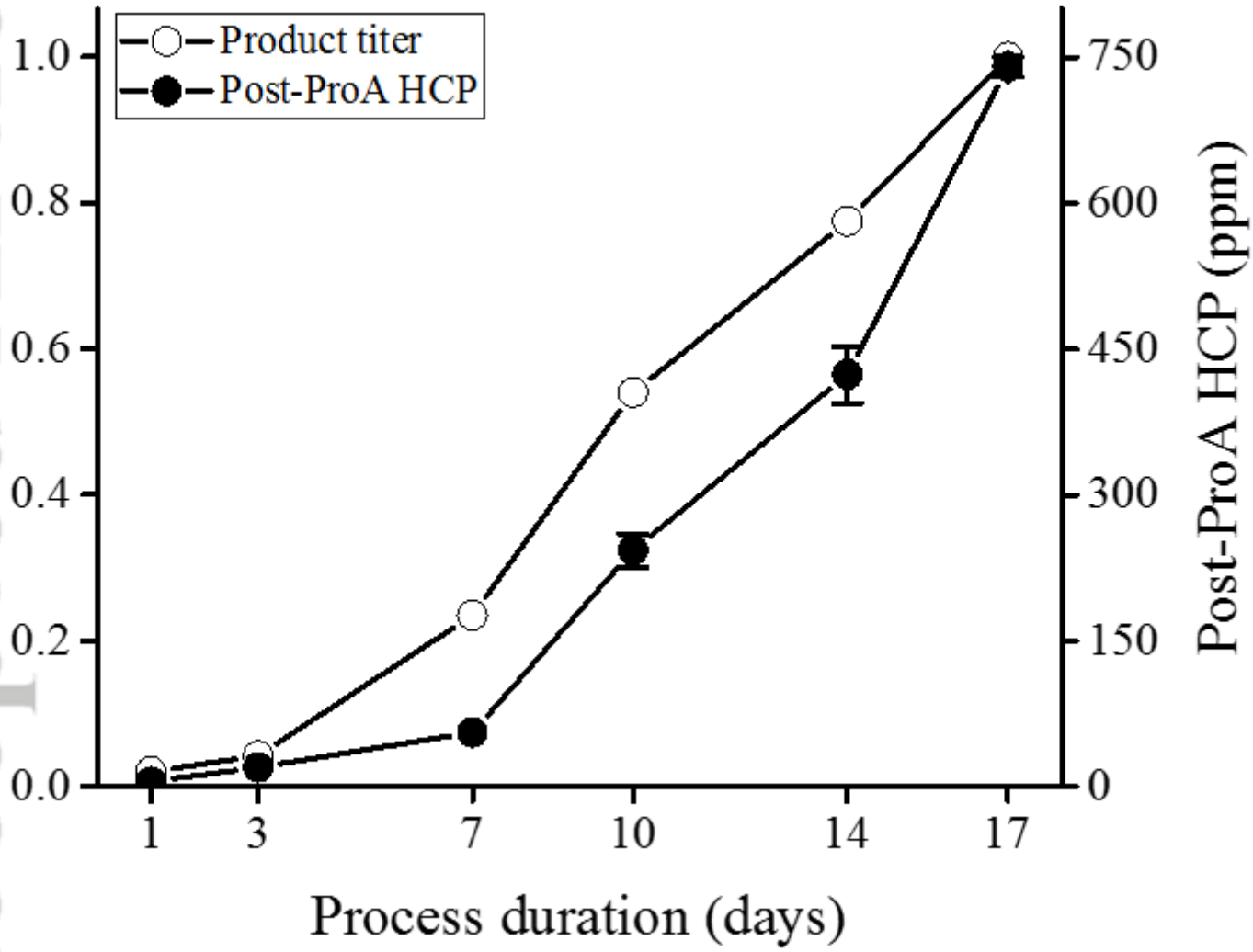
Figure 6. Changes to cell morphology upon shear exposure (12,000 rpm for 20 seconds) (Case Study III). Vi-Cell cell count images. Material from day 10 prior to shearing **(A)** and afterwards **(B)**, in comparison to material from day 17 prior to shearing **(C)** and afterwards **(D)**. Whereas the total cell count has been dramatically reduced by 60% in day 10 material, no obvious breakage has occurred in day 17 and subsequent days (images from days 20 and 24 not shown).

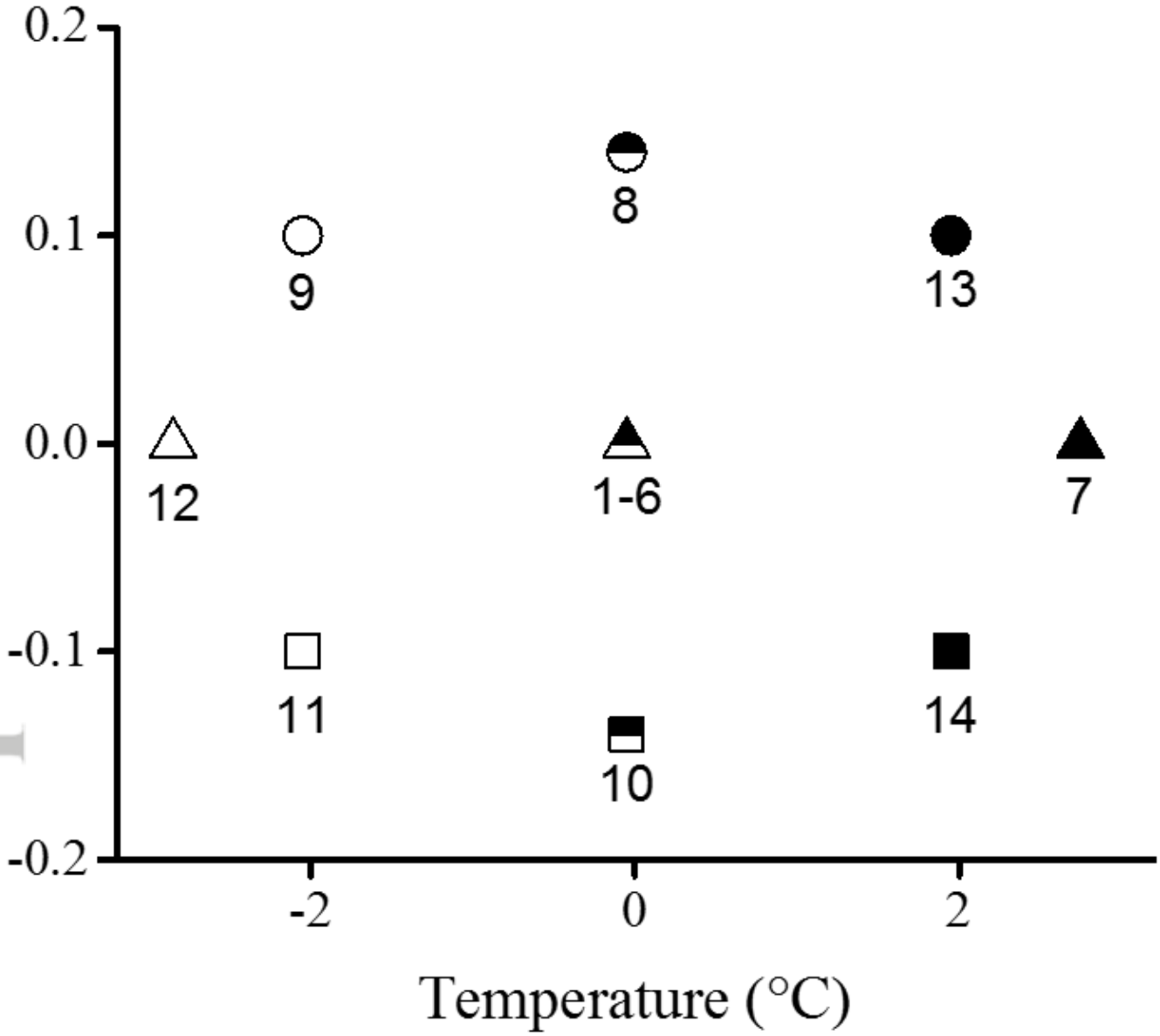
Figure 7. Effects of shear damage on product quality in early stage cell culture samples (Case Study III). Material from day 10 was processed as described in the legend for figure 1 (non-sheared vs. sheared) and aggregate / fragment levels were determined by analytical SEC after protein A purification.

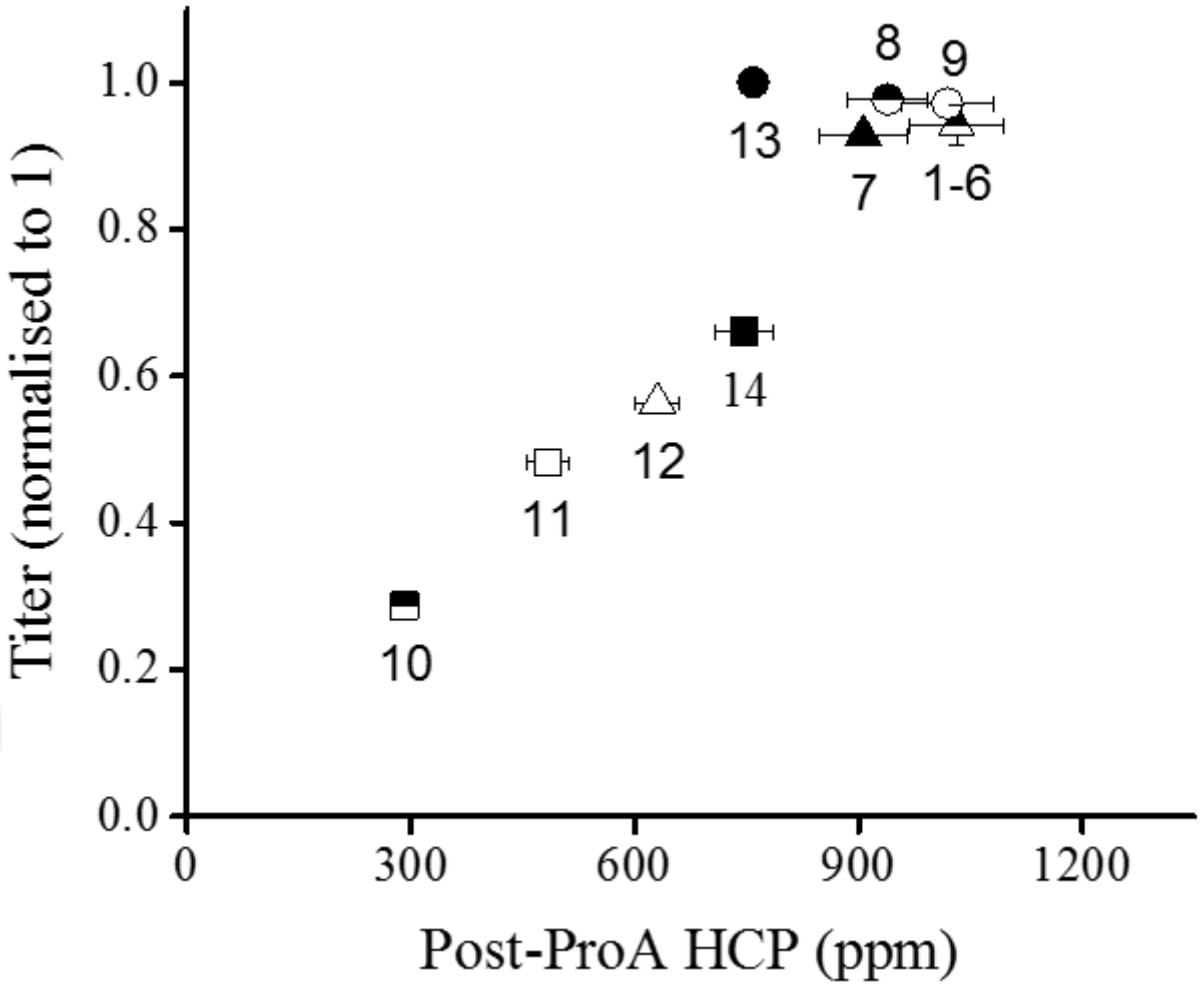
Figure 8. Effects of a polishing chromatography step on product quality (Case Study III). **(A)** HCP levels in non-sheared samples before and after cation exchange chromatography, as determined by ELISA. HCP data point for day 10 after CEX is approximated based on titer as HCP levels were below detection (< 2 ng/mL). Descriptive error bars are based on HCP assay serial dilutions and show one standard deviation. **(B)** Fragmentation percentages in non-sheared and sheared samples before and after cation exchange chromatography, as determined by analytical SEC.

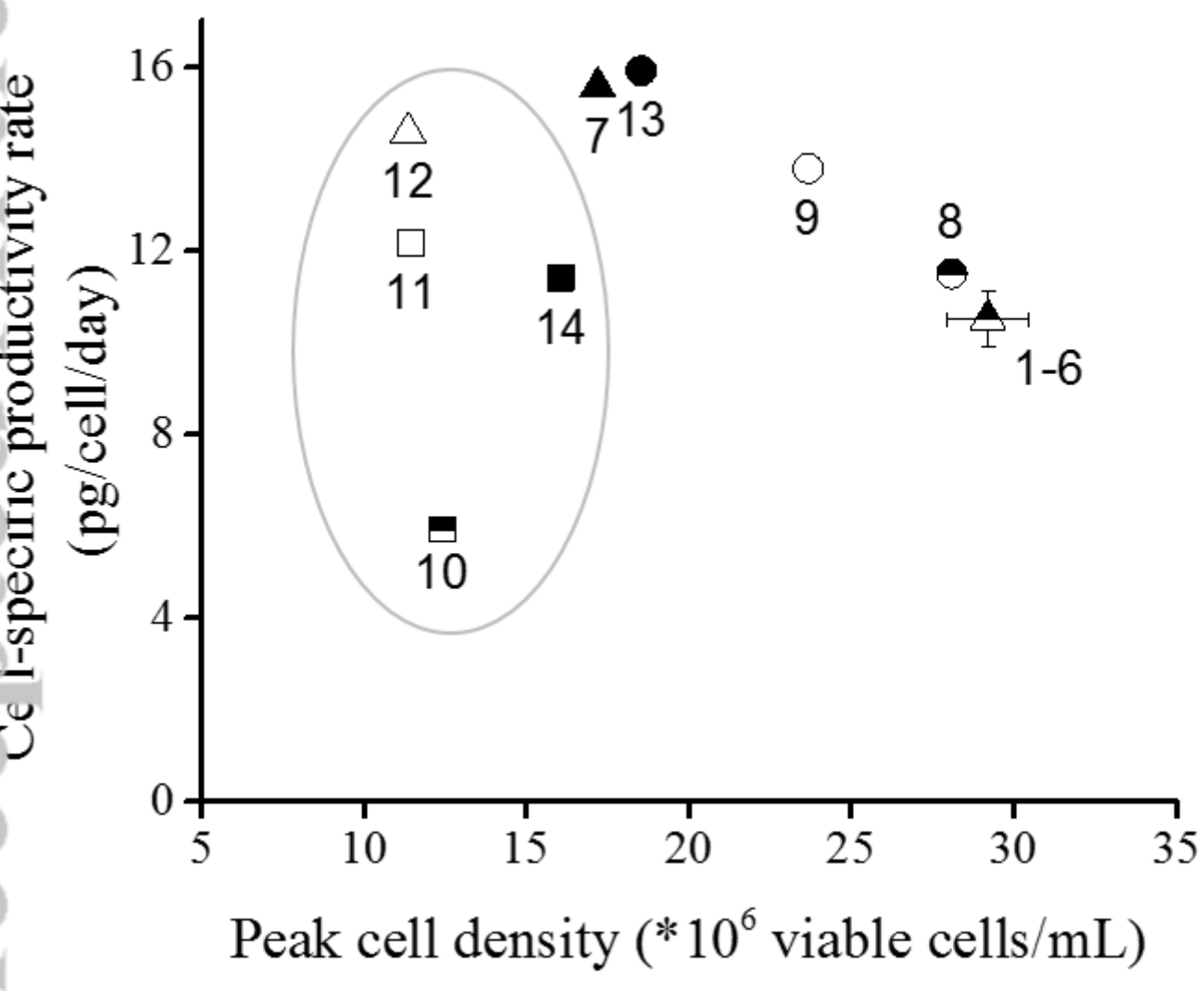
Table 1. Summary of effects of harvest conditions.



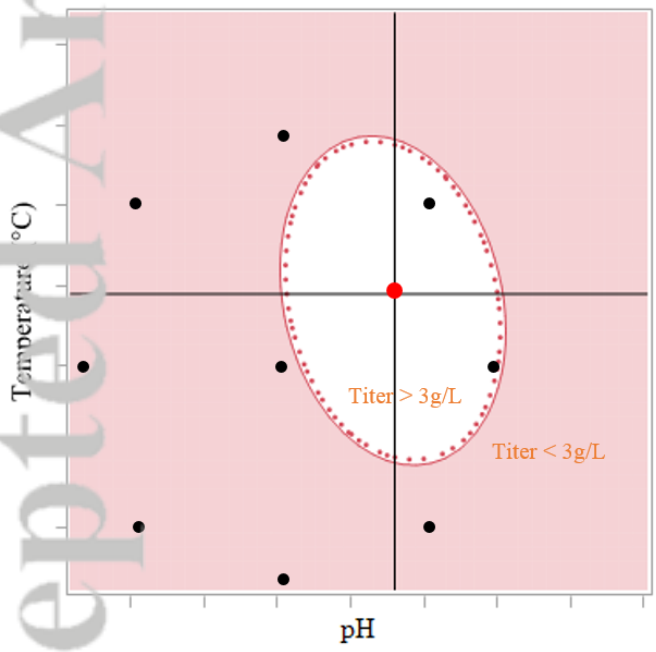








(A)



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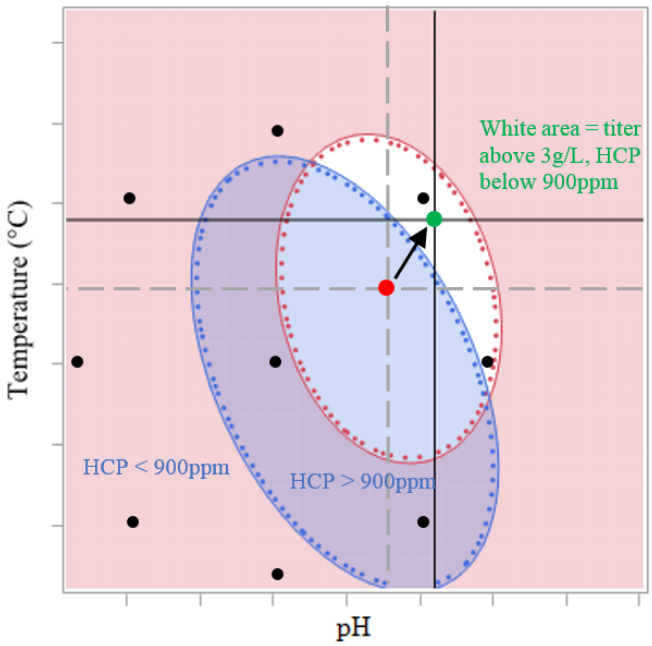

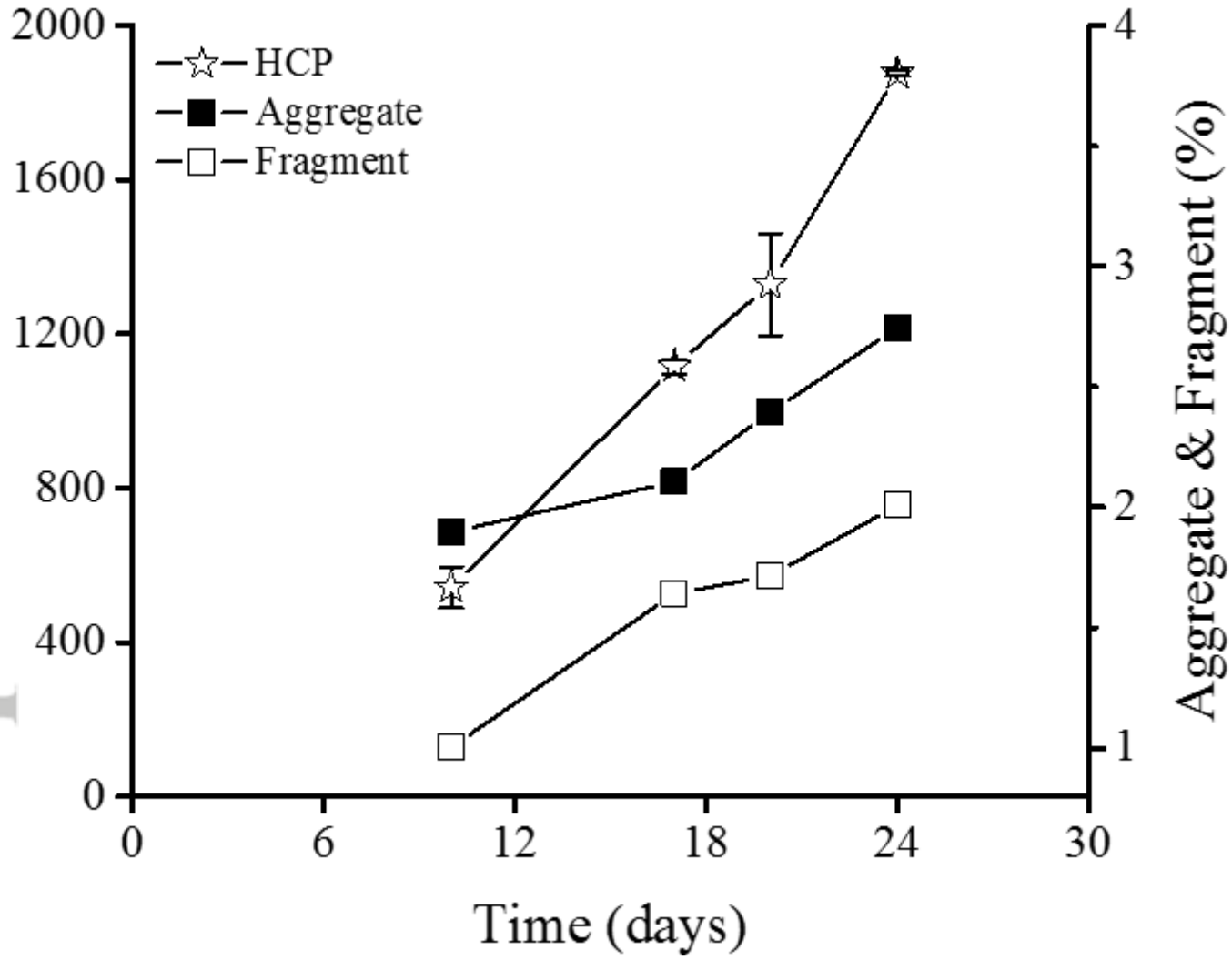

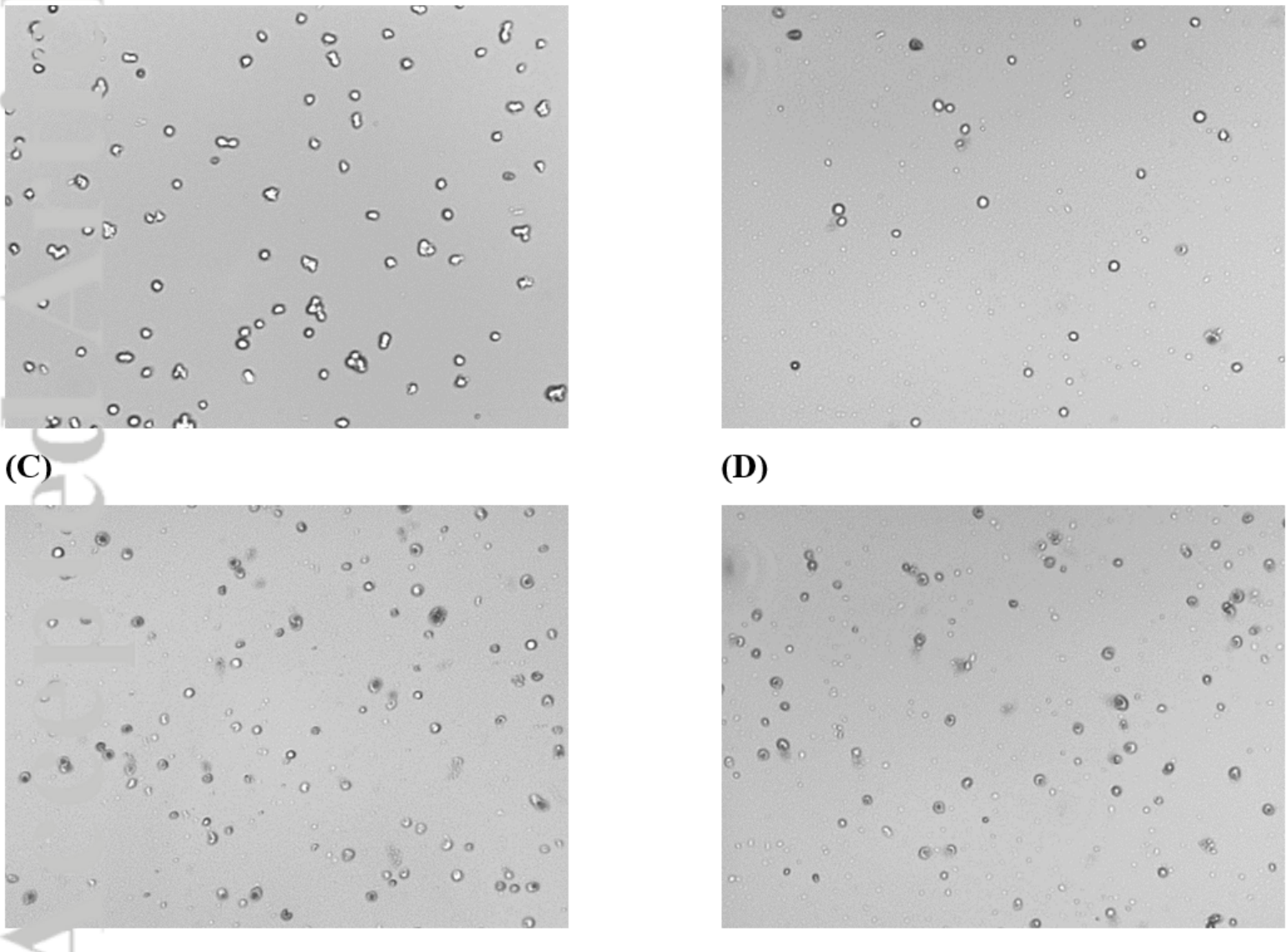

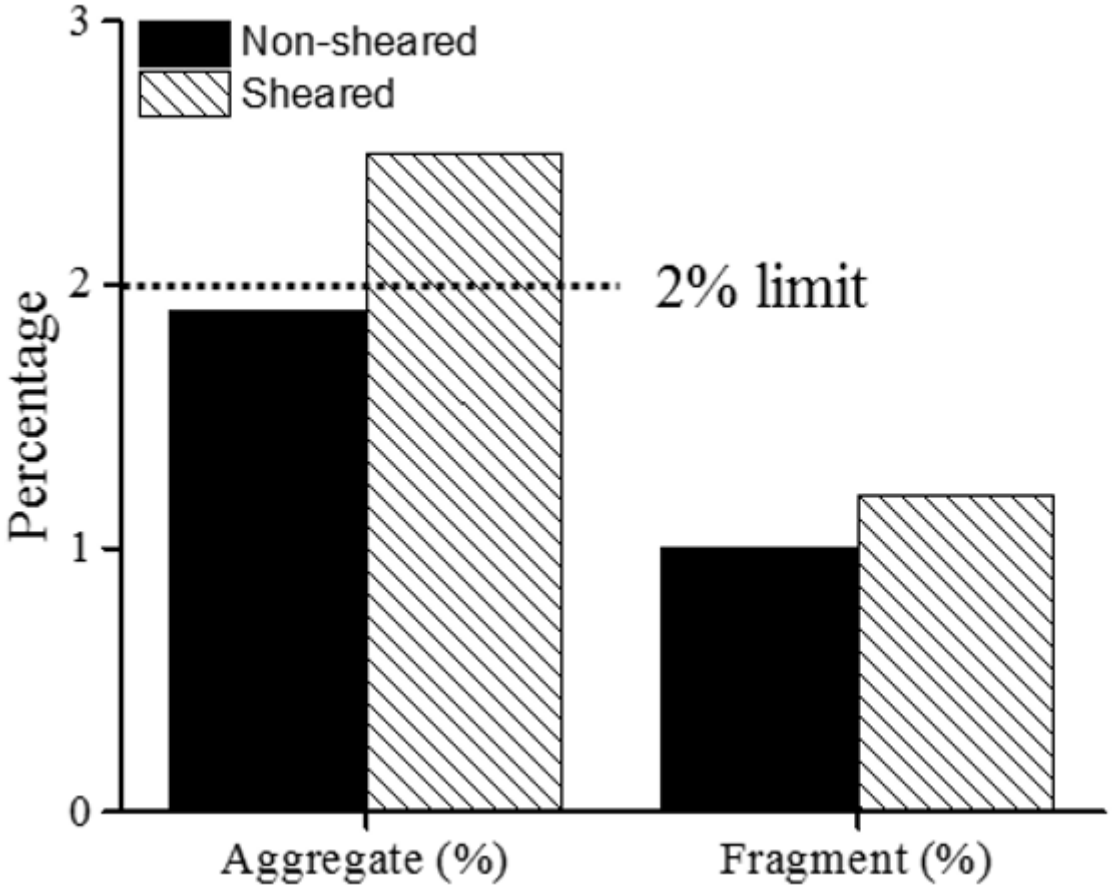

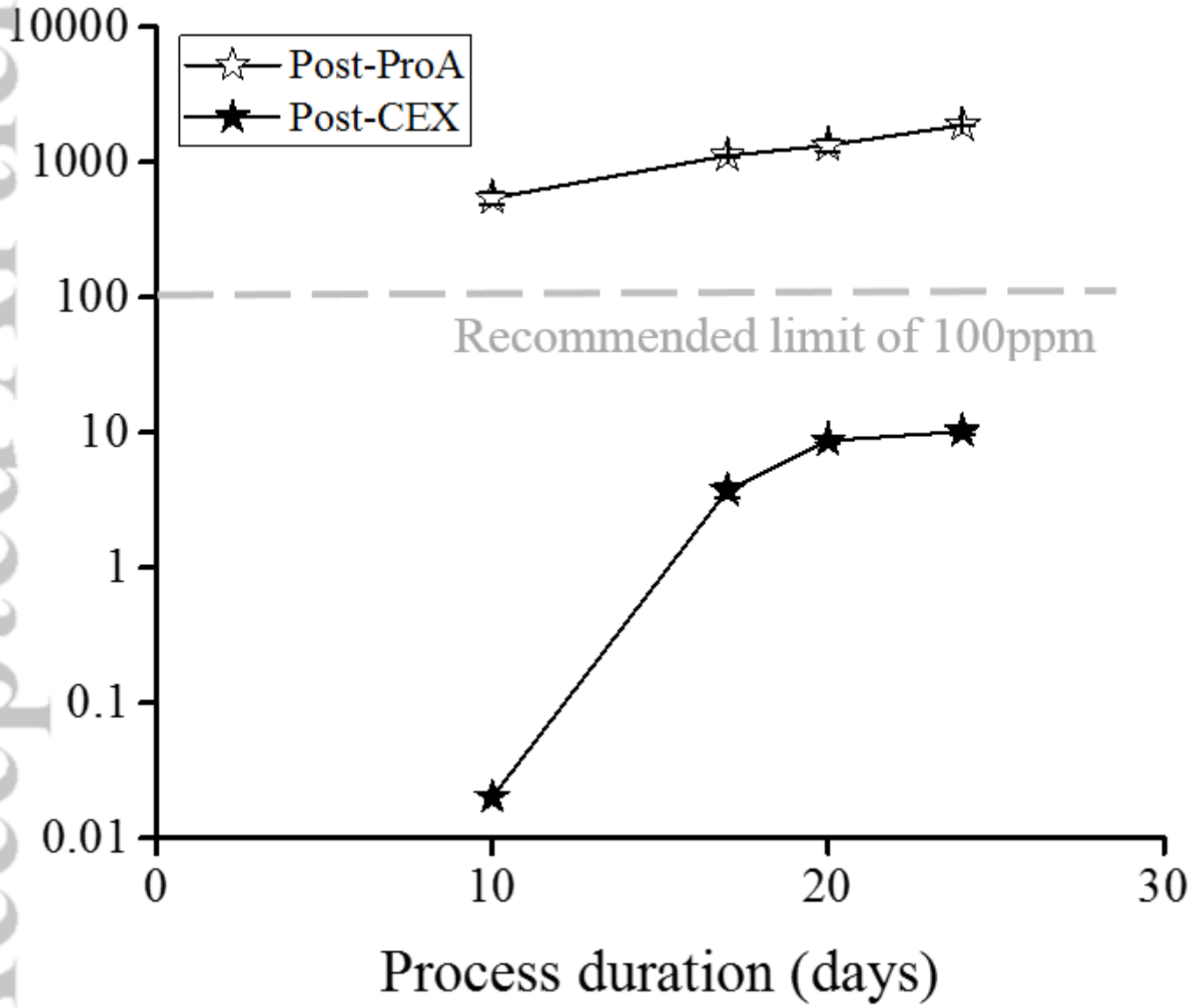

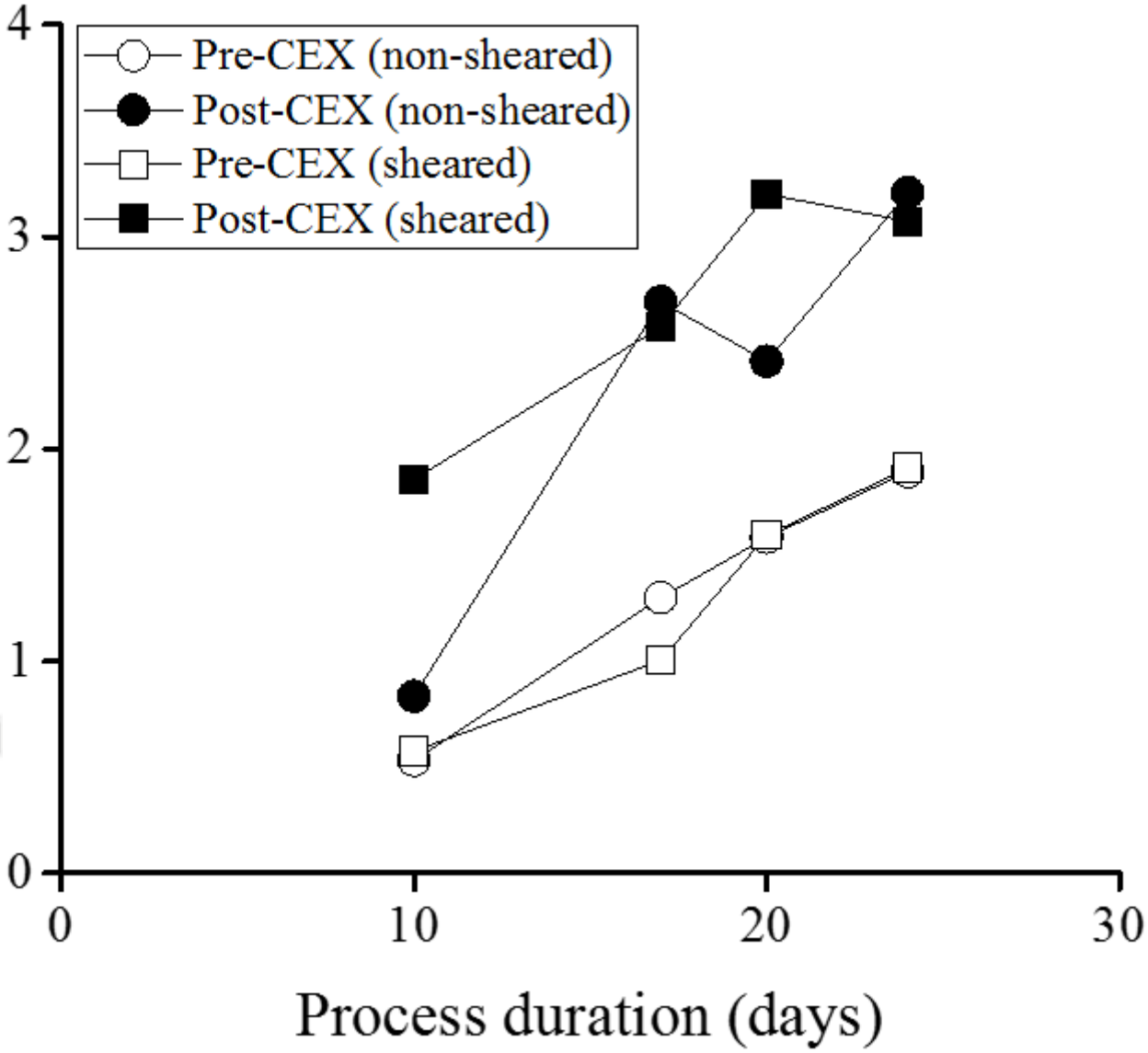

	Positive effects	*Negative effects*
Later harvest time	Increased titer; Cells are more shear resistant	Decreased product purity including higher HCP levels; In rare cases, presence of proteases may affect subsequent processing steps
Culture conditions	Increased titer	Decreased product purity including higher HCP levels