Investigating the influence of the *Chlamydomonas reinhardtii* cell wall on downstream processing for recombinant protein production.

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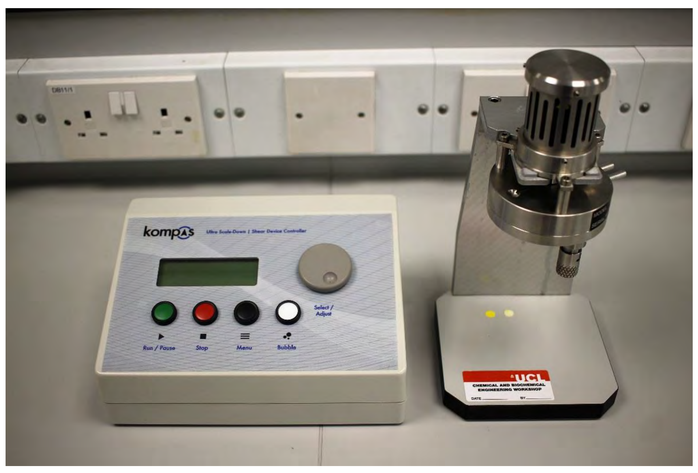
Introduction.

Microalgae hold promise as a simple, low cost and benign production system for the manufacture of recombinant proteins. In particular, the chloroplast of *Chlamydomonas reinhardtii* has been used successfully to express a number of proteins, including human erythropoietin, fibronectin and proinsulin [Rasala et al., 2011]. To date, there have been few studies on the recovery of recombinant proteins from microalgal hosts with the aim of identifying process challenges for scale up. The influence of host cell selection has significant implications on downstream processing. A key question remains for *C. reinhardtii*, is there an advantage to choose a cell wall-less mutant over a cell walled strain as a production system? Using a model system of *C. reinhardtii* engineered to produce endolysin antimicrobial proteins, process parameters relevant to downstream processing were investigated to understand the impact of host system on the yield and integrity of product. Cell walled (BCi) and Cell wall-less (TCii) mutants were engineered to express the endolysin Cpl-1.

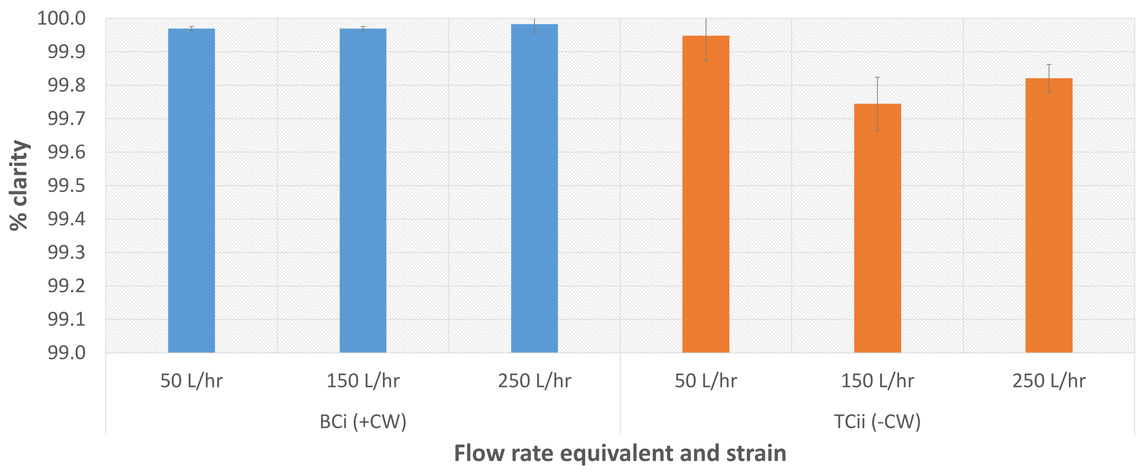
The use of Ultra Scale-Down (USD) technology has allowed for a side-by-side comparison of BCi and TCii strains, highlighting the advantages of having a cell wall for particular downstream processing operations, and the lack of a cell wall for others. Knowing how to optimize these operations will enable effective scale up for larger scale microalgal processes in future.

Cell harvest.



USD shear device (right) and device controller (left). Up to 40mL of sample can be injected into the device. A spinning disk inside can be used to create shear. For the clarification step, an Eppendorf 5424R bench top centrifuge was used with 2mL microfuge tubes.

TCii samples all resulted in lower clarification for all flow rate equivalents – the lack of a cell wall resulting in greater cell disruption and hence, reduced clarification. Both strains, however, produced high clarification.

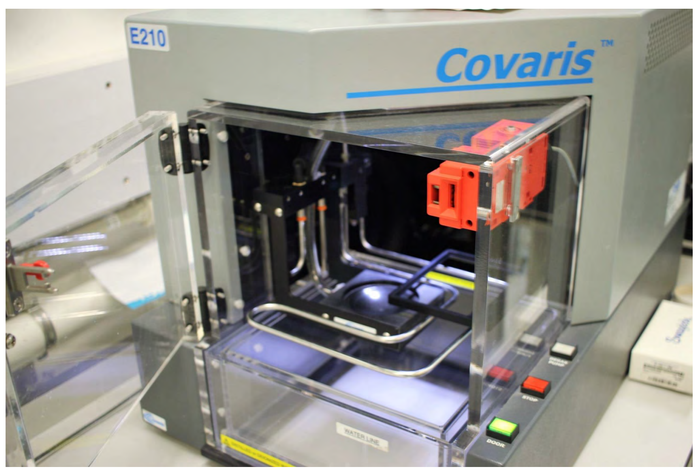


BCi (+CW) and TCii (-CW) were grown in a 1 L shake flask, inoculated 1:10 in TAP media, and were grown until each strain reached an optical density of ~1.8 at 750 nm.

Once at the correct optical density the samples were sheared using the USD shear device at 6000 rpm – shear speed which mimics entrance of a hydro-hermetic centrifuge. Once sheared the samples were then centrifuged for various times at 8000 rpm. Centrifugation times were calculated using Sigma theory, [Boychyn et al., 2004] ; 50, 150, and 250 L/hour were successfully mimicked.

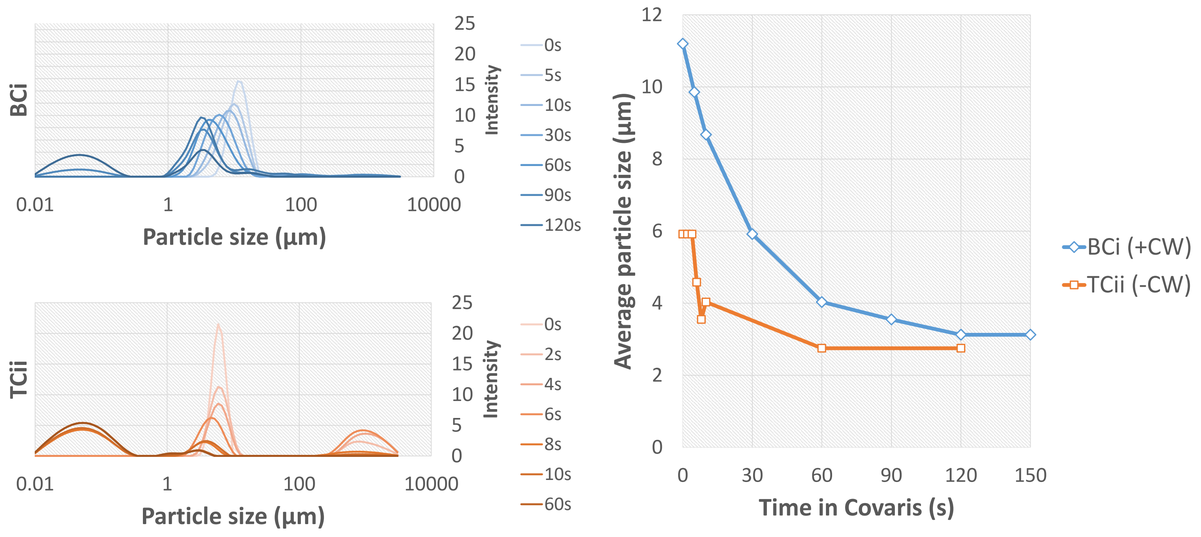
The supernatant was collected from these samples and the optical density at 750 nm was measured, % clarification was then calculated.

Cell rupture.



Covaris E210 focused ultra-sonication device uses focused acoustics to shear samples. Parameter selection and variable alteration is done through an attached computer.

BCi was shown to be more resilient to shear than TCii, taking longer for complete cell rupture.

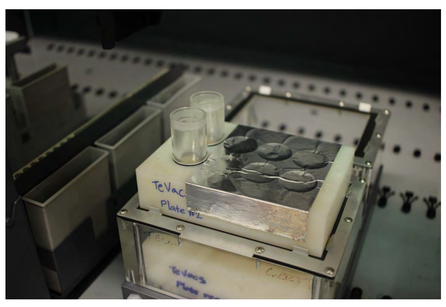


BCi (+CW) and TCii (-CW) samples were sheared at 6000 rpm and spun at 8000 rpm for 2 minutes – mimicking pilot scale operation of 150L/hour in a CSA-1 disk stack centrifuge at full power. The concentrated cells are then harvested and resuspended using TAP media to achieve a 1:50 concentration – again, mimicking pilot scale operation.

2mL of each strain was then placed inside the Covaris high frequency ultra-sonication device for varying amounts of time – the longer the cells are in the device, the more shear they are subject to. Each sample was then placed in the Mastersizer 3000 for the quantification of particle size distribution.

The results showed that, before shear, BCi is larger than TCii – 15 µm compared to 7 µm.
BCi also requires a greater amount of homogenisation in order to fully break the cells open to release the intracellular recombinant protein – 90 s compared to 10 s at full power in the Covaris.

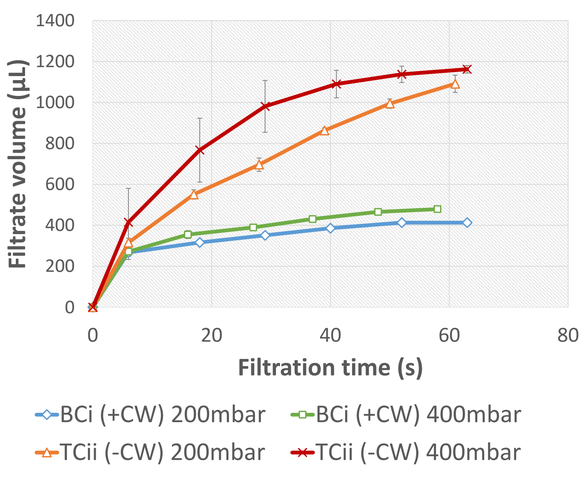
Filtration.



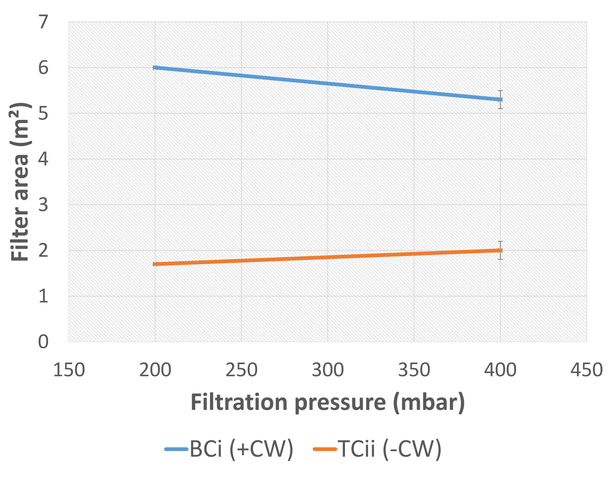
TECAN workstation adapted for USD filtration. Filter pods are loaded into air tight mould in preparation for application of a vacuum.

Close up of USD filter pod. The 0.28cm² filter is loaded into the left hand component and screwed into the right hand component. The rubber rings allow an air tight seal to form between the two components, as well as the filter pod and the mould.

Filtrate volume for TCii and higher pressures is greater than that of BCi and lower pressures.



Filter area required to filter 100L of clarified homogenate is greater for BCi than TCii.



BCi (+CW) and TCii (-CW) samples were, again, sheared at 6000 rpm, spun at 8000 rpm for 2 minutes, and then homogenised for 90 s and 10 s respectively in the Covaris high frequency homogeniser. The homogenate was centrifuged again, equivalent to 50 L/hour, and the supernatant loaded into prepared USD filter pods for filtration through a 0.28 cm² 30SP (2 µm pore size) filter from 3M. Two pressures were investigated, 200 mbar and 400 mbar, for each strain and the filtrate volume over time was measured [Zydney et al. 2002].

The charts opposite show that the greater the filtration operating pressure, the more filtrate that is collected. Also the presence of a cell wall reduces the amount of filtrate collected – the presence of cell wall debris causes blocking of the filter pores.

To filter pilot/process scale volumes of clarified homogenate a much larger filter area is required for the BCi strain, than the TCii strain. However, this filter area decreases with increasing filtration pressure.

Conclusion.

USD technologies [Li et al., 2013] enable small experiments to be used in order to try and predict large scale bioprocessing results with greater ease and reduced time. Using bench top shear devices and centrifuges in order to mimic the conditions undergone by the microalgae when entering/leaving large scale centrifuges, it was found that BCi were more resilient to the levels of shear that the cells are exposed to during the centrifugation process, resulting in less endolysin being released into the supernatant. Bench top focused acoustics were applied to effectively mimic the conditions of cellular disruption such as those encountered in larger batch or continuous cell homogenizers. USD experiments found that presence of a cell wall was predicted to require longer homogenization processing times in order to obtain the same degree of cell disruption achieved by cell wall-less variants in less time. Finally, the removal of cell debris post disruption was investigated using a USD depth filtration rig on a TECAN robotic lab workstation, it was found that using higher pressures and cell wall-less variants resulted in a greater volume of homogenate being able to pass through the filter before the filter pores became blocked.

These USD experiments have been replicated at pilot scale and similar results were achieved, proving the usefulness of USD experimentation when designing a bioprocess as a method to lower development costs and more rapidly establish parameters for operation.

References.

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