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C.BIRD™ | High-throughput Screening of Recombinant Clones in Microscale

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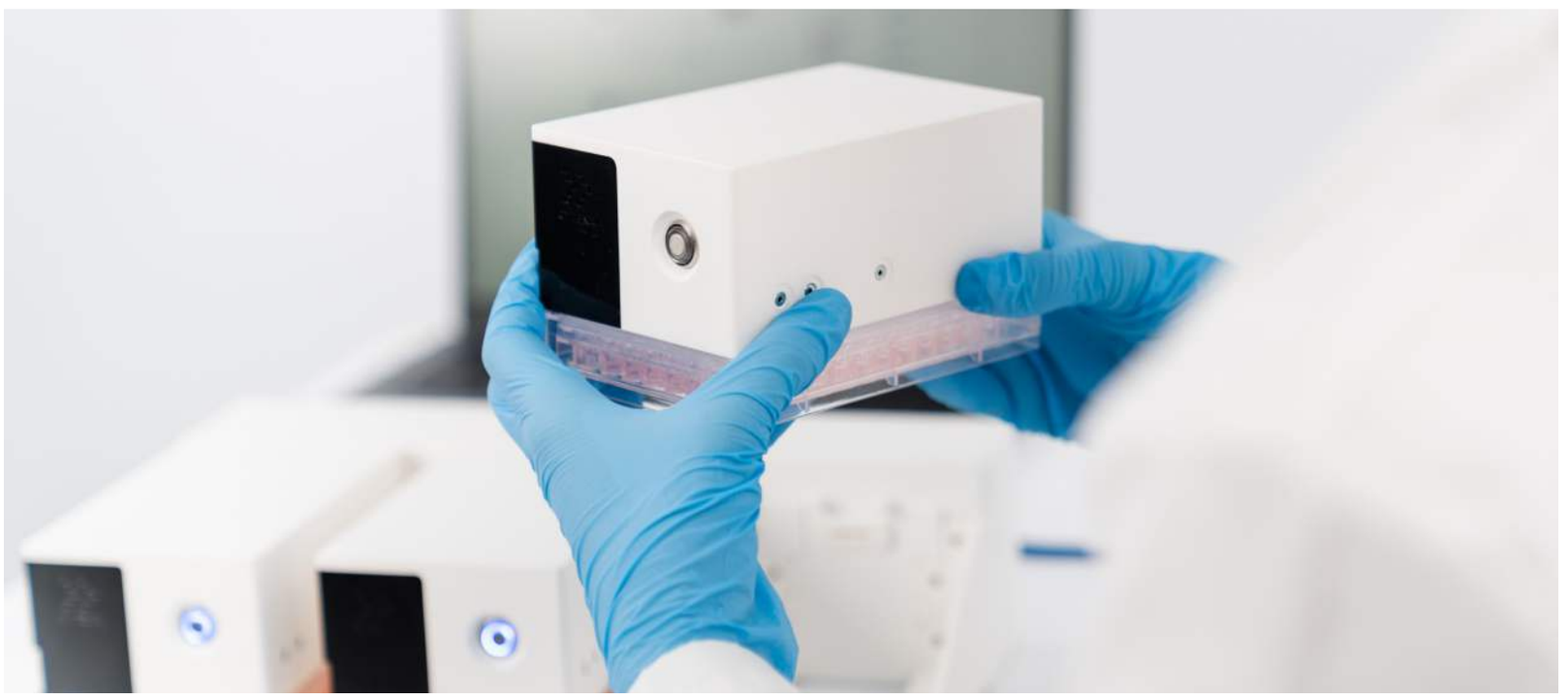


Abstract

High-throughput screening (HTS) has long been a method for selecting genetically modified clones from a pool of recombinants. To select clones with high protein and cell mass productivity for late-stage manufacturing, a need to provide comparable culture conditions between microscale and manufacture-scale has emerged. In this study, we present an innovative cell culture method for Pichia pastoris in 96-well plates and show a remarkable elevation in cell growth in microwells, compared to conventional static culture and aerated 96-deep-well (96-DW) shaking culture. The C.BIRD™ method, which provides tunable reciprocal agitation in microwells, offers suspension culture in 96-well plates with various mixing levels and, thus, introduces higher oxygen concentration in culture medium during the cell culture process. This flexible mixing method in microscale, with no compromise on high evaporation rate, sheds light on efficient HTS methods for aerobes and overcomes current limitations on protein engineering in biopharmaceutical industries as well as academic research.

Introduction

Pichia *pastoris*, a popular expression system for protein engineering, has found a place in commercial use in the past two decades. As a lower eukaryote, P. *pastoris* stands out because it allows for post-translational modifications as well as economical cell culture processing with high cell density outcomes. Diverse recombinant proteins are expressed in P. *pastoris*, such as antibodies, enzymes and vaccines. To attain recombinant proteins, DNA sequences of the desired proteins are integrated into the genome of P. pastoris for stable expression. However, this step inevitably results in clonal heterogeneity because of different numbers of expression copies integrated and off-targeted recombination. The need for an efficient method to screen for variants with high production of recombinant proteins, which are produced when P. *pastoris* reaches its stationary phase in cell growth, could not be overemphasized.

Materials and methods

The experiments were performed by Professor Guan-Chiun Lee's group at the School of Life Science of National Taiwan Normal University.
Yeast X-33 P. pastoris was used as the model in this study. To generate a library of stable clones, genetically modified plasmids of P. *pastoris* were integrated into the genome by electroporation. Various colonies were selected on YAG medium (20 g/L yeast extract, 5 g/L ammonium sulfate, 40 g/L glycerol) plates that contained 100 µg/mL of Zeocin, as the selection agent, and were inoculated into 1 mL of YAG medium (with 100 µg/mL of Zeocin) in 1.5 mL microcentrifuge tube. The transformants were then recovered for 3 days on a shaker at 225 rpm in a 30°C incubator.

For these experiments, 1 µL of each transformant was inoculated into individual wells in standard 96-well plates (or 96-DW plates), which contained 200 µL of YAG medium (with 100 µg/mL of Zeocin) and were incubated at 30°C under either static culture, aerated 96-DW shaking culture at 225 rpm, or C.BIRD culture with 13 seconds of mixing period. After 4 days of culture, absorbance at wavelength of 600 nm was measured in 96-well plates by Thermo Scientific's Multiskan GO Microplate Reader.

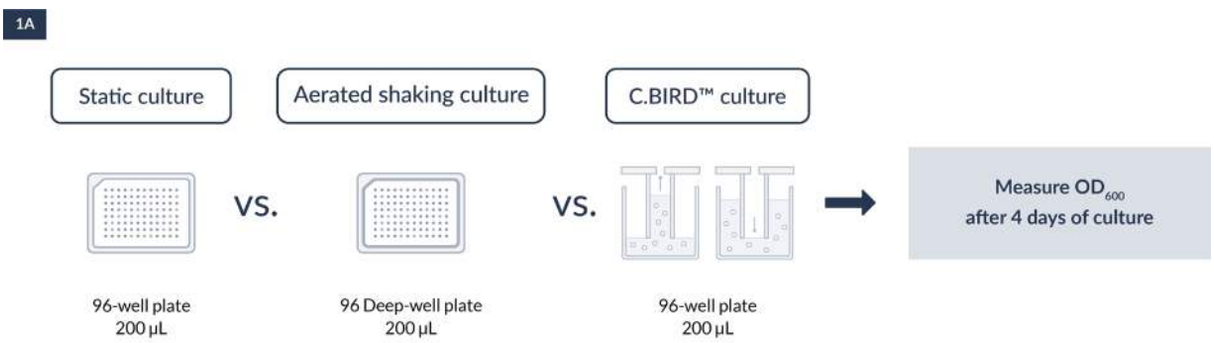


Figure 1. *Diagram of experiment design. The measurement of OD600 in each well indicated the cell growth under static, aerated shaking culture and the C.BIRD culture conditions*

Results and discussion

The experiments were designed as shown in **Figure 1**, and the absorbance in each well of static culture, aerated 96-DW shaking culture, and C.BIRD culture was measured by microplate reader on day 4. The results showed that the OD600 in the static group remained low at an average of 0.14, while that of the C.BIRD and aerated 96-DW shaking groups were at 0.43 and 0.29, respectively (**Figure 2A-C**). In the same clones, the C.BIRD group got 3.10-fold and 1.55-fold augmentation in OD600 value on average, compared to static and aerated 96-DW shaking groups. This indicated that the C.BIRD cell culture method could efficiently promote the cell growth of P. *pastoris*, compared to other conventional culture methods. Furthermore, the C.BIRD culture method provided a way to select the potential high-growth rate clones in microscale, such as clones A7, A8 and B3 in **Figure 2C**, and enabled an efficient and low-labor HTS method with more comprehensive outcomes. Additionally, with the overall elevation of OD600 value in the C.BIRD group, more clones that harbored high cell mass productivity were observed, compared to other culture method groups. This showed that the C.BIRD culture method could not only significantly increase cell growth in P. *pastoris* but also help selecting potential highly productive clones from the pool.

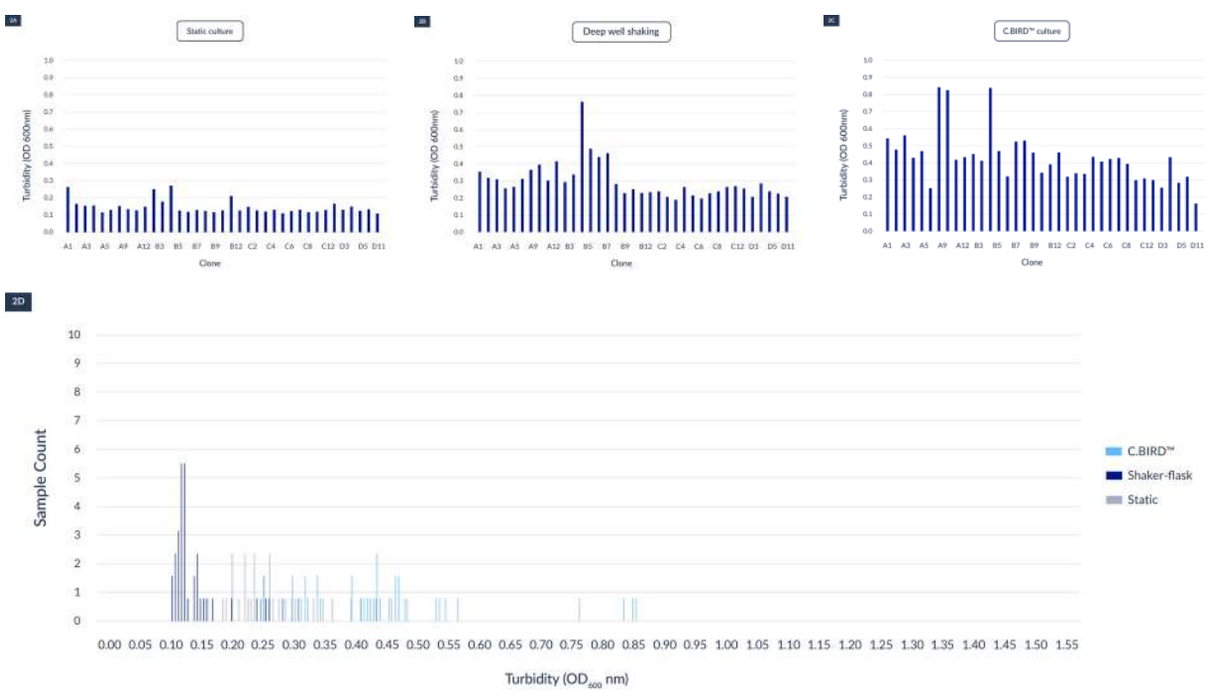
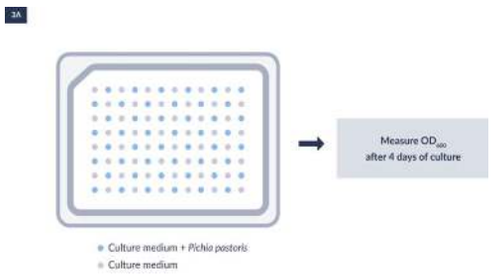


Figure 2. *The cell densities under three culture methods after 4 days of culture. The x-axis represented the name of the clones. A) static culture, B) aerated 96-DW shaking culture, and C) C.BIRD culture were shown. D) Integrated data showed the distribution of turbidity and its sample counts of the three culture methods.*

In parallel, a cross-contamination test was performed to secure the independence of each clone as well as the following measurements. Different clones were inoculated into 200 µL of YAG culture medium in the wells of a 96-well plate, while 200 µL of sterile culture medium was placed in the adjacent wells without inoculum, as shown in **Figure 3A**. After 4 days of continuous culture with the C.BIRD device, it showed that the OD600 value of the sterile wells remained the same as on day 1, which indicated that there was no well-well cross-contamination (**Figure 3B**). On the other hand, the wells, inoculated with transformants, showed increasing OD600 values during 4 days of culture, therefore demonstrating the capability for cell division of the clones (**Figure 3C**).



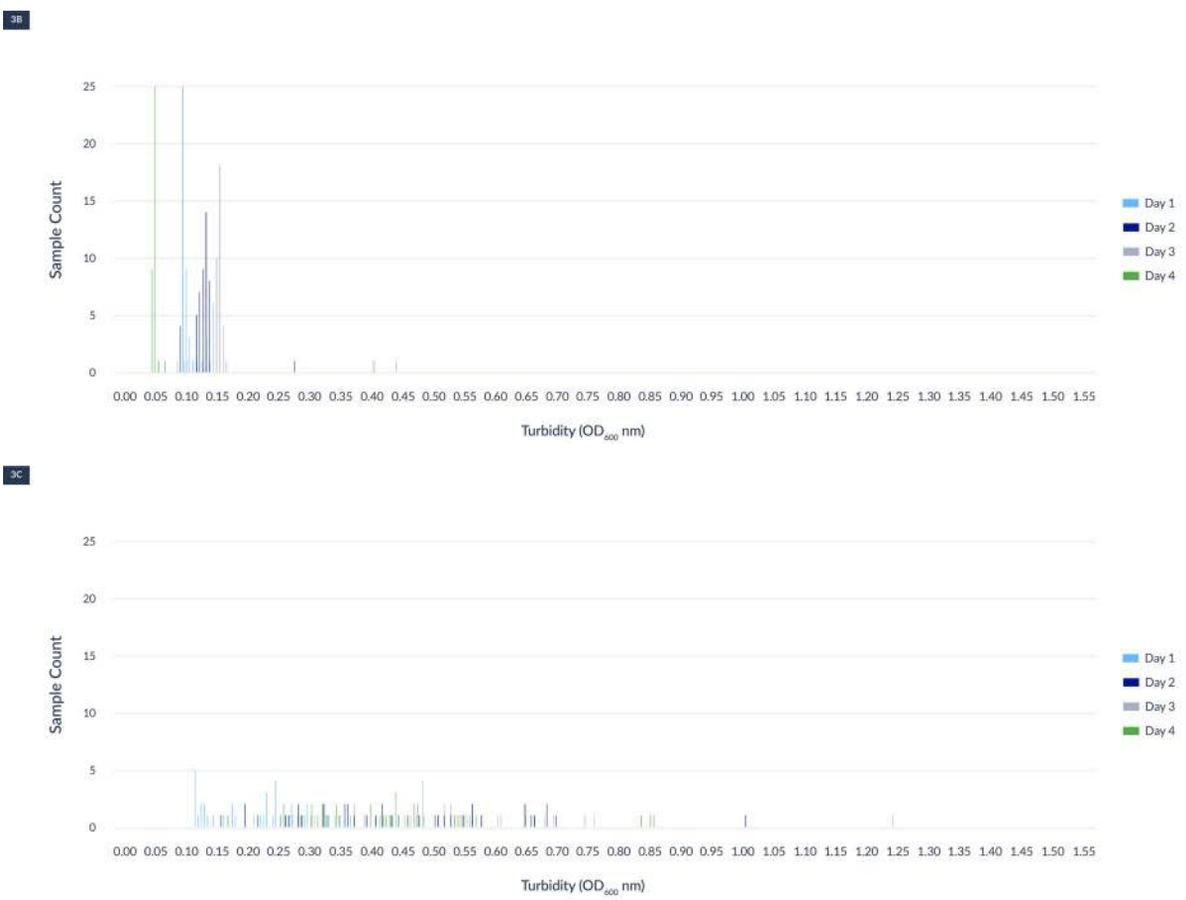


Figure 3. *Cross-contamination test results. A) The experiment design of cross-contamination test. B) In culture medium group, it showed that there was no cross-contamination between adjacent wells, while C) the cell division of P. pastoris remained active.*

Conclusion

Introducing the C.BIRD method to standard 96-well plates provided higher oxygen transfer rates, which furnished the aerobic cell culture with better culture conditions. Our results show that efficient mixing during the P. *pastoris* cell culture process dramatically and evenly elevated cell density, allowing researchers to select highly productive clones in a high-throughput fashion, with no need for laborious process. Moreover, the results also ensured the independence of each well during long-term culture. This undoubtedly fortified the strength and reliability of the C.BIRD method on selecting clones from genetically modified transformants.

The C.BIRD method refined the conventional HTS methods in P. *pastoris* by enabling a well-mixed and aerated culture condition in 96-well plates and increased the average cell density of each clone in comparison with other methods. The C.BIRD method provides an ideal cell culture condition for aerobic organisms and offered a more efficient way for HTS in P. *pastoris*.

Reference

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