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Ectoine enhances recombinant antibody production in Chinese hamster ovary cells by promoting cell cycle arrest

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ABSTRACT

Chinese hamster ovary (CHO) cells represent the most preferential host cell system for therapeutic monoclonal antibody (mAb) production. Enhancing mAb production in CHO cells can be achieved by adding chemical compounds that regulate the cell cycle and cell survival pathways. This study investigated the impact of ectoine supplementation on mAb production in CHO cells. The results showed that adding ectoine at a concentration of 100 mM on the 3rd day of cultivation improved mAb production by improving cell viability and extending the culture duration. RNA sequencing analysis revealed differentially expressed genes associated with cell cycle regulation, cell proliferation, and cellular homeostasis, in particular promotion of cell cycle arrest, which was then confirmed by flow cytometry analysis. Ectoine-treated CHO cells exhibited an increase in the number of cells in the G0/G1 phase. In addition, the cell diameter was also increased. These findings support the hypothesis that ectoine enhances mAb production in CHO cells through mechanisms involving cell cycle arrest and cellular homeostasis. Overall, this study highlights the potential of ectoine as a promising supplementation strategy to enhance mAb production not only in CHO cells but also in other cell lines.

Introduction

Since most commercial therapeutic monoclonal antibodies (mAb) are produced by Chinese hamster ovary (CHO) cells [1], several strategies to increase their productivity have been studied [2–4]. These include optimization of feed medium or the use of supplementations to increase viable cell density (VCD), and/or culture duration, and/or to reduce the accumulation of toxic metabolites [5–7]. Several chemical compounds have been reported to positively affect the productivity of recombinant proteins in CHO cells by regulating cell proliferation, specifically by reducing cell division and inducing cell cycle arrest. Examples of such compounds include nucleosides and nucleotides [8], valproic acid [9], valeric acid [10], and catechins [11]. However, there is inherent variability in cellular responses to supplementation across cell lines, the discovery of alternative supplementations remains interesting for their application in mAb production in different cell lines.

Ectoine (Fig. 1), was first discovered in 1985 in the halophilic phototrophic bacteria *Ectothiorhodospira halochloris* [12]. This molecule exhibits high water solubility (~ 4 M) [13] and has a predicted pKa of 3.14 [12,14]. Ectoine is classified as a biocompatible solute due to its compatibility with cellular metabolism without adverse effects on physiological processes [15]. It serves as a cell protectant, enabling halophilic organisms to survive under extreme conditions, including high temperatures, freezing, dryness, and osmotic stress [15]. It has been reported that ectoine at a concentration ranging from 10 – 1000 mM, can stabilize lactic dehydrogenase and phosphofructokinase

Abbreviations: BP, Biological Process; CDK, Cyclin-Dependent Kinases; CHO, Chinese Hamster Ovary; DEG, Differentially Expressed Gene; GPCR, G-Protein Coupled Receptor; IVCD, Integral Viable Cell Density; mAb, Monoclonal Antibodies; RT-qPCR, Reverse Transcription Quantitative Polymerase Chain Reaction; VCD, Viable Cell Density; VL, Variable Fragment Light Chain; VH, Variable Fragment Heavy Chain.

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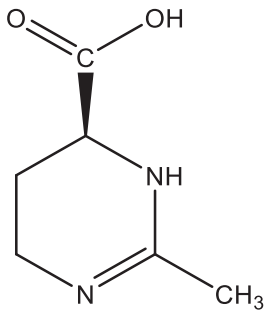

Fig. 1. Chemical structure of ectoine (Synonym: (S)– 2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid).

enzymes from freeze-thawing, heating, and freeze-drying [16], phytase enzymes from heating [17], and zymogens from proteolysis by enteropeptidase [18]. Additionally, it can improve cryopreservation of human endothelial cells [19], and inhibit thermal-induced aggregates of recombinant human interferon alpha2b [20]. Additionally, ectoine is widely used in cosmetics to prevent skin dehydration [15] and alleviate skin inflammation [21], as well as in medical formulations such as mouthwash [22] and eye drops [23]. Based on the beneficial effects of ectoine, this study aims to investigate its potential to prolong the survival of CHO producer cells, thereby increasing mAb production. The effects of ectoine on cell growth profiles which include cell viability, VCD, integral viable cell density (IVCD), and cell diameter, as well as on mAb production were determined. In addition, gene expression and cell cycle analysis of CHO cells treated with ectoine were performed using RNA sequencing and flow cytometry, respectively, to study the mechanism of action of ectoine on CHO cells.

Materials and methods

Materials

All cell culture reagents which were Dynamis (catalog no. A2661501), L-glutamine (catalog no. 25030081), and anti-clumping agent (catalog no.0010057AE) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A conical 50 mL-Tubespin® bioreactor tube (catalog no. BS-010158-CK) was purchased from Biosan (Riga, Latvia). Ectoine (catalog no. E1372) was purchased from Tokyo Chemical Industry (Saitama, Japan). For ELISA, Invitrogen™ Nunc MaxiSorp™ flat-bottom 96-well plates (catalog no. 44–2404-21) and 1-Step™ Ultra TMB solution (catalog no. 34028) were purchased from Thermo Fisher Scientific. Protein A (catalog no. GSZ02201) was purchased from GenScript (Piscataway, NJ, USA). Peroxidase AffiniPure F (ab')$_2$ fragment goat anti-human IgG (H+L) HRP (catalog no. 109–036-088) was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA).

Cell culture

A single clone of CHO-DHFR$^-$ cells (4G8) producing adalimumab has been developed [24] and used as a model of the study. The 4G8 cells were cultured in a 50 mL-TubeSpin® bioreactor containing 20 mL of cultured medium composed of Dynamis™ supplemented with L-glutamine (8 mM) and anti-clumping agent (1:100 dilution). Ectoine stock solution was prepared by dissolving the powder in 0.01 M PBS buffer (pH 7.4) to obtain a concentration of 5 M and filtered sterilized using a PES syringe filter with a pore size of 0.2 μm.

CHO cells were seeded at 3×10^5 cells/mL and cultured under the following conditions: temperature maintained at 37 °C, 7 % CO_2, and shaking at 200 rpm. On day 3, after cell sampling, the ectoine stock solution was added to obtain a final concentration of 100 mM. For the control group, sterile PBS solution was added in an equivalent volume. To compare the effect of 100 mM ectoine with the control group in terms of cell growth profiles and antibody production, we conducted two independent experiments, each experiment was conducted in duplicate. Samples were collected daily to monitor the cell growth profile, which was assessed using trypan blue staining and LUNA-IITM automated cell counter (Logos Biosystems; Gyeonggi-do, South Korea) technique. Additionally, the samples were used to monitor glucose levels, which were maintained at 2 g/L using Glucosure autocode test strips (Allwell Life Co., Ltd., Bangkok, Thailand). Cell cultures were stopped when the cell viability dropped below 20 %. Culture supernatants were aliquoted and stored at − 20 °C for further analysis.

Determination of antibody titer and productivity

The antibody titer was analyzed using ELISA-based method as previously described [25]. The mAb productivity (pg/cell/day) on the harvesting day was calculated based on the antibody titer and IVCD [26] according to the following equations;

$$IVCD_t = IVCD_{t_0} + \left(\frac{1}{2} \times VCD_t + VCD_{t_0}\right) \times \Delta t \quad (1)$$

$$\text{Productivity } (pg/cell/day) = \frac{\text{Antibody titer}}{IVCD} \quad (2)$$

where IVCD is integral viable cell density, VCD is viable cell density, t is cultured time, t_0 is initial cultured time, and Δt is time interval.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

On the 4th and 5th days of cultivation, RNA samples were collected following the method described for RNA sequencing. The collected RNA samples were subsequently converted to cDNA using the Maxime RT Premix kit (catalog no. 25082; iNtRON, Kyungki-Do, Korea). For qPCR analysis, the QuantStudio™ 5 System (Thermo Fisher Scientific) was utilized, and the data acquisition was analyzed using QuanStudio™ Design & Analysis Software v1.5.2 (Thermo Fisher Scientific). The thermal cycling steps consisted of an initial denaturation step of 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 63 °C. Subsequently, a melting curve step was performed to confirm the specificity of the primers. To determine the expression levels of VL (variable fragment light chain) and VH (variable fragment heavy chain) of adalimumab, normalization was conducted using the housekeeping gene GAPDH in order to account for any variations in RNA/cDNA quantity among the samples. Primer sequences for VL and VH of adalimumab genes, and GAPDH were presented in Table A.1.

RNA sequencing

On day 5 of cultivation, RNA samples were extracted from approximately 2×10^6 cells using an Easy-spin™ total RNA extraction kit (catalog no. REF17221; iNtRON). Subsequently, DNase (catalog no. AM1906; Thermo Fisher Scientific) was added to the extracted RNA samples to remove any contaminating DNA. The RNA samples were then preserved in GenTegra® RNA microtubes (GenTegra; Pleasanton, CA, USA), and shipped to Genewiz® Biotechnology Co. Ltd (Suzhou, China) via Getz Healthcare (Bangkok, Thailand) for RNA sequencing analysis. The RNA sequencing results were analyzed to identify genes with significant difference, based on the criteria of a fold change greater than 2 and a q value (fdr, padj) less than 0.05. Gene ontology functional enrichment analysis was performed using a filtering threshold of a p-value ≤ 0.05.

Cell cycle determination

Cell cycle distribution was determined using the Guava® Muse® cell analyzer (Luminex, Austin, USA). Sample preparation and analysis were

performed according to the manual of the Muse® cell cycle kit (catalog no. MCH100106).

Statistical analysis

Calculation of antibody titer and evaluation of the significant differences (with a 95 % confidence interval) between treatment groups were done with GraphPad (Prism version 9.4). Determinations of significant differences in antibody titers and cell cycle distribution between treatment groups were performed by unpaired t-test.

Results and discussion

Ectoine at 100mM improved cell viability, increased cell diameter, and enhanced mAb production

In previous reports, the effective concentration of ectoine for different applications was found to be in the range of 10 – 1000 mM. Thus, in a preliminary study, we examined the impact of four different concentrations of ectoine: 0, 10, 100, and 500 mM, under batch culture conditions. While the addition of ectoine at 10 mM did not enhance mAb production, a concentration of 500 mM was found to be toxic to CHO cells, resulting in cell viability of less than 20 % after one day of ectoine supplementation (Fig. A.1). Consequently, a concentration of 100 mM was selected for further investigation into the optimal timing of ectoine supplementation in fed-batch cultures. The findings indicated that ectoine supplementation on the 3rd day of cultivation was the optimal timing of supplementation rather than the 2nd day because mAb titer yield was higher (Fig. A.2). To confirm the beneficial effect of ectoine supplementation on the 3rd day of cultivation, two independent experiments with duplicate cultures of each group were tested in fed-batch cultures with glucose feeding, which is the standard condition for manufacturing therapeutic mAb [27].

As illustrated in Fig. 2A, the results indicated that CHO cells treated with ectoine exhibited better performance, as evidenced by % cell viability. The cell viability remained above 90 % until day 8 of cultivation, after which it dropped to 80 %. In contrast to the control group, which was not treated with ectoine, the cell viability declined to 86.5 % on day 7 of cultivation. Since ectoine-treated CHO cells were able to survive one day longer than the control group, an overall IVCD was 6.4 % higher than that of the control. (Fig. 2B). Additionally, the addition of ectoine seemed to have minimal impact on VCD, with values of 1.4 % and 6.1 % lower than the control group after one (Day 4) and two days (Day 5) of supplementation, respectively (Fig. 2C). At the later stage of cultivation, starting on day 8, CHO cells treated with ectoine exhibited higher VCD compared to that of the control group. This observation corresponded with the higher cell viability as shown in Fig. 1A. In this study, the minimal impact of ectoine on VCD was

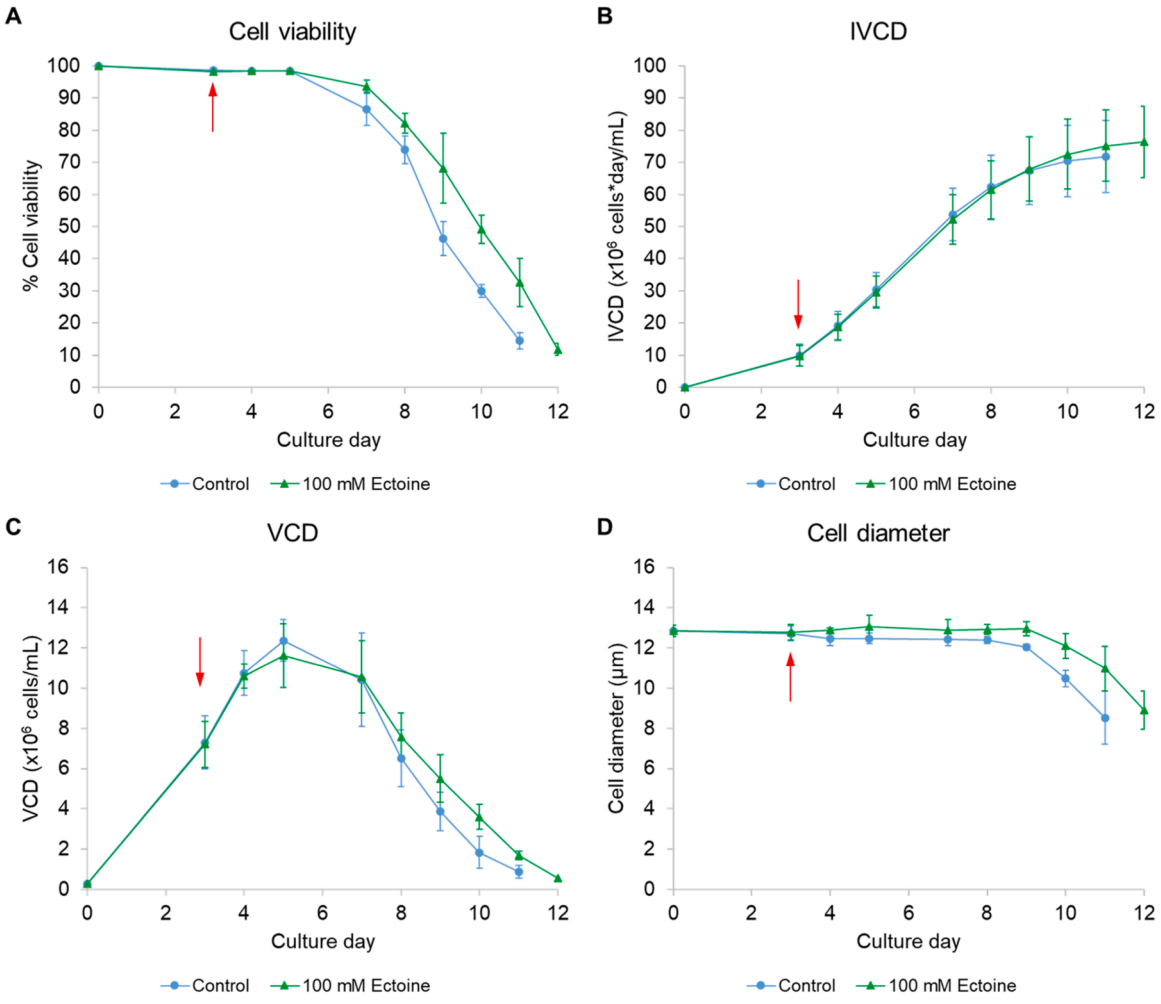

Fig. 2. Effects of ectoine on CHO cells in fed-batch cultures. Effect of ectoine in terms of A) % cell viability, B) IVCD, C) VCD, and D) cell diameter. The blue circle represents the control group, while the green triangle represents CHO cells treated with 100 mM ectoine. Each data point represents the average of two independent experiments, of which each experiment was conducted in duplicate. The error bars indicate ± SD of the measurements. The red arrow denotes the third day of cultivation when 100 mM ectoine was added.

observed in comparison to those observed when 50 μM resveratrol was used as a supplement on the third day of cultivation [28], of which approximately 30 % and 50 % reduction in VCD was observed after one and two days of addition, respectively.

In addition, we found that ectoine caused an increase in cell diameter (Fig. 2D), similar to what was found in CHO cells treated with 1 mM adenosine [29] and 50 μM catechin [11]. The increase in cell diameter has been reported to have an association with cell cycle arrest, which is a recognized approach for boosting mAb production [30].

Evaluation of mAb productivity, as shown in Fig. 3, revealed that ectoine could significantly increase both mAb titer and productivity, up to 29 % and 17 %, respectively, compared to those of the control group (Fig. A.3). The observed enhancement in mAb titer was attributed to the extension of culture duration and an associated increase in overall IVCD. Therefore, it can be concluded that ectoine supplementation on the 3rd day of cultivation had a positive impact on mAb production in CHO cells.

Investigations on the addition of various compounds that could interfere with the regulation of cell proliferation to improve mAb production from CHO-DHFR system have been reported. When compared to sodium valproate, ectoine demonstrated superior performance in both mAb titer and productivity [9]. As for catechin [11], resveratrol [28], and adenosine [31], ectoine exhibited a comparable benefit in terms of mAb titer but lower effects on productivity levels, i.e., the increase in productivity levels upon addition of ectoine, resveratrol, catechin, and adenosine was at 1.2-, 1.5-, 1.9-, and 2.5-folds, respectively. However, variations in productivity may be attributed to factors such as the types of CHO cells, the culture conditions, and the duration of the culture. Specifically, our results were obtained under fed-batch cultures, leading to higher maximum VCD. On the contrary, the results of the studies using resveratrol, catechin, and adenosine were conducted under batch cultures. The difference in experimental setups might explain why ectoine yielded a comparable mAb titer but exhibited lower productivity when compared to the aforementioned additives.

RT-qPCR analysis indicated that ectoine didn't induce the expression of VL and VH genes

We next investigated whether an increase in adalimumab production by ectoine supplementation was the result of an increase in the expression of VL and VH genes. In this experiment, RNA levels of VL and VH of adalimumab were normalized to the housekeeping gene, GAPDH. As illustrated in Fig. 4, there was no significant difference regarding the expression of VL and VH genes of adalimumab between control and ectoine-treated CHO cells after 1 and 2 days of supplementation. Thus, our findings suggested that the enhancing effect of ectoine on antibody

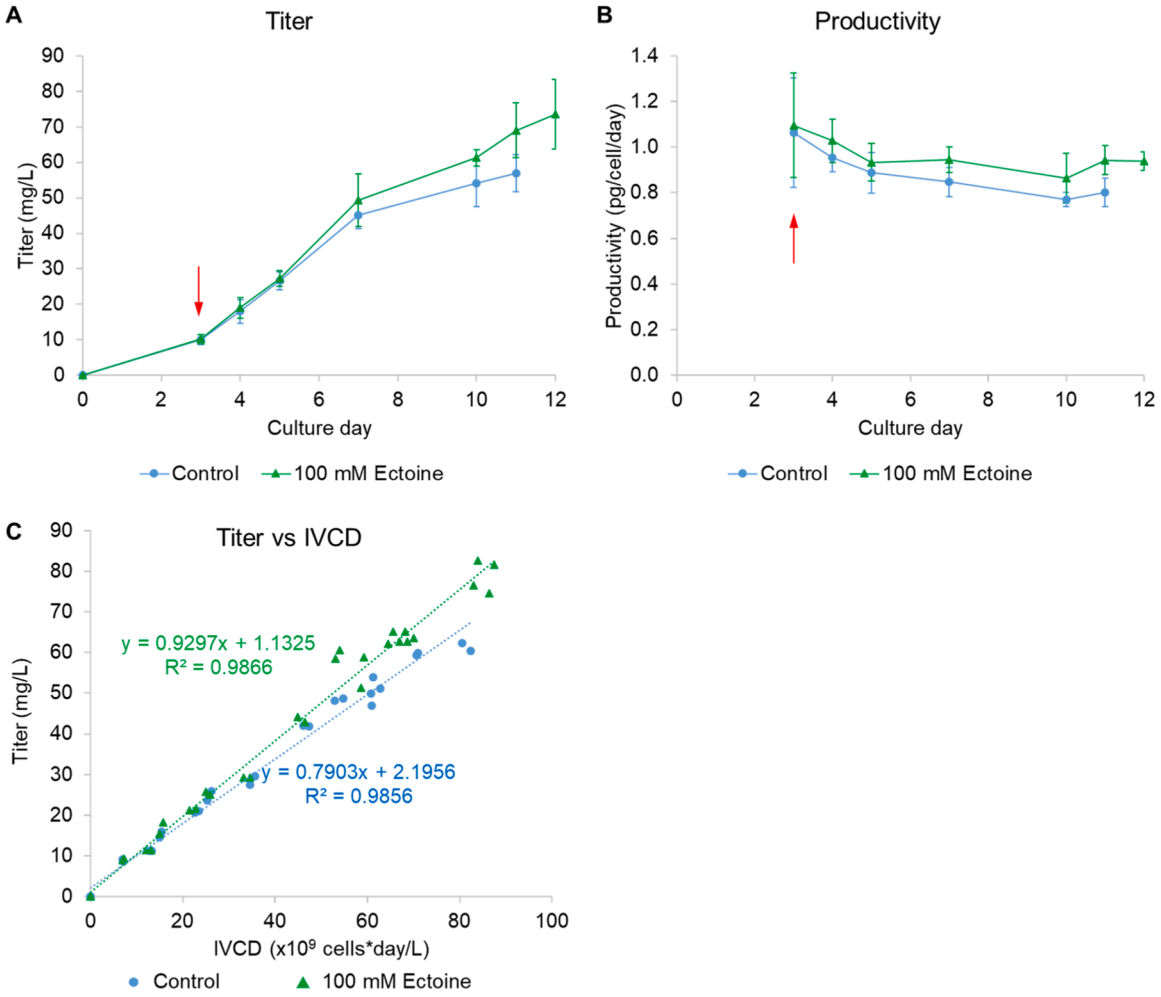

Fig. 3. The impact of 100 mM ectoine on mAb production in CHO cells during fed-batch cultures. A comparison of mAb titer (A) and productivity (B) over the culture duration between ectoine-treated CHO cells and the control group. C) The correlation between mAb titer and IVCD for CHO cells treated with 100 mM ectoine, compared to the control group. The slopes of the linear equations denote the productivity of CHO cells in each respective group. The blue circle and line represent the control group, whereas the green triangle and line represent CHO cells treated with 100 mM ectoine. Each data point represents the average of two independent experiments, of which each experiment was conducted in duplicate. The error bars indicate ± SD of the measurements. The red arrow denotes the third day of cultivation when 100 mM ectoine was added.

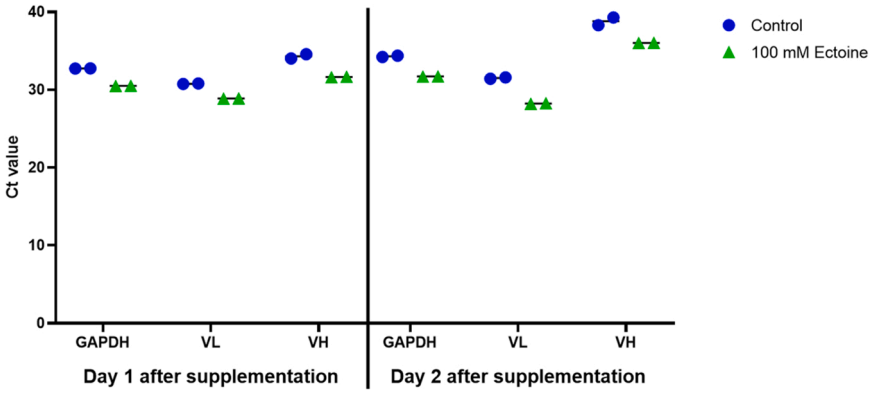

Fig. 4. RT-qPCR analysis of cDNA samples converted from RNA of CHO cells on Day 1 and 2 after adding ectoine (culture days 4 and 5). The cycle threshold (Ct) represents the cycle number at which the fluorescence signal reaches a detectable threshold in qPCR, indicating the relative abundance of the target genes which were variable fragment light chain (VL) and variable fragment heavy chain (VH) of adalimumab gene compared to GAPDH. The blue circle represents the control group, whereas the green triangle represents CHO cells treated with 100 mM ectoine. The black line connected to each data point indicates the average value obtained from two replicates of each sample.

production was not due to an increase in mAb gene expression level.

Differentially expressed gene (DEG) profile of ectoine-treated CHO cells indicated changes in genes involving in cell cycle regulation, in particular cell cycle arrest

Since an increase in mAb production was not based an increase in antibody gene expression, we performed RNA sequencing analysis to try to understand how ectoine could increase the production of mAb from CHO cells. DEG profiles analysis revealed that the expression of 301 genes in CHO cells treated with the optimal concentration of ectoine (100 mM) were significantly different from those of the control cells (Fig. A.4). Among these genes, 73 genes were up-regulated, and 228 genes were down-regulated. A cluster analysis of these 301 genes was used to assist in predicting the function of unknown genes (Fig. A.5).

A list of the top 10 of up-regulated genes found in ectoine-treated cells is reported in Table 1. These 10 genes have been linked to various pathways associated with the biological process (BP) of cell cycle regulation, cell proliferation, and cell homeostasis. The gene demonstrating the most significant up-regulation, namely *LOC100762114*, is implicated in the regulation of the cell cycle, potentially through ubiquitination [32], which is also linked to gene *Asb16*. Another pathway that may be linked to cell cycle regulation is the G-protein coupled receptor (GPCR) signaling pathway [33] involving gene *Actl10*. Additionally, the gene *LOC113837292* plays a role in the cell cycle regulation, specifically in G1 arrest, but conversely, in promoting G1/S progression and G2/M arrest, depending on the specific type of Rho proteins involved [34,35]. Moreover, the gene *B4galnt2*, which relates to negative cell-cell adhesion, and *Mmp11*, which relates to negative regulation of fat cell differentiation, may also contribute to the observed slight decrease in cell proliferation.

The second most up-regulated gene, namely *Gltpd2*, is associated with ceramide transport, and such transport has been reported to be related to the cell cycle, cell differentiation, and cellular homeostasis [36]. In particular, ceramide has been reported to induce G0/G1 arrest [37,38]. Additionally, gene *Pced1b* and *LOC103160255* are related to the cell cycle, specifically in the process of existing from mitosis. Notably, gene *LOC103160255* plays a role in the process of peptidyl-serine dephosphorylation, which contributes to the exit from mitosis [39]. These up-regulated genes from the DEG results support the idea that one mechanism of ectoine to increase adalimumab production might involve the regulation of the cell cycle, similar to using nucleoside supplementation to improve recombinant protein production [8].

In the case of the down-regulated genes, eight out of the top 10 most down-regulated genes are linked to the cell cycle or cell proliferation through various pathways. The highest down-regulated gene in ectoine-treated CHO cells was *Ralgps2*. Silencing this gene has been reported to induce cell cycle arrest at the G0/G1 phase in lung cancer cells [40]. Additionally, the gene *Togaram*, which is involved in mitotic spindle assembly, may also be related to cell cycle arrest in the G0/G1 arrest, along with other down-regulated genes (Table A.3), specifically, *LOC103159011* which is involved in G1/S transition. The gene *Apoh*, *Slc23a3*, *Sgcg*, and *LOC113838085* that were down regulated are also involved in cell proliferation. The *Napsa* and *Qrfp*, the second and third most down-regulated genes, respectively, may potentially be involved in the cell cycle or cell division through the proteolysis process [41,42] and neuropeptide signaling pathway by binding to GPCR, respectively [43].

Table 1
Top 10 log2 fold-change of up-regulated genes affected to biological process in ectoine treated CHO cells.

Gene symbol	Log2 fold-change	Gene functions in biological process (BP)
LOC100762114	4.700	cell cycle; proteasome-mediated ubiquitin-dependent protein catabolic process; protein ubiquitination
Gltpd2	4.000	Ceramide transport
Actl10[a]	3.907	*Not available information for BP in gene ontology database*
LOC100750613	3.807	positive regulation of macromitophagy; anion transport; negative regulation of apoptotic process; epithelial cell differentiation; mitochondrial calcium ion transport
Pced1b[b]	3.807	*Not available information for BP in gene ontology database*
LOC113837292	3.585	positive regulation of Rho protein signal transduction; adenylate cyclase-activating G-protein coupled receptor signaling pathway; regulation of cell adhesion
LOC103160255	3.585	cell division; exit from mitosis; peptidyl-serine dephosphorylation
B4galnt2	3.585	negative regulation of cell-cell adhesion; lipid glycosylation; protein glycosylation; UDP-N-acetylgalactosamine metabolic process; UDP-N-acetylglucosamine metabolic process
Asb16	3.585	intracellular signal transduction; protein ubiquitination
Mmp11	3.585	basement membrane organization; extracellular matrix organization; collagen catabolic process; negative regulation of fat cell differentiation

[a] According to the cluster analysis results, *Actl10* involves with G-protein coupled receptor signaling pathway.
[b] According to the cluster analysis results, *Pced1b* involves with axon transport of mitochondrion, cell division (exit from mitosis)

Overall, these findings suggest that ectoine's ability to enhance mAb production is attributed to its regulation of the cell cycle, specifically through the induction of cell cycle arrest in the G0/G1 phase, which has been reported as the optimal stage for cell arrest to maximize mAb production [3]..

Flow cytometry analysis confirmed the promotion of cell cycle arrest in ectoine-treated CHO cells

The DEG profile and noticeable increase in cell diameter after ectoine treatment as described above suggested that cell cycle arrest might be the key mechanism of ectoine enhancement of mAb production. This observation prompted us to investigate cell cycle distribution of CHO cells upon ectoine treatment in comparison with non-treated cells. As expected, a significant increase in CHO cells in the G0/G1 cell cycle phase was observed after one day of ectoine supplementation, in comparison to the control group (Fig. 5). These results were consistent with the study conducted by Rieckmann et al. [44], which found that two types of head and neck squamous cell carcinoma cells exhibited a higher fraction of cells in the G1 phase when incubated with ectoine at concentrations of 70 and 140 mM for 2 h.

The increase in cell population in the G0/G1 phase in ectoine-treated CHO cells was similar to that observed when CHO cells were treated with various additives, such as valeric acid [10], cyclin-dependent kinases (CDK) inhibitors [45], and sodium butyrate [46]. Conversely, other additives, such as adenosine 5′-monophosphate (AMP) [8], resveratrol [28], and adenosine [29], induced cell arrest in the S phase of CHO cells. Numerous studies have indicated that the G1 phase is considered the ideal phase for enhancing recombinant antibody production [3,47,48]. This preference arises because cells arrested in the G1 phase exhibit increased metabolic activity [49]. Remarkably, the increase in cell diameter was also observed in ectoine-treated CHO cells which were arrested in the G1 phase (Fig. 2D). Therefore, these results corresponded to the DEG profile and suggest that the mechanism underlying the improvement of mAb production involves the implementation of a cell cycle arrest strategy, which is a recognized strategy for enhancing the production level of mAb [30].

While there was no significant difference in the cell cycle distribution on the fifth day of cultivation (two days after the supplementation of ectoine), RNA sequencing of samples obtained on the same day revealed that several genes related to cell cycle process in the ectoine-treated group were significantly different from the control group. These results were possibly due to the complexity of the cell cycle process and the fact that not all cells were in the same phase at the time of supplementation. Cells already committed to division continued to progress until reaching the next G1 phase [50]. This might explain the lack of significant overall difference in cell cycle distribution using flow cytometry, despite the observed changes in gene expression related to cell cycle arrest.

The duration of cell cycle arrest varies depending on the type of chemical additives used. For example, a selective cyclin-dependent kinase 4/6 small molecule inhibitor induced a sustained effect for at least four days [45], while resveratrol exhibited a temporary effect lasting for 48 h [28]. In the case of ectoine, which showed a significant cell cycle arrest for only one day, the underlying molecular changes, as evidenced by the observed gene expression changes and continued influence on cell diameters, were observed for a more prolonged period of time.

Regarding antibody production, a clear improvement in titer was observed on the seventh day of cultivation, potentially linked to an initial decrease in cell viability in the control group. Therefore, it is assumed that ectoine might enhance antibody production through multiple mechanisms in addition to cell cycle arrest. RNA sequencing results indicated that genes involved in cell homeostasis, as well as cell cycle and cell proliferation processes were affected. These findings support the hypothesis that ectoine employs various mechanisms, beyond cell cycle arrest, to improve antibody production.

Further DEG analysis indicated other molecular mechanisms of ectoine on mAb production in addition to cell cycle arrest

In addition to cell cycle arrest, an increase in adalimumab production in ectoine-treated CHO cells might be due to better protection from toxic effects, leading to a prolonged culture duration and thus overall increase in mAb titer. This assumption was supported by the up-regulation of gene *LOC100750613*, which is involved in the positive regulation of macromitophagy. This process functions to remove dysfunctional mitochondria that could potentially release harmful reactive oxygen species and other species leading to cell death [51].

Furthermore, our DEG results revealed several up-regulated genes (Table A.2) associated with cell proliferation and survival pathways, including MAPK, PI3-Atk, ERK, p53, TORC1, and Ras. These pathways play a role in facilitating growth and maintaining cellular functions including an increase in cell diameter [52,53]. Importantly, previous studies have reported a positive correlation between the activation of these pathways and high productivity in CHO cells [54–56]. Additionally, an increase in cell diameter, which indicates a higher cell volume, has been demonstrated to correlate with enhanced specific productivity [56,57], similar to what observed in this study.

Among the 73 up-regulated genes and 228 down-regulated genes identified in ectoine-treated CHO cells, both groups exhibited the presence of genes involved in the cell cycle, cell proliferation, and survival pathways, which sometimes provide conflicting effects. These observations could be because the regulation of cell cycle and cell proliferation is a complex process influenced by multiple factors and pathways, including ubiquitination and GPCR pathways, operating across all phases of the cell cycle. Further investigations into the DEG profiles during the later stages of fed-batch cultures supplemented with ectoine may offer deeper insights into the mechanisms underlying ectoine's enhancement of mAb production.

Finally, down-regulation of *LOC113833508* and *Nyx*, which are required for the activation of immune responses in cells to various stimuli have been shown to result in the reduction of energy consumption and nutrient uptake [58,59]; therefore, more extra energy supply could be available for more mAb production. Moreover, the reduction in nutrient uptake may decrease the production of toxic metabolites,

Table 2
Top 10 log2 fold-change of down-regulated genes affected to biological process in ectoine treated CHO cells.

Gene symbol	Log2 fold-change	Gene functions in biological process (BP)
Ralgps2	-5.322	regulation of Ral protein signal transduction
Napsa	-4.907	membrane protein proteolysis
Qrfp	-4.700	neuropeptide signaling pathway
Apoh	-4.459	positive regulation of lipoprotein lipase activity; triglyceride metabolic process; negative regulation of endothelial cell proliferation; negative regulation of myeloid cell apoptotic process
Slc23a3[a]	-4.459	*Not available information for BP in gene ontology database*
Sgcg[b]	-4.459	*Not available information for BP in gene ontology database*
Togaram2	-4.459	mitotic spindle assembly
LOC113838085	-4.322	negative regulation of RNA splicing; positive regulation of insulin secretion; positive regulation of protein dephosphorylation; positive regulation of transcription from RNA polymerase II promoter; regulation of cell differentiation
LOC113833508	-4.170	immune response
Nyx	-4.170	response to stimulus

[a] According to the cluster analysis results, *Slc23a3* involves with cell differentiation, lipid transport and carbohydrate metabolic process

[b] According to the cluster analysis results, *Sgcg* involves with actin filament organization and cell proliferation

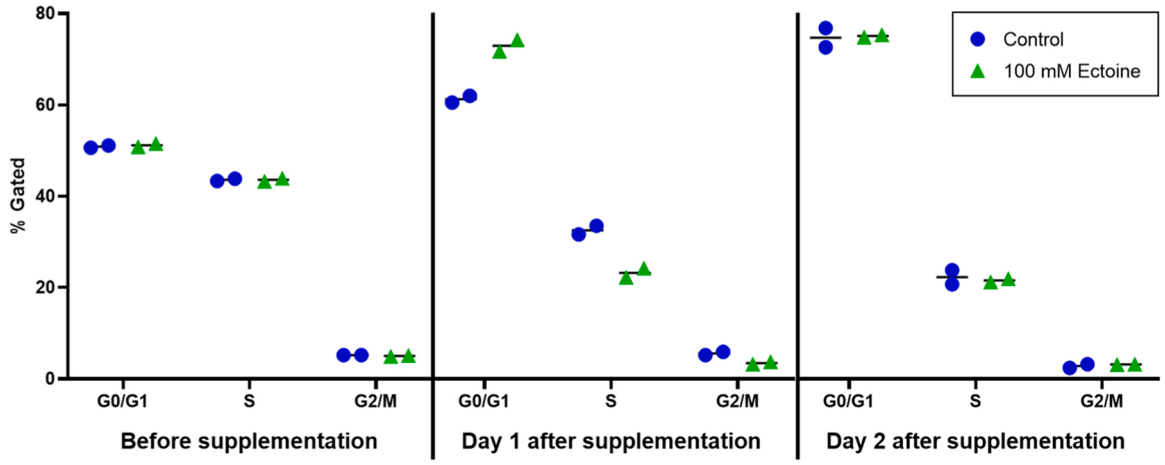

Fig. 5. Effect of ectoine on cell cycle distributions of CHO cells (* indicating a p-value < 0.05). The yellow, red, and green colors represent the cell proportions in G0/G1, S, and G2/M phases, respectively. The blue circle represents the control group, whereas the green triangle represents CHO cells treated with 100 mM ectoine. The black line connected to each data point indicates the average value obtained from two replicates of each sample.

potentially extending the culture duration.

To gain deeper insights into key functions and pathways affected by ectoine supplementation, gene ontology (GO) functional enrichment analysis of the top 30 most prominent GO categories comprising biological process, cellular component, and molecular function, are performed. The results of GO enrichment analysis are displayed as a bar plot in Fig. 6. The most enriched GO term was the one referring to the extracellular region, which falls under the category of cellular

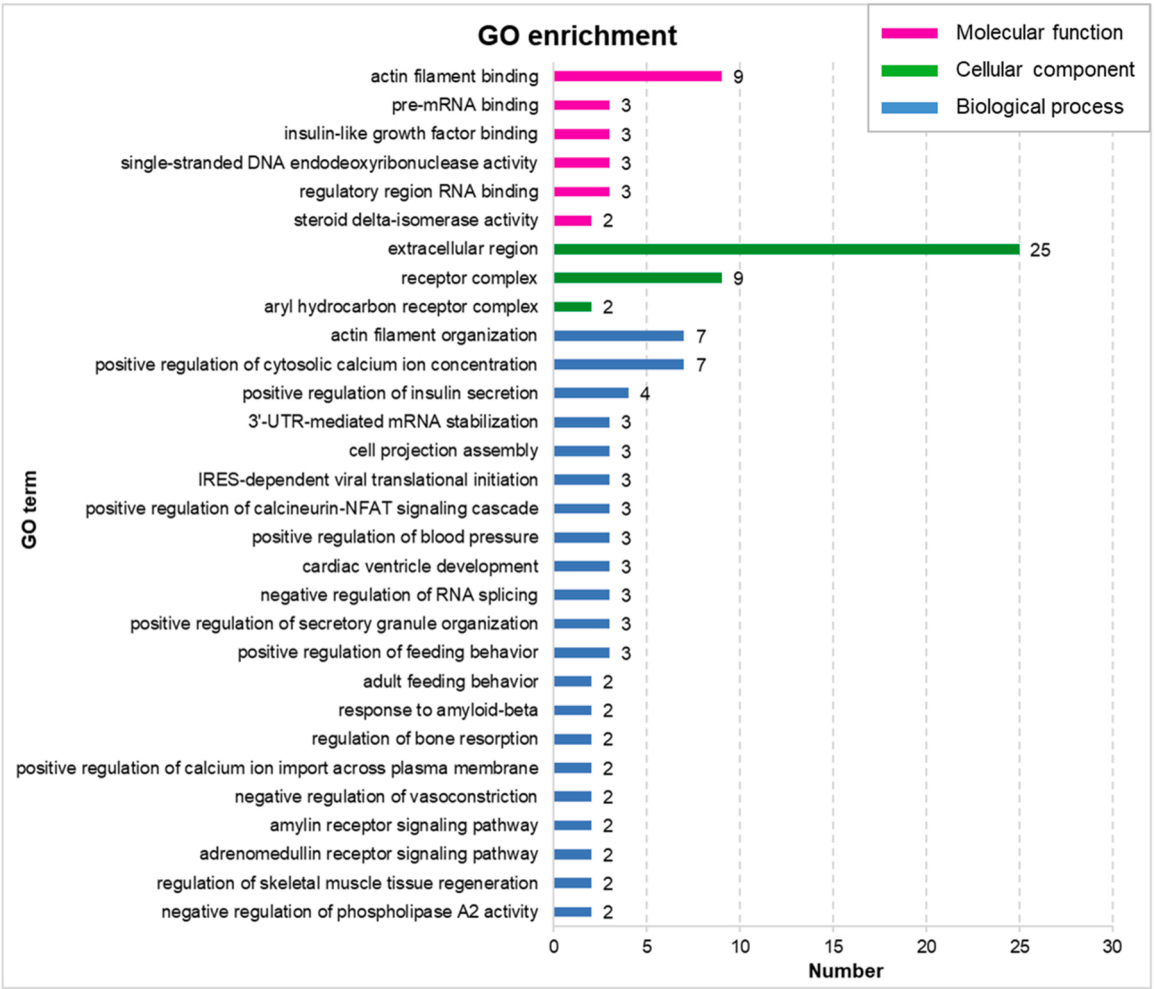

Fig. 6. A bar plot of GO functional enrichment analysis. The x-axis represents the number of differentially expressed genes in each GO term, while the y-axis represents the GO terms. The color coding distinguishes between GO categories: pink color represents molecular function, green color represents cellular component, and blue color represents biological process.

component. The extracellular region includes locations outside the plasma membrane such as the extracellular matrix (ECM). Proteins or molecules within the ECM typically participate in cellular processes, including cell communication, cell behavior, and intracellular signaling pathways. These processes include cell adhesion, cell migration, and MAPK activity, which is related to cell proliferation and survival [60]. To illustrate the significance of this enriched GO term, specific gene examples associated with the extracellular region were examined. For instance, the down-regulation of genes *Apoh* and *Qrfp*, implicated in cell proliferation and migration, suggests an impact on these processes. Conversely, the up-regulation of genes *Mmp11* and *Corin*, involved in cell differentiation, indicates a shift toward differentiation. These changes in gene expression highlight the potential effects of ectoine on cellular processes related to the regulation of cell cycle, including proliferation, differentiation, and homeostasis.

The most enriched GO terms categorized in molecular function and biological process were actin filament binding and actin filament organization, respectively. These results indicate that ectoine significantly influenced actin filaments, which relate to cell morphology, motility, and intracellular transport. Additionally, actin filament organization is associated with cytoskeletal organization, which may influence the cell cycle and proliferation of CHO cells, potentially impacting protein production [61]. Moreover, actin filament involvement extends to signal transduction in the AMP biosynthesis process and metabolic processes for aspartate and purine nucleotide, as evidenced by the significant down-regulation of *Adss1*. Furthermore, a specific example of a gene associated with the enriched GO term of actin filament organization was the down-regulated gene *LOC100767225*, involved with cell proliferation and Rac protein signal transduction. Rac protein is involved in various cellular functions such as cell movement, and cell cycle progression [62].

Moreover, among the top 30 enriched GO terms, two terms under the biological process category were involved in the positive regulation of cytosolic calcium ion and the positive regulation of calcium ion import across plasma membrane. These findings suggest that ectoine might influence calcium ion levels, impacting several cellular functions such as proliferation, and homeostasis, including programmed cell death [63]. Therefore, the results of the GO term enrichment analysis indicate that ectoine affects cell proliferation, cell cycle distribution, and cellular homeostasis.

Cellular metabolism analysis

In certain halophilic bacteria, ectoine is hydrolyzed to Nα-acetyl-L-2,4-diaminobutyric acid. This compound subsequently undergoes several reactions and is ultimately metabolized to aspartate [64,65]. Conversely, *Escherichia coli*, which is unable to synthesize ectoine, accumulates ectoine in its cytoplasm through osmoregulated transporters, where it remains unmetabolized [66]. Similarly, due to their inability to naturally synthesize ectoine and potentially lacking specific metabolic pathways presenting in halophilic bacteria, CHO cells may also accumulate unmetabolized ectoine. Further research, such as metabolic flux analysis, could provide deeper insights into ectoine's metabolic pathways related to cell growth and cell viability for enhancing mAb production in CHO cells. The biosynthesis and the degradation pathways of ectoine in halophilic bacteria suggest potential alterations in glycolysis, the TCA cycle, and amino acid metabolism pathways [65,67]. According to results obtained from RNA sequencing, ectoine-treated CHO cells showed significant changes in genes involved in various metabolic processes. For example, genes associated with the amino acid metabolic process, such as *Spaar* and *Ggt1*, were up-regulated. Conversely, the down-regulated gene *Slc7a10*, links to the transport of D-serine, which plays a role in succinate secretion and connected to the TCA cycle was found [68]. In the context of carbohydrate and glucose metabolism, two genes (*Sybu* and *LOC113838748*) were up-regulated, while ten genes (*Aldoc*, *Pgam2*, *Slc37a1*, *LOC100770118*, *LOC113838085*, *Igfal*, *LOC100756544*, *Ghrl*, *LOC113838083*, and *Ccn2*) were down-regulated. The changes influence glucose uptake, affecting the glycolysis pathway, which forms pyruvate. Pyruvate can be converted to lactate, potentially acidifying culture medium and inhibiting growth [69]. In the case of lipid metabolism, gene *LOC100762115* was up-regulated, while genes *Apoh* and *Plcb4* were down-regulated. These changes in lipid metabolism influence energy sources through the TCA pathway, thereby affecting cell growth [70]. Overall, ectoine may impact metabolic fluxes related to both the TCA and glycolysis pathways.Moreover, the analysis of ectoine consumption will be useful to evaluate whether all administered ectoine is utilized and to determine the duration required for complete consumption. These data are crucial for designing strategies to use ectoine as a supplement to enhance antibody production. This study can be conducted by profiling the concentration of ectoine in both cellular extracts and supernatants, especially after ectoine supplementation on day 3 of cultivation. The concentration of ectoine in cellular extracts indicates the amount consumed and the time required for complete consumption by CHO cells. Meanwhile, the concentration of ectoine in supernatants reveals the remaining ectoine that CHO cells have not consumed, potentially, allowing for the study of ectoine degradation in the supernatant. The concentration of ectoine can be measured using chromatographic methods, such as high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS).

Based on the current findings, further exploration of the effects of ectoine on different types of CHO cells, such as those employing different selection strategies, e.g., glutamine synthetase system, as well as on other cell lines for mAb production, would be of great interest. Investigating the impact of ectoine supplementation in these alternative cell lines may offer valuable insights into the broader applicability of ectoine as a supplementation strategy for enhancing therapeutic protein production from animal cell lines. Moreover, examining ectoine's benefit in larger-scale fed-batch cultures should also be conducted to validate its application in mAb manufacturing.

In terms of the potential industrial applicability of ectoine, factors such as cost, practical concentration, and effectiveness in enhancing antibody production are significant considerations. Currently, the cost and the optimal concentration of ectoine appear to be higher than those of other reported additives, such as adenosine, catechin, resveratrol, and valproic acid, potentially limiting its utility on an industrial scale. Therefore, further studies are required to maximize its efficacy while minimizing costs. For instance, the utilization of response surface methodology to explore the potential interaction between ectoine and addition timing could assist in designing or optimizing ectoine supplementation strategies for greater cost-effectiveness. Moreover, ectoine has been more extensively studied, particularly in the aspect of use in pharmaceutical products such as medicines, mouthwashes, and cosmetics [71]. Eventually, this broader aspect may encourage increased efforts towards developing scalable production methods for ectoine to reduce its overall cost and facilitate widespread applications.

Conclusions

This study represents the first report on the positive impact of ectoine on mAb production in CHO cells. The optimal concentration of ectoine was determined to be 100 mM, added on the 3rd day of cultivation. RNA sequencing analysis revealed that the top 10 up- and down-regulated genes are involved in various pathways associated with the regulations of the cell cycle, cell proliferation, and cellular homeostasis. Flow cytometry analysis confirmed an increase in the number of cells in the G0/G1 phase, indicating that ectoine can induce cell cycle arrest, which is a known mechanism for enhancing mAb production. In conclusion, this study provides novel insights into the positive impact of ectoine on mAb production in CHO cells and highlights its potential as a supplementation strategy for improving biopharmaceutical manufacturing processes.

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CRediT authorship contribution statement

Montarop Yamabhai: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Salinthip Jarusintanakorn:** Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Enrico Mastrobattista:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2024.06.006.

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