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RESEARCH PAPER

Enhanced production of recombinant proteins by a small molecule protein synthesis enhancer in combination with an antioxidant in recombinant Chinese hamster ovary cells

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Abstract The improvement in the production of recombinant proteins has been linked in a number of small molecules such as carboxylic acids to the inhibition of histone deacetylase, leading to increased transcription of genes. However, carboxylic acids such as pentanoic acid and butanoic acid have been shown to promote an apoptotic response in Chinese hamster ovary (CHO) cell culture. Supplementation of cultures with antioxidants has shown the ability to reduce the apoptotic response of carboxylic acid supplementation, leading to increased therapeutic protein production. In this study, we showed that pentanoic acid reduced the number of cells entering early apoptosis relative to butanoic acid by 15.4%. Additionally, supplementation of butanoic acid- and pentanoic acid-treated cultures with *N*-acetyl cysteine (NAC) reduced the population of cells entering early apoptosis by 5.3 and 10.0%, respectively, while increasing productivity by 19.5% in the presence of pentanoic acid and NAC. Conversely, a decrease of 5.7% in production was observed in response to combined butanoic acid and *N*-acetyl cysteine treatment. The results presented herein provide evidence that a culture supplementation method is critical for optimization of biopharmaceutical manufacturing processes.

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Introduction

The high cost of monoclonal antibody (MAb) treatment can be attributed to its low potency and short half-life, which necessitate large and frequent dosages. As a result, large amounts of MAb are needed to treat patient populations. It is estimated that approximately 90% of the worldwide production capacity using mammalian cell culture is utilized for MAb production [1], much of which requires the use of large volume bioreactors at the 10,000 L scale. One of the primary objectives of process development and scale-up is to optimize the production process to minimize manufacturing costs. Accordingly, increasing the volumetric titer of recombinant therapeutic proteins is a key objective in bioprocess manufacturing development.

Carboxylic acids have demonstrated the ability to improve synthesis of recombinant proteins in industrially important cell lines such as Chinese hamster ovary (CHO) cells. One of the most studied molecules involved in stimulation of protein production is butanoic acid, which is a short-chain fatty acid (SCFA). SCFA have been shown to regulate gene transcription [2] and inhibit histone deacetylase [3]. A number of SCFA are important small molecule enhancers in protein production from cells, including acetate (C2), propionate (C3), butyrate (C4), pentanoate (C5), and caproate (C6). The most widely studied of these is C4, which has been found to have a wide range of effects on protein production from cells. Additionally, butyrate has been implicated in cell proliferation, gene transcription regulation, differentiation, and apoptosis

[3–5]. Butyrate has been shown to stimulate the gene coding of metallothioneins, which are small metal-binding proteins that are cysteine rich. Metallothioneins play an important role in maintenance of zinc and copper homeostasis in cells and can detoxify harmful metals such as cadmium [6].

Histone deacetylase inhibition is one of the most widely studied effects of butanoic acid treatment. Inhibition of histone deacetylase leads to hyper-acetylation of histone molecules, a class of DNA packaging molecules. This opens gene regions, making them readily available for transcription and leading to increased mRNA levels, which increases translation and productivity. However, butanoic acid is known to kill the cells treated with it. Butanoic acid has been shown to block cell cycling in the G1 cell cycle phase, which may also be a source of increased productivity in some cell lines. A limitation to the use of carboxylic acids is increased levels of apoptosis in treated cultures. Use of alternative carboxylic acids, particularly pentanoic acid, has shown reduced levels of apoptosis with improved productivity. Additionally, the use of short-chain carboxylic acids such as pentanoic acid [7] has been shown to enhance the volumetric productivity of recombinant proteins produced in bioprocesses. Moreover, the addition of antioxidants to cultures treated with butanoic acid has resulted in reduced levels of apoptosis [8]. Here, we report the combined effects of pentanoic acid and the antioxidant, *N*-acetyl cysteine (NAC), on recombinant protein yield improvements. Pentanoic acid has also been shown to be a histone deacetylase inhibitor [2]. Therefore, in this study, we investigated the effects of two SCFAs, 4-carbon butanoic acid and 5-carbon pentanoic acid alone and in combination with NAC on cell growth and IgG production in recombinant CHO cells.

Materials and methods

Cell quantitation

Cells were enumerated using an automated Trypan Blue dye exclusion method on a Roche Cellavista (Penzberg, Germany) brightfield automated imaging system. Cells were injected into Trypan Blue solution diluted to 0.02% in PBS and aliquoted into microtiter plates and then imaged and counted using the Cellavista.

Product titer

Product titer was determined by biolayer interferometry using a Protein A biosensor with a Fortebio Octet QK384 (Menlo Park, CA, USA). A capture molecule layer is immobilized to a biosensor. As molecules bind to the capture molecule the thickness of the biolayer increases, resulting in a phase shift of light that can be measured. The binding between a capture molecule and an analyte in solution increases the optical thickness of the biosensor, which causes a shift of wavelength that can be correlated to the increasing thickness of the analyte layer. For this study, a reference standard was generated using human IgG (Sigma-Aldrich, St. Louis, MO, USA) in the range of 3.16–1000 μg/mL. Crude samples of 40 μL from various culture conditions were diluted in phosphate-buffered saline (PBS) in standard 96-well microtiter plates for analysis.

Cell line

The recombinant Chinese hamster ovary cell line (CHO-HYC1) producing a monoclonal antibody was used in this study. This line was adapted to suspension cell growth in a serum-free cell culture medium, HyQ CDM4CHO. Cells were adapted to suspension by direct adaptation methods and then maintained in 200-mL shake flask cultures at 37 °C under 5% CO_2. Cells were passaged every 3–4 days during culture.

Culture bioprocess

Conditions were studied in 1-mL microscale cultures in 96-deep-well microtiter plates. Cultures were maintained at 37 °C under 5% CO_2 at a relative humidity of 80%, during which time they were shaken at 1000 RPM on a platform with a 3 mm orbit. Culture sterility was maintained using a rayon fabric to seal the plates. Samples were taken for cell quantitation and product titer on specified days.

Component preparation

Pentanoic acid (Sigma-Aldrich, St. Louis, MO, USA) was prepared at a concentration of 250 mM in deionized water, while *N*-acetyl cysteine (Sigma-Aldrich, St. Louis, MO, USA) was prepared at a concentration of 500 mM and butanoic acid was prepared at a concentration of 250 mM in deionized water.

Induction experiments

Experiments were performed in Deepwell 96 microtiter plates (Nunc, Thermo Fisher Scientific, Massachusetts, USA). All experiments were randomized and conducted in triplicate. The initial cultures contained 250,000 cells/mL on day 0 and were induced with small molecules between days 3 and 5. Cultures were sampled on days 0, 3, 5, 7, and 10 for cell quantitation and product titer. Aliquots for cell enumeration were counted immediately. Samples for

product titer were plated in 96-well plates and stored at −20 °C until further analysis.

Apoptosis analysis by flow cytometry

Apoptosis was analyzed following treatment of cells with butanoic acid or pentanoic acid combined with treatment by *N*-acetyl cysteine. Early apoptosis was monitored by phosphatidylserine exposure on the cell surface using a fluorescein isothiocyanate-labeled Annexin V. To distinguish dead cells from apoptotic cells propidium iodide was used to label dead cells. Propidium iodide is a fluorescent molecule that intercalates in dead cell and binds with nucleic acids. Annexin V, propidium iodide-treated cells were analyzed using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA) with a HyperCyt® Autosampler (Intellicyt Corporation, Albuquerque, NM).

Statistical analysis

The cell viability and volumetric productivity analyses were independently repeated three times, and the results are presented as a mean ± standard error of the mean. A Student's *t* test (two-tailed, equal variance) was used to determine the significance of differences between experimental groups. A $p < 0.05$ was considered statistically significant.

Results

Effects of butanoic and pentanoic acids in combination with *N*-acetyl cysteine on cell growth and volumetric productivity

The treatment of a CHO cell line producing a recombinant IgG with small molecule enhancers and the antioxidant *N*-acetyl cysteine was analyzed in a scaled-down model using deep-well microtiter plates. The cultures demonstrated expected growth responses to treatments. Loss of viable cells was observed in cultures treated with higher concentrations of small molecule enhancers. Higher peak growth was observed in cultures treated with pentanoic acid relative to cultures treated with butanoic acid (Fig. 1). Interestingly, growth of CHO cell cultures treated with pentanoic acid improved relative to the untreated control sample, but this did not occur in butanoic acid-treated samples (Fig. 1). Comparison of the cultures treated with various levels of pentanoic acid and butanoic acid with those treated with antioxidants revealed no major differences in volumetric productivity (Fig. 2), but did show variations in concentration for peak productivity. Peak productivity for butanoic acid and NAC was observed at 0.625 and 1.25 mM, respectively, while it was observed at 1.25 and 1.25 mM for pentanoic acid and NAC, respectively (Fig. 2).

Effects of butanoic and pentanoic acids in combination with *N*-acetyl cysteine on apoptosis

The CHO IgG cell line producing a recombinant IgG1 was cultured in small-volume shake flasks and treated with different levels of butanoic acid, pentanoic acid, and *N*-acetyl cysteine (NAC) on day 3. On day 7, samples were analyzed for apoptosis by flow cytometry using Annexin V and propidium iodide. Annexin V was used to identify cells that were entering early apoptosis and propidium iodide to identify cells that had died (Fig. 3). The control culture had a viability of 86.8% (Fig. 1) and showed normal growth characteristics (data not shown).

The culture treated with *N*-acetyl cysteine showed only minimal improvements, with a 1.4% increase in viability and a 1.3% decrease in early apoptosis (Table 1). Treatment of cultures with butanoic acid and NAC showed minimal improvement in viability, but a decrease in the number of cells entering early apoptosis of 5.3% (Table 1). Pentanoic acid- and NAC-treated culture showed a 6.4% improvement in viability and 10.0% improvement in prevention of cells from entering early apoptosis (Table 1). All treatment conditions induced improved productivity relative to the untreated control (Table 2; Fig. 4c). In this study, butanoic acid induced a 5.7% loss in productivity when treated with NAC; however, pentanoic acid treatment with NAC demonstrated an improvement in productivity of 19.5% (Fig. 4c).

Comparison of viability, early apoptosis and productivity improvement

Treatment of pentanoic acid-containing culture with *N*-acetyl cysteine demonstrated a 19.5% increase in productivity and a 7.3% increase in viability (Fig. 4a, c). A reduction of 5.72% in IgG production was observed in butanoic acid cultures treated with the antioxidant *N*-acetyl cysteine. Reduced levels of apoptosis were observed in cultures of both pentanoic acid and butanoic acid (Fig. 4b).

Discussion

Researchers have employed multiple strategies to achieve higher product titers in an attempt to drive down costs. A key target is arresting the cells in G1 cell cycle phase. The methods most often used to arrest growth are temperature shift, cell engineering, and bioactive chemicals. Temperature shifting resulting in cell cycle arrest can improve

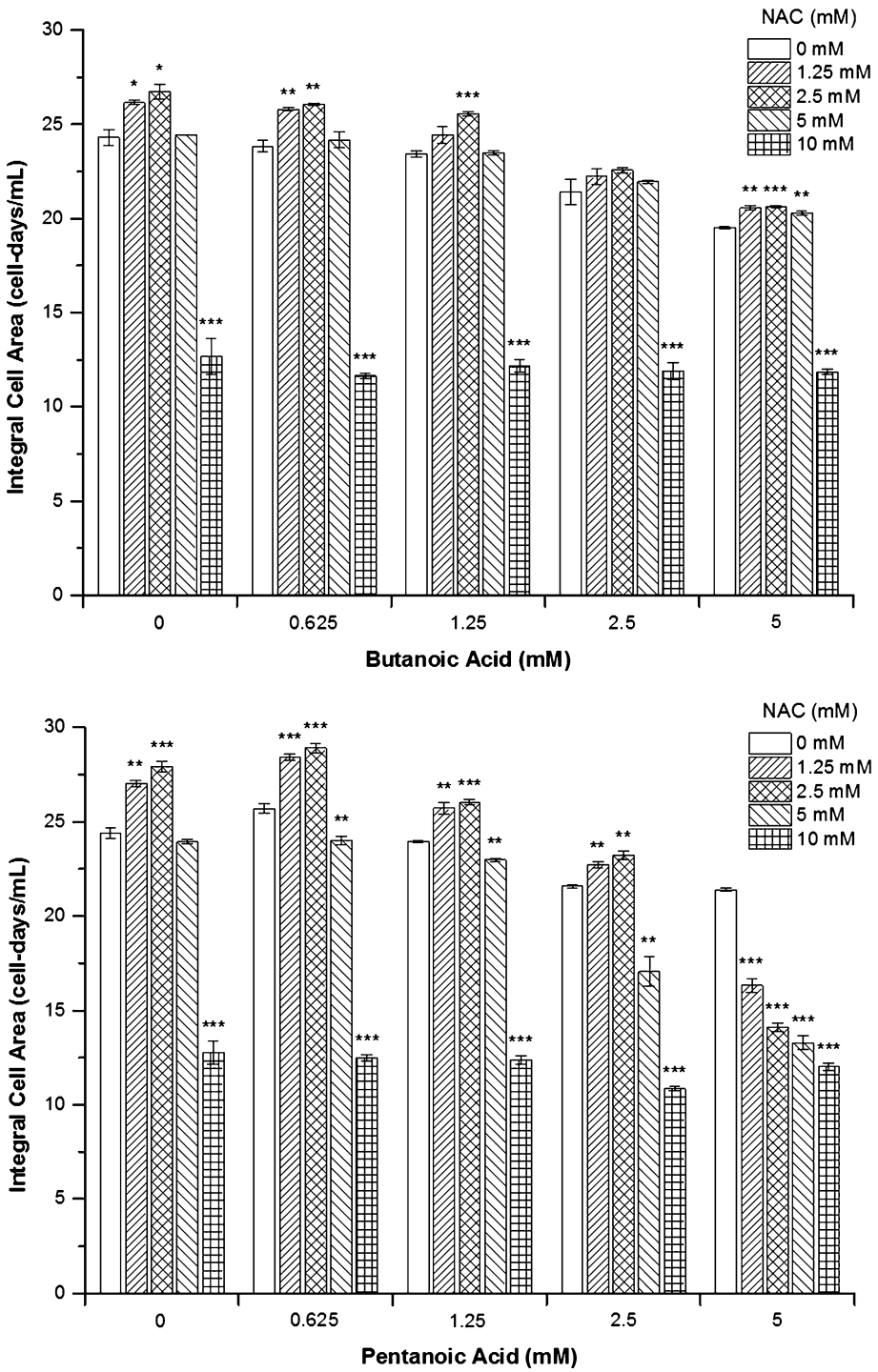

Fig. 1 Integral cell area of time courses of small molecule treatment of Chinese hamster ovary cells, **a** butanoic acid and **b** pentanoic acid ($n = 3$, mean ± SEM. *, **, *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively)

transcription, translation, and cytoskeletal rearrangement, with reduced metabolic activity. The reduced metabolism in the cells is reflected in reduced glucose metabolism and oxygen consumption [9, 10]. Cell engineering approaches can target the integration of genes for overexpression such as Bcl-2, a regulatory protein involved in apoptosis-mediated cell death [11]. Bioactive chemicals can target many cellular mechanisms, with inducing upregulation of protein production. A wide variety of molecules have been used to accomplish this, including quinoline thioethers [12], dimethyl sulfoxide [3, 7, 13–15], and aurintricarboxylic acid [7]. Additionally, broad classes of small molecules including aromatic carboxylic acids, hydroxamic acids, and acetamides have been studied [16].

Butanoic acid is a well-known small molecule enhancer of protein production that induces apoptosis in many

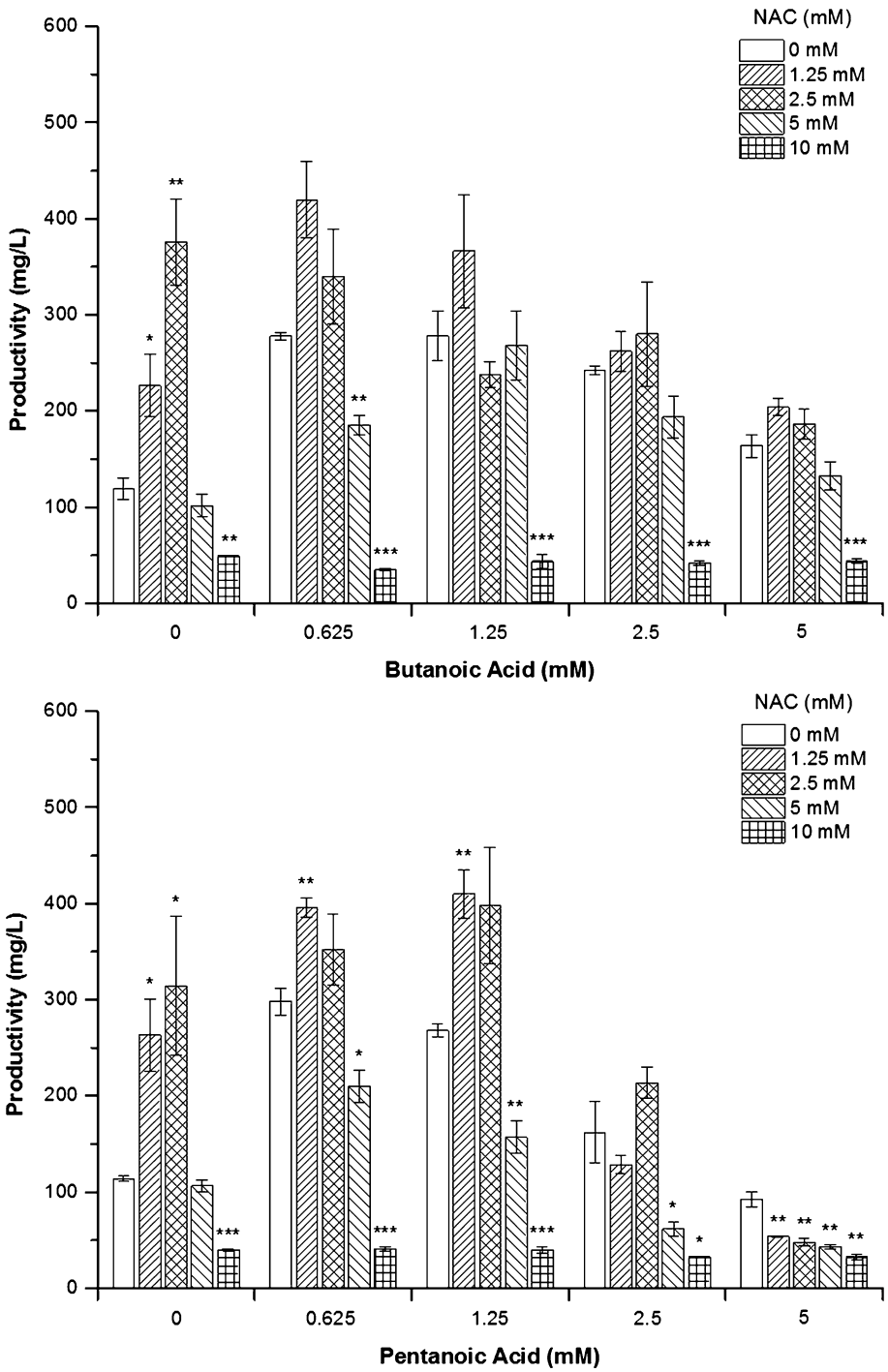

Fig. 2 Volumetric productivity of cultures treated with small molecule enhancers, **a** butanoic acid and **b** pentanoic acid ($n = 3$, mean ± SEM. *, **, *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively)

different cell types. *N*-acetyl cysteine is a thiol-containing antioxidant that has been shown to reduce the induction of apoptosis by butanoic acid. Pentanoic acid has been shown to have similar properties for inducing increased production of proteins in this study. We demonstrated that pentanoic acid can improve protein production while inducing increased apoptosis, which can be delayed by treatment with *N*-acetyl cysteine. In this study, pentanoic acid was shown to improve the volumetric production of IgG in CHO cells treated during exponential phase of growth. Although this stimulation was positive, it did not achieve the productivity levels obtained in response to butanoic acid supplementation. Pentanoic acid experiments showed similar improvements in productivity to those observed by Liu et al. [7].

We identified apoptosis indication following treatment with pentanoic acid and butanoic acid (Table 1; Fig. 3). Although supplementation with *N*-acetyl cysteine resulted

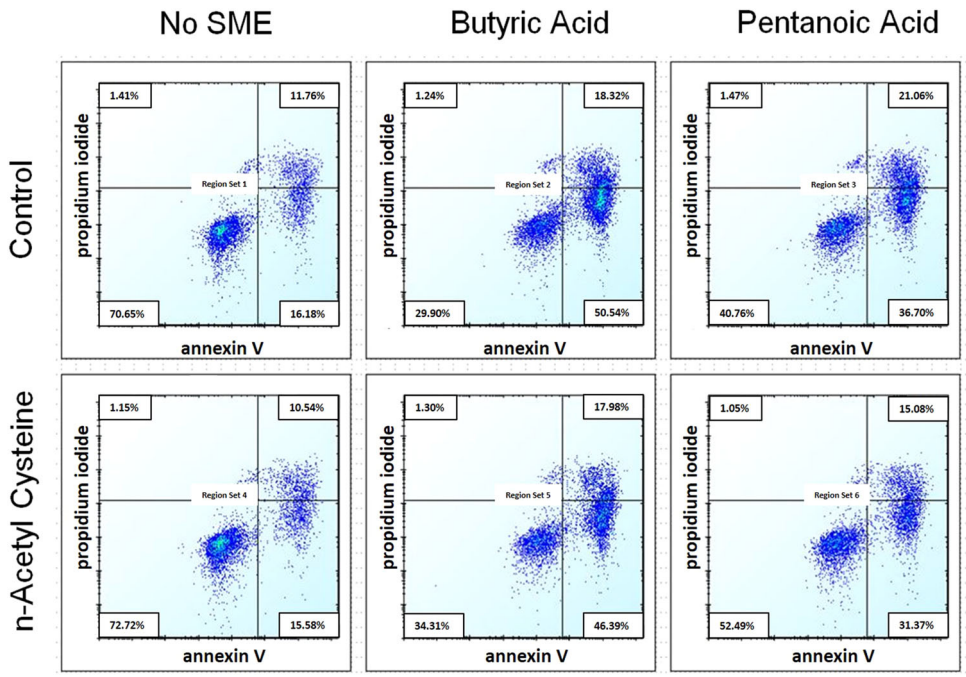

Fig. 3 Flow cytometric analysis of apoptosis of small molecule enhancers with and without co-supplementation with the antioxidant *N*-acetyl cysteine

Table 1 Summary of results of flow cytometric analysis of viability and percentage of cells entering early apoptosis

Viable % (early apoptosis %)	No SME	Butanoic acid 0.625 mM	Pentanoic acid 0.625 mM
0 mM NAC	86.9 (19.0)	80.4 (62.8)	77.5 (47.4)
1.25 mM NAC	88.3 (17.7)	80.7 (57.5)	83.9 (37.4)

Table 2 Summary of productivity, normalized to untreated negative controls

Normalized productivity	No SME	Butanoic acid 0.625 mM	Pentanoic acid 0.625 mM
0 mM NAC	1.0	2.56	1.44
1.25 mM NAC	1.12	2.42	1.71

in improved cell viability in both treatments, only pentanoic acid resulted in improved productivity. We also observed further performance increases relative to previous studies testing the addition of antioxidants [8]. However, the overall volumetric productivity was 1.7-fold higher in the butanoic acid-treated cultures. Although pentanoic acid led to decreased growth under most conditions, a significant improvement in productivity was also observed. However, butanoic acid led to greater protein production than pentanoic acid, even though it induced apoptosis.

Conclusions

In this study, we showed that pentanoic acid or butanoic acid improved production, and that this improvement was further enhanced by supplementation with the antioxidant, *N*-acetyl cysteine (NAC). Specifically, pentanoic acid reduced the number of cells entering early apoptosis relative to butanoic acid by 15.4%. Additionally, supplementation of butanoic acid- and pentanoic acid-treated cultures with NAC reduced the

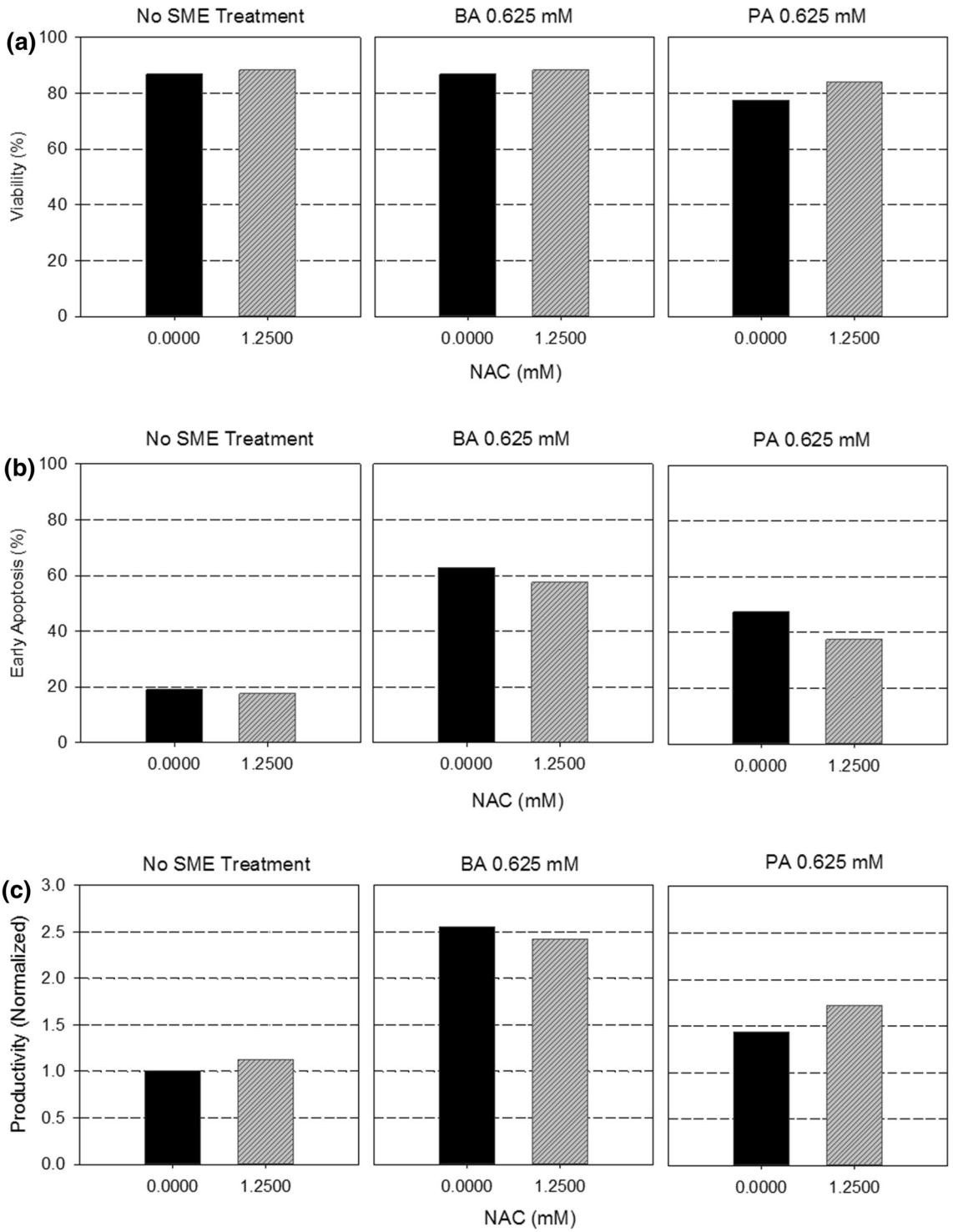

Fig. 4 Comparison of viability, cells in early apoptosis and improvement in productivity ($n = 3$)

population of cells entering early apoptosis by 5.3 and 10.0%, respectively, while increasing productivity by 19.5% in the presence of pentanoic acid and NAC. Conversely, a decrease of 5.7% in production was observed in response to combined butanoic acid and *N*-acetyl cysteine treatment. The results presented herein provide evidence that a culture supplementation method is critical for optimization of biopharmaceutical manufacturing processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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