Conversion of spent CHO cell culture media waste to new fermentation feed efficiently supports production of recombinant protein by *Escherichia coli*

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Abbreviations: CHO, Chinese hamster ovary; LB, lysogeny broth; CDSM, chemically defined spent media;

Summary

Deriving new value from waste streams is a central aim of the circular bioeconomy. In this study we investigate whether chemically defined spent media (CDSM) waste from cell culture bioprocess can be effectively recycled and used as a feed in microbial fermentation to produce new recombinant protein products. Our results show that 1) CDSM supplemented with 2% glycerol supported a specific growth rate of *E. coli* cultures equivalent to that achieved using a nutritionally rich media (LB) used as a baseline reference. 2) The amount of recombinant protein produced following induction in an expression screen was approximately two-fold higher in the CDSM fed cultures than that of baseline. 3) Mass spectrometry analysis of the proteome of *E. coli* cultures fed in CDSM revealed a greater or lesser differential protein expression pattern depending on supplementation conditions. Further, in a 16 hr fermentation the optimised CDSM-fed culture delivered a protein yield of more than double that achieved by the baseline media. We conclude that spent cell culture media, which represents millions of litres of waste generated by the bioprocessing industry annually, has the potential to be a valuable feed resource for the production of recombinant proteins in secondary microbial fermentation.

1 Introduction

Diversion of waste streams from bio-industries to new processes to generate valuable products through microbial and chemical engineering has become a central pillar of the circular bioeconomy (Stegmann et al., 2020). One example of a waste stream from bioindustry that has yet to be diverted to the creation of new valuable products in this way is the cell culture media used in the bioprocessing of high value recombinant proteins from Chinese hamster ovary (CHO) cell culture. This media is itself a carefully developed chemical formulation that represents a significant waste product in downstream processing. For example, three countries including Ireland, India and the US, each contribute greater than 1 million litres of bioreactor capacity per annum to the global bioprocessing industry (Rader et al. 2018). Despite these very high volumes, systematic approaches to valorize this waste as part of a commitment to a circular bioeconomy have not been investigated.

Previous studies conducted into the potential use of spent media focused mostly on reuse using the same cell lines (Riese et al. 1994, Wu et al. 1998, Ogawa et al. 2009, Beas-Catena et al. 2013). Studies into the recycling of yeast, microalgae and insect cell culture media have yielded improvements in protein expression and growth rates in those culture systems (Yano et al. 1980, Wu et al. 1998, Beas-Catena et al. 2013). A smaller number of studies have been conducted into recycling spent mammalian cell culture media (Riese et al. 1994, Ogawa et al. 2009). These studies showed that reusing media as a supplement in cell culture led to an increase in protein production by those cells. For example, IgG production was increased by as much as 50% by mouse hybridoma cells that were fed with media supplemented with spent media that they had already grown in (Ogawa et al. 2009). While this increase was achieved through nutrient supplementation and dilution with fresh media, feeding the cells with 100% spent media led to a significant decrease in growth and protein production by the mammalian cells. This decrease was attributed to the build-up of auto-inhibitory metabolites and lack of nutrients.

Microbial fermentation has the potential not only to overcome the inhibition from autoinhibitory metabolites but to use them as a metabolic fuel. Genetic engineering of a wide array of bacteria has been a central pillar of the development of the circular bioeconomy, enabling new product development from diverse organic waste streams including food oils, agrochemical waste and used bioplastics (Guzik et al. 2014, Cerrone et al. 2015, Narancic et al. 2018, Gervasi et al. 2018, Ruiz et al. 2019). *Escherichia coli* is a classic example of a robust and versatile bacteriological expression system that has been employed commercially to produce recombinant proteins of value in fermentation systems at large scale for global markets, with the production of recombinant human insulin in *E. coli* a major milestone in human drug production (Kroeff EP et al. 1989, Storrs et al. 1991). *E. coli* can utilize breakdown metabolites such as lactate present in spent culture medias, indicating that cell culture waste is a potentially useful food for fermentation (Caglar et al. 2017). It has been shown previously *E. coli* can grow successfully in spent media from the fermentation of other cell types (Yano et al. 1980). In addition to this, supplementation with waste media has the potential to yield higher amounts of protein in fermentation systems (Yano et al. 1980, Rechlin et al. 2014). We hypothesise that spent media from CHO cell culture has the potential to support *E.*

coli fermentation with increased recombinant protein yields when recycled from bioprocessing systems.

To test the growth rates and the protein yield from *E. coli* expressing a recombinant fusion protein, expression cultures were first grown in a nutrient rich media (lysogeny broth, LB) and in a nutrient limiting minimal media (M9) to establish baseline growth rates. Titration of the carbon sources glucose and glycerol as supplements to minimal media were then studied to measure the potential benefits of supplementation prior to the analysis of chemically defined spent media (CDSM) from CHO cell culture. Our growth rate and protein yield analysis revealed CDSM as a viable food source for bacterial fermentation with or without supplementation with glycerol or glucose, and was superior in recombinant protein production with double the protein yield obtained over baseline rich media culture conditions.

2 Materials and methods

2.1 Mammalian Cell Culture

Chinese Hamster Ovary (CHO) cells were incubated in 20 ml of serum-free CHOgro® Expression media supplemented with 4 mM L-Glutamine in T75 adherent cell line flasks, at 37°C with 5% CO₂ and 95% O₂. Cells were split at 70-80% confluency every 3-4 days. Spent media was harvested at each splitting, and was clarified of cells and cellular debris at 300 x g for 4 minutes prior to storing at 4°C for up to 14 days.

2.2 Growth and Expression Cultures of *E. coli*

E. coli BL21 Gold was transformed with a 6His-mCherry-EF2 fusion protein expression construct. A 10 ml starter culture of BL21 transformed *E. coli* was grown by incubating a single, isolated colony in Lysogeny Broth (LB) media, 2% glucose, and 50 µg/ml ampicillin. The culture was grown in a shaking incubator at 250 rpm, 37°C for 16 hours. Expression cultures were grown in 50 ml cultures in triplicate in 250ml flasks. The flasks were grown in a shaking incubator at 250 rpm, 37°C until the OD₆₀₀ reached 0.6. OD₆₀₀ was measured every half an hour for growth curve plotting. Expression of the fusion protein was induced by addition of 1 mM IPTG. Cultures were then incubated on a shaking incubator at 250 rpm, 30°C for 4 hours. Cultures were then spun at 4°C, 1700 x g for 20 minutes to harvest the pellets which were weighed prior to protein purification. To perform a 16 hour expression analysis with a strong T7 promoter, we expressed a second mCherry-EF2 fusion protein construct in LB, glycerol supplemented CHOgro®, and glycerol supplemented Expi-CHO® CDSM. IPTG induction was performed at OD₆₀₀ of 0.6. Expression cultures were then incubated at 30°C for 16 hours, at 250 rpm. Cultures were collected as above, and weighed prior to protein purification.

2.3 Calculations of Specific Growth Rate

Specific growth rate (k) was calculated by the equation: $(\log_{10} [X_t] - \log_{10} [X_0]) / 0.301 \times t = k$, where k is generations per hour and t is time in hours. Plots of growth curves of OD₆₀₀ (nm) versus time (hrs) were generated to gather this data in Excel. X_t is one OD₆₀₀ value at a later position, and X₀ is another OD₆₀₀ value taken from an earlier position.

2.4 Recombinant Protein Purification

Cell pellets from the 4 hour screen were re-suspended using a lysis buffer containing 10 mM imidazole and lysed by sonication. The 6 histidine tagged fusion protein was captured using Immobilised Metal-Affinity Chromatography (IMAC). 2 ml of 50% Ni-NTA (nickel-nitrilotriacetic acid) was used to isolate the protein from 150 ml of culture. 4 ml of eluate in 250 mM imidazole were collected for further purification by Size Exclusion Chromatography (SEC), using Hepes running buffer containing 10 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH 7.4. Protein concentration was measured with a DeNovix DS-11 Spectrophotometer. The purification protocol for the 16 hour expression was changed to replicate larger scale preparation without affinity chromatography. Briefly, culture pellets were resuspended in 10 mM Tris buffer + 2 mM CaCl₂ at pH 8, sonicated, and then boiled at 95°C to precipitate *E. coli* proteins leaving the calcium-binding fusion protein in the supernatant. This phase was then further purified by running through the SEC as above using PBS as a running buffer, and then measured on the DeNovix DS-11 Spectrophotometer as before. Visual comparison was also performed with polyacrylamide gel electrophoresis and Coomassie staining.

2.5 Proteomic Analysis

4 mls of *E. coli* culture were harvested after reaching OD₆₀₀ of 0.6, but before IPTG induction. These cultures were re-suspended in 8M urea. 5mM DTT was then added, and samples were incubated at room temperature for 10 minutes. After incubation, 10mM iodoacetamide was added, and samples were incubated in the dark for 10 minutes at room temperature. Samples were brought to neutral pH by addition of 150 µl of 200 mM NH₄HCO₃. Samples were then digested with trypsin at 37°C and 300 rpm on a thermomixer overnight. Samples were spun at 4000 x g for 2 mins to prevent ZipTip® from getting blocked in the next steps. Digested peptides were filtered through ZipTip® columns and eluted using 70% acetonitrile in acidic water (0.1% formic acid). The elution was dried off by speed-vac at 45 °C. The dried peptides were then re-suspended in 30 µl Buffer A (5% acetonitrile and 0.1% TFA). Analysis of proteomic data files was carried out using MaxQuant (Cox et al. 2008) to assemble peptides using the E.coli BL21 proteome from Uniprot (UP000002032). The software Perseus (Tyanova et al. 2016) was used to analyse the resulting dataset for student's t-tests and heatmap generation. A Python programme was generated to give the remaining graphs, using the t-test results generated from Perseus and annotation information from Uniprot for *E. coli* strain BL21 (available on GitHub at https://github.com/Ciara-Lynch/Mass_Spec_Analysis.git).

3 Results

3.1 Measuring the growth rates of *E. coli* BL21 mCherry/EF2 in minimal media, baseline rich media and chemically defined spent media.

Gradients of glucose additions were tested to find the optimal concentration for maximum growth rate. *E. coli* BL21 Gold was grown in 50 ml cultures in triplicate, using M9 minimal media with glucose supplementations of 1%, 2%, 3% and 4%. Cultures grown in 3% glucose had the fastest specific growth rate of 0.649 generations per hour compared to 1.07 generations per hour for baseline rich media (lysogeny broth), and 0.433 generations per hour for M9 minimal media controls. The growth rate fell sharply at any concentrations higher than 3%, with the specific growth rate of 4% at 0.305 (Figure 1 A). Concentrations of 6% and 9% glucose appeared to inhibit culture growth with lower specific growth rates than the minimal media (data not shown).

Gradients of glycerol additions were tested at concentrations of 1%, 2%, 3% and 4%. In these experiments, 2% glycerol supplementation was found to perform optimally, with a specific growth rate of 0.673 generations per hour, as compared to the growth rates of 1%, 3% and 4% at 0.578, 0.647, and 0.415 respectively. Once again, volumes over 3% appeared to significantly slow down the growth rate (Figure 1B). 2% glycerol addition to the reference LB media did not affect the growth rate seen in LB alone.

Fig 1. Growth curves from *E. coli* in nutrient rich, minimal and CDSM growth conditions. (A) Growth curves during titration of glucose concentration in M9 minimal media; ■ = baseline media, ● = minimal media, ◆ = 1% glucose, □ = 2% glucose, △ = 3% glucose, ○ = 4% glucose. (B) Growth curves using a titration of glycerol concentration in M9 minimal media; ■ = baseline media, ● = minimal media, ◆ = 1% glycerol, □ = 2% glycerol, △ = 3% glycerol, ○ = 4% glycerol.

The chemically defined spent medium used was CHOgro® spent media harvested from CHO (Chinese Hamster Ovary) cell culture. The specific growth rate of the *E. coli* in the CDSM was 0.704 gen/hour, approximately 70% of the rate achieved in the baseline LB media. Supplementing with either 3% glucose or 2% glycerol identified as optimum supplementations in the minimal media screen, led to a further increase in growth rates with 3% glucose supplementation equivalent to ~86% compared to the baseline, while supplementation with 2% glycerol led to an increase to ~94% compared to the baseline (Fig 2).

Figure 2. Growth curves for unsupplemented and supplemented spent media. ■ = baseline media, ● = M9 media, ◇ = chemically defined spent media (CDSM) unsupplemented, △ = CDSM with 3% glucose, ○ = CDSM with 2% glycerol. All time points were completed in triplicate with standard deviation as error bars.

3.2 Protein expression screen in supplemented and un-supplemented media.

The recombinant protein expression was tested in expression cultures grown in CDSM and CDSM supplemented with 3% glucose or 2% glycerol. Without supplementation a protein production level of 49.94 mg/L was reached compared to 28.35 mg/L in LB media. Supplementation with 3% glucose achieved a protein yield of ~49 mg/L and with 2% glycerol a yield of ~60 mg/L of recombinant protein was measured. That represents a 2-fold increase over that achieved with LB (Table 1).

Measurement	LB Media	M9 Media	CDSM	CDSM with 3% Glucose	CDSM with 2% Glycerol
Specific growth rate (k)	1.070	0.433	0.704	0.860	0.944
R-squared coefficient	0.972	0.935	0.906	0.934	0.955
Doubling time (hrs)	0.935	2.309	1.420	1.163	1.059
Biomass (g) ($\pm$ SD)	0.243 ($\pm$ 0.083)	0.086 ($\pm$ 0.036)	0.179 ($\pm$ 0.057)	0.331 ($\pm$ 0.129)	0.385 ($\pm$ 0.034)
Recombinant protein (mg/L)	28.350	14.050	49.940	49.143	60.760

Table 1. Table of growth characteristics, biomass and recombinant protein yield. LB media; Lysogeny Broth baseline rich media. M9 media; minimal salts media. CDSM; chemically defined spent media. R^2 coefficient is the percentage of variability in the growth curve dataset that is accounted for by linear correlation between the OD600 (nm) and the time (hr). For equation for the specific growth rate, see Methods. Biomass is represented with +/- the standard deviation of the triplicates.

We next tested a 16 hour expression post-induction with IPTG using CDSM, and included a second chemically defined spent medium, Expi-CHO® (termed CDSM II), to examine the robustness of our finding with widely used commercial medias.

Both CDSM samples were supplemented with the optimum 2% glycerol addition. The CDSM I condition outperformed the LB media with a protein yield of 185.57 mg/L, with a near two-fold increase in protein yield quantified by visual analysis on an SDS-PAGE gel (figure 3). The CDSM II condition delivered a yield of 68.37 mg/L, lower than that obtained by the LB baseline media, at 79.38 mg/L or 86% of the yield achieved at baseline. These yields were determined by SDS-PAGE analysis using a known protein standard.

Figure 3. Protein quantification from size exclusion chromatography. (A) Overlaid chromatograms from the SEC. Green = CHOgro® spent media (CDSM I), red = Expi-CHO® spent media (CDSM II), blue = baseline (LB media). (B) SDS-PAGE gel of samples harvested between the two dashed lines on (A). (1). Protein marker (2). mCherry-EF2 peak CDSM I (3.) mCherry-EF2 peak CDSM II (4.) mCherry-EF2 peak LB media.

3.3 Analysis of *E. coli* protein expression at the whole proteome level

Proteomic analysis using mass spectrometry was carried out on *E. coli* collected from five growth conditions including CDSM, CDSM + 3% glucose, CDSM + 2% glycerol, LB and M9 media. A tryptic digest of cellular proteins was carried out for each condition in triplicate (biological replicates). Analysis of LFQ (label-free quantitation) intensity values for peptides identified significant differences in the number of upregulated and down regulated proteins depending on the media used for culture. Using the expression pattern for LB media as a baseline reference, a student's t-test with a false discovery rate of <0.05 identified 655 differentially expressed proteins in unsupplemented CDSM. In the 2% glycerol and 3% glucose CDSM conditions there were 167 and 168 proteins differentially expressed by comparison (Figure 4).

Figure 4. Number of statistically significant differentially expressed proteins compared to the baseline condition in LB media. Orange bars represent the number of downregulated proteins while blue represents all upregulated proteins compared to baseline media after a student's t-test with an FDR of < 0.05.

Pathway protein analysis of each sample was carried out using a de novo script written in Python code that converts tabulated MS data to graphical visualisation of significant up and down regulation compared to the baseline media conditions, which we have termed a pool table plot. In CDSM supplemented with 2% glycerol, 87 proteins were identified as upregulated and 80 proteins as downregulated and within these a cut-off significance value of >0.5 or <-0.5 was used to identify the most significantly changed expression levels (figure 5). Proteins upregulated in the CDSM + 2% glycerol condition were principally in the amino-acid and purine biosynthesis pathways, while proteins that were most significantly downregulated were in the carbohydrate metabolism pathway (figure 5). 45 proteins in the amino acid biosynthesis pathway were up-regulated in the CDSM unsupplemented condition, while most of the down-regulated pathways were involved in pathways such as lipid metabolism and pyrimidine metabolism, as well as tRNA modification.

Figure 5. Pool-table plot of the proteomic analysis of the significantly dysregulated proteins from the sample condition of CHOgro® with 2% glycerol supplementation. This graph was constructed using the Matplotlib library in Python. Significant upregulation of expression is shown to the right of the central threshold divider (-0.5 to 0.5), with significant downregulation to the left. Significance in this case was defined as having a mean difference in intensity compared to the baseline media of more than 0.5 in either direction after a student's t-test and with an FDR of <0.05. All plots for other sample conditions are contained in the supplementary materials. Top 3 most significantly upregulated and downregulated proteins are labelled with their corresponding protein ID.

Amino acid biosynthesis and purine metabolism pathways were up-regulated in all conditions compared to the baseline. Carbohydrate metabolism by contrast was mainly downregulated in both of the supplemented conditions, CDSM with glucose and CDSM with glycerol. The three most significantly upregulated proteins are the biosynthetic enzymes Phosphogluconate dehydratase (A0A140NAT6), Amidophosphoribosyltransferase (A0A140N9W7) and Aspartate-ammonia ligase (A0A140ND59) with a mean difference in LFQ intensity over 2.5. The three most significantly downregulated proteins include a TCA cycle protein homolog identified as a probable malate:quinone oxidoreductase (A0A140N9V4), Phospho-2-dehydro-3-deoxyheptonate aldolase (A0A140N8U6) and D-amino acid dehydrogenase, a D-alanine catabolic process protein (A0A140NCJ4) with a mean difference in LFQ intensity below -2.0 (Figure 5).

4 Discussion

4.1 Can spent media from CHO cell culture be reused to feed *E. coli* fermentation?

The efficient production of human therapeutic proteins from bioprocess reactors has been optimised via the intensification of upstream processes that include the engineering of the CHO cell to maximise culture density and product yield. There has been a complementary set of developments in the creation of chemically

defined culture media for increasing recombinant protein titres and protein quality (Huang et al. 2010, Xu et al., 2018). These successful developments have led to this cell culture media becoming a significant waste stream with many millions of litres sent for disposal annually. A recent report on global biopharmaceutical production states that around \$1.4 billion is spent annually on cell culture media per year (Langer et al. 2020). Harnessing waste as a source of renewable building blocks is a goal of the emerging bioeconomy that seeks to generate value more than once in the lifecycle of a product (McCormick & Kautto 2013). A scientifically interesting question we sought to address here is can we realise new value with this sophisticated culture material before it is consigned to waste.

Our experiments indicate that chemically defined spent media (CDSM) is a potentially valuable resource for producing new recombinant proteins in useful quantities when compared with nutritionally rich media prepared with casein digests and yeast extract. We studied the expression of a recombinant protein ligand (EF2) derived from the calcium binding protein Calbindin D9k, also known as S100G that has a highly specific and high affinity complementarity to another domain from this protein (EF1) (Berggård T et al, 2001; Dell'Orco et al, 2005). In previous studies we have immobilised a chemically synthesised EF2 peptide onto agarose beads in affinity purification protocols for the purification of EF1-tagged proteins from microbial and mammalian cell expression cultures (Ni Mhurchu 2018; McGauran 2020). To produce this EF2 affinity ligand at a larger scale we developed recombinant fusions to the fluorescent beta-barrel protein mCherry and here we used the expression of this protein as a reporter to measure the utility of CDSM as a food source for shake flask fermentation cultures of E.coli BL21/pETmCherry-EF2.

We first analysed the growth rate in log phase of this expression strain in baseline rich media (LB) or in minimal M9 media alone and supplemented minimal media with the carbon sources glycerol and glucose. From a titration series we identified that 2% glycerol and 3% glucose additions were optimal for increasing the culture growth rate toward that of the baseline rich media (Figure 1A and 1B). In previous studies on carbon supplementation in minimal media, glucose generally performed better than glycerol in terms of growth when used at the same concentration of 0.05% w/v (Calgar et al. 2017). Glycerol usually performed the best of any other gluconeogenic sources tested such as pyruvate, succinate or acetate in equal amounts of carbon atoms (Paily et al., 2007). Using an autoinduction model, one study found that the optimal glycerol concentration was 0.75% w/v, while glucose was 0.03% w/v for production of a nanobody (Rezaei, L. *et al.* 2020).

Interestingly we show that the CDSM without any carbon source supplementation is a useful feed supporting a specific growth rate approaching 70% of that which was achieved with baseline media conditions. Supplementation of the CDSM with either carbon source led to an increase in growth rate to between 85-95% of baseline (Figure 2). Glycerol is a common waste product from biobased industries and as such is an attractive supplement from the perspective of valorizing waste cell culture media (Tan H. W. et al., 2013). Having determined that the expression host cells can grow in CDSM alone we next employed a rapid inducible expression screen where the cultures were induced with IPTG and the recombinant protein was expressed from the lac operon for a maximum of 4 hours. Strikingly we measured a 1.75 fold higher yield of the protein in un-supplemented CDSM over

the baseline LB media, while upon supplementation with 2% glycerol there was a two fold increase in recombinant protein production over baseline (Table 1). This data confirmed that the expression strain can grow quickly and produce recombinant protein in CDSM validating the investigation of this waste as a potential food for microbial fermentation.

4.2 Analysis of the nutritional content of CDSM

We next sought to determine the nutritional value of CDSM using mass spectrometric analysis of the protein content of the spent media. Chemically defined medias are not supplemented with complex protein additives such as animal serum and in these experiments the sole addition to the media was 4 mM glutamine. We expected to see some contribution of CHO host cell protein in the spent media that was harvested from CHO cell culture. CHO cell host proteins are a prominent feature of downstream processing due to cell lysis in high density cultures and purification steps are monitored carefully for contaminating proteins during product capture and elution (Levy et al. 2016).

Upon mass spectrometry analysis of the CDSM alone, it was found that 879 CHO host cell proteins are present in the spent media after culturing (see Table S1). These proteins were identified from all cellular compartments, suggesting they are debris from CHO cell lysis in addition to any active secretion by the growing cells. These host cell proteins are a source of building blocks for the increased recombinant protein production capabilities of the *E. coli* cultures.

CHO cells generally produce approximately 1,400 host cell proteins (HCPs) which are detectable in the clarified spent culture media, with ~80% of the top 1000 of these HCPs in common across multiple cell lines (Yuk, I. H. et al., 2015, Valente, K. N. et al., 2018). This mass spectrometry based analysis has been widely employed to characterise HCP's across a number of studies (Tscheliessnig, A. L. et al., 2013; Hogwood, C. E. et al., 2014).

4.3 Analysis of proteomic adaptation by *E.coli* BL21/pETmCherry-EF2 in CDSM

To study the adaptive changes made by the expression strain in CDSM we performed mass spectrometry analysis of the *E. coli* proteome in baseline media conditions, in CDSM alone and in CDSM supplemented with either 2% glycerol or 3% glucose. Our results showed the proteomic profile of the CDSM alone has changed much more significantly from the baseline than that of the two CDSM supplementation conditions (figure 4). This suggests that the addition of carbon sources to the spent media leads to a reduction of stressful conditions for the bacterium. Despite the reduction of proteomic dysregulation, the supplemented conditions are distinct from the baseline (~150 dysregulated proteins each). Therefore the recombinant protein expression increases seen in this study are likely to be due to the repertoire of proteins that are upregulated and downregulated. The number of proteins in common between the two supplemented conditions that are statistically, significantly dysregulated compared to baseline is 103, 72 of which are also dysregulated in the CDSM alone condition. Further analysis of these proteins will help to explain the mechanisms behind the increases in production seen (see Table S2).

The most statistically significant upregulated proteins observed include Phosphogluconate dehydratase, the only upregulated protein involved in carbohydrate metabolism (figure 5). This enzyme has been shown to be sensitive to superoxide levels in media and is most likely upregulated in the supplemented CDSM with 2% glycerol as it is known to be involved in the breakdown of the glycerol (Gardner PR, 1991). Amidophosphoribosyltransferase is part of the biosynthetic pathway that catalyzes the formation of phosphoribosylamine from phosphoribosylpyrophosphate (PRPP) and glutamine. CDSM I was supplemented with 4 mM glutamine prior to the culturing of CHO cells, as per manufacturer instructions. This upregulation appears to provide the bacterium with the necessary fuel to drive the formation of IMP, inosine monophosphate, a key factor in the purine metabolism pathway (Gots JS et al., 1977; Messenger LJ, 1979). Aspartate-ammonia ligase is a class-II aminoacyl-tRNA synthetase family member catalyzing the production of L-asparagine. This protein is involved in step 1 of the subpathway that synthesizes L-asparagine from L-aspartate (ammonia route). We propose that the CHO host cell proteins that we identified in the media (Supplementary Table S1) are providing the L-aspartate substrate for the production of L-asparagine. L-asparagine biosynthesis is dependent on glutamine uptake, which is supplemented in the CDSM (Lomelino CL, 2017). Amino acid accumulation such as increased L-asparagine is a known feature of the *E. coli* stress response, in order to make available the building blocks needed for synthesis of stress response proteins (Jozefczuk S et al., 2010). Our observation that the level of recombinant protein expressed in the CDSM media, which was double that of the LB condition, appears to be related to a significant increase in overall amino acid synthesis by the bacterium that may reflect a stress response adaptation.

Significantly downregulated proteins included the probable malate:quinone oxidoreductase that is involved in step 1 of the subpathway that synthesizes oxaloacetate from (S)-malate (quinone route). This subpathway is part of the tricarboxylic acid cycle a part of general carbohydrate metabolism (Van der Rest ME, 2000). Phospho-2-dehydro-3-deoxyheptonate aldolase is a downregulated protein involved in chorismite biosynthesis, an aromatic compound (Flores N, 1996). These carbohydrate metabolism enzymes are likely downregulated where the bacteria are not utilising the glucose metabolism pathway, with no glucose added to this growth condition of CDSM + 2% glycerol. We can conclude that the available glucose levels are reduced by the prior metabolism of the CHO cells in the media before fermentation, reflecting the glycolytic respiration of these cells. Finally, D-amino acid dehydrogenase is involved in step 1 of the subpathway that synthesizes NH_3 and pyruvate from D-alanine. This subpathway is part of the amino acid degradation pathway and its downregulation likely complements the observation of the enhanced amino acid production of the cells. The higher expression of this enzyme in lysogeny broth (LB) is associated with the catabolism of D-amino acids present in LB that are not available in CDSM I (Baev MV et al., 2006; Franklin FC, 1976).

One of the main metabolic pathways that was upregulated in the CDSM media compared to LB was the amino-acid biosynthesis pathway. The baseline condition of LB contains tryptone, a source of nitrogen-containing peptides for *E. coli* growth,

whereas the spent CHOgro media must rely on the proteins produced by the CHO cell culture conditions (Sezonov et al. 2007). Furthermore, this increase in amino-acid production aligns to the significant increase in recombinant protein yield seen in his study. Purine breakdown is also upregulated in these conditions. *E. coli* does not generally metabolize purines as a nitrogen source, though it can catabolise certain purines to supply intermediates for other biological processes (Xi et al. 2000). The increase in amino acid synthesis demands more components which the cells could be supplying by breaking down purines such as adenosine. This is of interest when considering that the growth rate was decreased yet protein production highly increased in the CDSM supplementation experiments.

CHO cell growth in chemically defined media produces metabolites such as ammonium and lactate that can inhibit further growth, (Pereira et al. 2018). These factors however can be used by *E. coli* to grow, utilising ammonium as its preferred nitrogen source and can break down lactate as a possible carbon source if needed (Merrick et al. 1995). Mammalian cells such as CHO cells can secrete a metabolite known as methylglyoxal (MG) toxic to CHO cells (Chaplen et al. 1998). *E. coli* can detoxify MG by converting it to D-lactate using an enzyme known as glyoxalase III (Misra et al. 1995). We see that this protein is upregulated in the CDSM unsupplemented experiments, though not statistically significant, and is not upregulated in the supplemented experiments. Other toxic metabolites produced by CHO cells include adenine monophosphate, sorbitol, and other growth cycle intermediates which inhibit further growth in CHO culture, which may fuel faster growth and protein production by *E. coli*.

4.4 Analysis of a 16 hour fermentation model

The results of the protein expression screen prompted us to study a longer expression duration of 16 hours as a model of scaled up fermentation scale expressions. The CDSM I condition (CHOgro spent media + 2% glycerol) produced a yield of recombinant protein of 185.57 mg/L, more than twice the recombinant protein yield that was obtained using the LB media over the longer period of time, at 79.38 mg/L. This result was similar to that seen in the four-hour screen of the same condition. This indicates that any factors within the spent media that are responsible for the protein increase are not exhausted within a short time but can continue to support an increased protein production rate in overnight expressions. This is an important finding toward the wider development of valorization of cell culture waste streams by research and development laboratories to biopharmaceutical production facilities.

The alternate media type tested, Expi-CHO with 2% glycerol (CDSM II), also performed well for a waste media, achieving 86% of the protein production achieved by the LB media (figure 3). This is a very interesting result, as it shows that with the simple addition of carbon sources potentially any chemically defined culture media used to grow mammalian cells, can be valorized to support *E. coli* culture for new protein expression. The identification of waste culture media as a renewable building block in this study aligns very well with the aspirations of the emerging circular bioeconomy, generating value more than once in the lifecycle of chemically defined cell culture media.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Author Contributions

CL performed the experiments, CL and DJO'C analysed the data, CL and DJO'C wrote the paper. DJO'C designed the study.

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