Redesigning Spent Media from Cell Culture Bioprocess to Feed New Bacterial Fermentations

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Abbreviations

BE Bioeconomy
CE Circular Economy
CBE Circular Bioeconomy
MAb Monoclonal Antibody
CHO Chinese Hamster Ovary

1 Introduction

A key goal of the emerging bioeconomy is the selective separation and extraction of valuable compounds from renewable materials, converting those resources into novel bio-based products and processes, with the potential to deliver market and industry-scalable sustainable resources. When we consider what is meant by a bioeconomy (BE) we can look to the European Commission who have been championing the development of this idea for the past decade. Their specific definition of the BE is *"the production of renewable biological resources and the conversion of these resources and waste streams into value added products, such as food, feed, bio-based products and bioenergy"* [1] Concurrently there has been a development of related terminology with regard to the Circular Economy (CE) with this being defined as *"minimizing the generation of waste and maintaining the value of products, materials and resources for as long as possible."* [2]. The

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parallel descriptions of efforts to promote sustainability with industrial practice have then led to the adoption of the holistic term, the Circular Bioeconomy (CBE) [3]. The circular bioeconomy may then be thought of as a circular economy where non-renewable inputs to industrial systems are replaced by renewable biological resources with the European Commission defining this as the application of the CE concept to biological resources, products and materials [4–5]. It is also possible to look at this slightly differently and consider a CBE where non-renewable inputs are re-evaluated such that they are not replaced but redesigned such that they may become renewable to have a second life in delivering new products and materials.

The production of human monoclonal antibodies (MAb) for the treatment of chronic diseases has become the most substantial sector of the bioprocessing industry in the past decade, representing just over a quarter (27%) of all first-time biologic approvals from 2010 to 2014. This number rose to over half (53%) of all first-time approvals between 2015 and 2018. Production of these approved drug products increasingly relies on bioprocessing with mammalian cell lines. By 2018, 62 of 71 new biopharmaceutical active ingredients on the market were recombinant proteins, of which 52 (84%) are expressed in mammalian cell lines, with nine produced in microbial expression systems: Escherichia coli (five) or yeast (four) [6]. This has led to a significant intensification in activity of the bioprocessing industry globally with the consumption of millions of litres of cell culture media leading to the production of millions of tonnes of bioprocessing waste in a processing system, where once the drug product is extracted by affinity chromatography or related separation method, the liquid culture media is sent as waste to biokill containment. In 2018 over 16.5 million litres of active production capacity was estimated at more than 1,500 facilities worldwide including 6 million litres (37%) in the United States and Canada, 5.5 million litres in Western Europe (33%), and 4.7 million litres (25%) in the Asia–Pacific region, with a further 870,000 litres in China and 941,000 litres in India [7]. Approximately 10.2 million litres is based on the bioprocessing of mammalian cells primarily for MAb manufacture and nearly all using Chinese hamster ovary (CHO) host cells. At present the consignment of these millions of litres of cell culture media to waste immediately after the initial production of the drug product runs counter to the aims of the CBE, with a potentially valuable opportunity to investigate this waste stream, with its high water content and nutritional content being lost to containment as a hazardous material. The same systems biology approaches that have been employed to intensify the production of MAb products from CHO for example, may now be employed by researchers interested in developing the CBE to encompass the biopharmaceutical sector with a view to redesigning this waste as a valuable entity and converting this resource into a feed for new bio-based product manufacture.

2 Valorization of Waste with Bacteria

The successful application of systems biology approaches to the development of the CBE is exemplified by the engineering of the soil bacterium species *Pseudomonas putida*, which has been intensively developed through metabolic engineering strategies to take advantage of its robust nature to become a highly valuable tool for the conversion of waste streams to new value [8]. The manipulation of the genome of this organism through classical genetics approaches and more recently through the advent of genome editing technologies such as the CRISPR-Cas9 system has facilitated the suppression of undesirable characteristics and the amplification of properties that facilitate new biotechnology [9]. Genetic engineering of the metabolism of the Pseudomonas strain P. putida KT2440 is a particularly good example, resulting in the production of a wide range of target compounds of industrial value from native compounds such as polyhydroxyalkanoate for bioplastic manufacture (Fig. 1) to completely novel molecules [10]. The application of systems biology approaches in the past decade using new powerful analytic techniques for studying cellular metabolism, including accessing the central design elements of genomic/ transcriptomic/ proteomics as engineering instructions, have driven new efforts to modify the entire cell as well as cellular pathways used by the cell. This systems metabolic engineering opens up extraordinary possibilities in the use of bacteria for production of new products and also the use of waste streams as high value feed to generate these new products [11–12]. This synthetic biology knowhow driven by systems biology can support highly ambitious efforts such as the recycling of plastic waste, an enormous burden on the planet's ecosystem and an urgent problem for this and future generations [13]. Recycling of plastic and other non-conventional feedstocks arising from industrial production, including millions of litres of bioprocessing waste produced annually by the bioprocessing industry, is now not only of scientific interest but is demanded by the focus on developing the CBE.

3 Heterologous Expression of Recombinant Human Proteins Provides Alternative Waste Streams

In addition to the application of engineering strategies for recycling of environmental waste by bacterial species such as Pseudomonas, there is a long history of successful recombinant DNA engineering strategies that have led to the overproduction of human proteins for biotherapeutic application using the classical microbial 'cell factories' *Escherichia coli* and *Saccharomyces cerevisiae* [14]. Heterologous overexpression of recombinant human insulin for the treatment of diabetes was a major milestone in the use of DNA engineering. This process evolved from the initial production of each individual chain of the hormone protein in separate processes with subsequent chemical crosslinking to the production of the

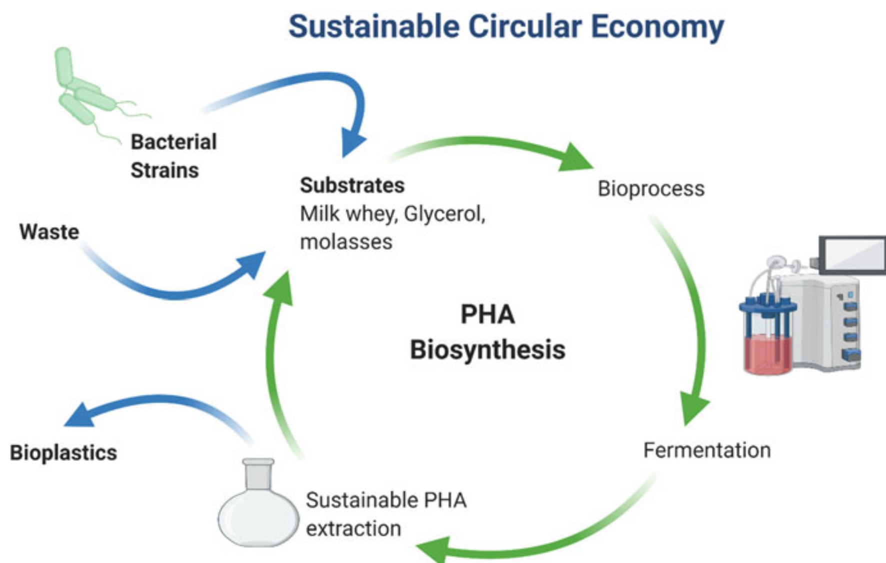

Fig. 1 Schematic diagram depicting the valorization of waste streams as feed for bacterial production of new products. Example shown is the production of the bioplastic polymer poly-hydroxyalkanoate (PHA) using bacterial strains such as Pseudomonas species. Created with BioRender.com

authentic human proinsulin molecule [15–16]. Manipulating the microorganism to produce a human hormone that regulates a chronic human disease was the launchpad of the modern bioprocessing industry which has quickly gained on the traditional pharmaceutical sector in terms of value since the early 1980s. *Saccharomyces cerevi*siae, with eukaryotic cell architecture and efficient folding and secretory pathways, has also been further engineered for the successful preparation of some of the world's biggest selling replacement therapies with short acting insulin lispro and long acting insulin detemir [17]. However for complex macromolecules such as immunoglobulin G, the cell factory of choice is undoubtedly the CHO cell.

3.1 Expression of Human Proteins in CHO Cells

Chinese Hamster Ovary cells have been the principle system for the expression of human macromolecules such as immunoglobulins over the past 30 years, with 6 of the top 10 selling or blockbuster MAbs produced by these cells in regulated bioprocessing systems (Table 1). Key features of CHO cell bioprocessing include (i) the ability to grow in suspension culture to very high cell density (ii) production of high titres of product. Growth in serum-free, chemically defined media is an important enabler in determining reproducibility across different batches of cultures

Table 1 The top 10 biotherapeutic MAbs by value in the period 2014–2017 and their method of production

Rank	International Non-proprietary Name	Cumulative sales 2014–17 ($ billions)	Approved	Company	Type	Cell line
1	Adalimumab	62.6	2002	Abbvie	IgG1	CHO
2	Infliximab	35.6	1998	J&J	IgG1*	Sp2/0
3	Rituximab	29.1	1997	Roche	IgG1*	CHO
4	Trastuzumab	27.1	1998	Roche	IgG1	CHO
5	Bevacizumab	27.0	2004	Roche	IgG1	CHO
6	Ranibizumab	14.3	2006	Roche	Fab	E.coli
7	Denosumab	11.6	2010	Amgen	IgG2	CHO
8	Nivolumab	11.4	2014	BMS	IgG4	CHO
9	Eculizumab	10.7	2007	Alexion	IgG2/4	NS0
10	Golimumab	9.7	2009	Merck	IgG1	Sp2/0

*Indicates the molecule is chimeric

and possesses a much better safety profile than in media containing human or animal-derived proteins [18–19]. The intensification of upstream processing in the production of drug substance with this cell type began with mutations to suppress deleterious gene mediated events such as apoptosis [20]. Further mutational analysis helped to improve the titre and quality of antibody secreted by the cells through the silencing of selected genes. The incorporation of mutations in the dihydrofolate reductase (dhf r) gene led to the production of two of the most widely used CHO cell lines in the biopharmaceutical industry, CHODXB11 (also known as CHO-DUKX) and CHO-DG44 [21]. This event coincided with arguably the most significant step forward in the evolution of the systems biology-led approach to intensification of CHO cell bioprocessing, the sequencing of the CHO-K1 cell line [22]. This and the later advent of genomic engineering technologies has paved the way for maximising the productivity of this non-human cell line through systematic engineering of pathways and genes to drive production of human biotherapeutic proteins at multi-gram per litre scales resulting in multi-tonne yields of product annually [11, 23].

3.2 Expression of Human Proteins in HEK293 Cell Culture

The human cell line HEK293 has been used extensively in research and development laboratories and is very often used for functional interaction screening between human proteins, however it is also widely employed for the production of vaccines, anticancer drugs, and other clinically relevant drugs. Transient transfection of HEK293 cells is the most commonly used method for the commercial production of recombinant adeno-associated viruses (AAV) [24–25]. In addition advances in cell line development technology has allowed for increased productivity with human

cell lines, leading to approved recombinant biotherapeutic products produced from HEK293 including agalsidase alfa for the treatment of Fabry disease and the first recombinant human coagulation factor VIII for the treatment of haemophilia [26–27]. Of particular interest during the ongoing COVID-19 pandemic is the production of new vaccines against this virus but also against other members of this virus family that cause SARS diseases. Research into the CoV spike receptor-binding domain in HEK293 cells has opened up the possibility of producing more effective immunogens that produce higher antibody titres and which can be produced in high yield bio process [28]. The versatility of this full human cell line model should see a significantly increasing volume of bioprocessing for human protein production in the coming decades.

3.3 Expression of Human Proteins in Non-mammalian Cell Culture

The Structural Genomics Consortium (SGC) is a group of laboratories in institutes and companies around the world that are focused on exploring less well-studied areas of the human genome. The baculovirus insect cell eukaryotic expression system was established in 2007 at the SGC as a measure to tackle challenging kinases, RNA-DNA processing proteins and integral membrane protein families [29]. The system has been used successfully with difficult to express proteins ranging from membrane bound proteins to complex enzymes that have proved intractable with other methods [30–31]. The success of this heterologous system is not confined to assisting with the resolution of crystal structures of proteins, but has been highly effective in the production of virus-like particles (VLPs) for the development of vaccines [19, 32].

The industrial scale of production of a growing array of proteins, from biotherapeutic drug molecules to VLPs, and structural genomics efforts using animal cell culture systems to eukaryotic insect cell expression systems, has led to a huge expansion of bioprocessing capability whether in small scale facilities for research and development or in the hundreds of thousands of litres capacity in bioprocessing facilities across the globe. This clearly opens up new opportunities into the research of how we should deal with this waste and identify new opportunities to use it as part of a circular bioeconomy.

4 Recycling of Waste Culture Media – Lessons from the Literature

A limited amount of research into reusing cell culture media has been explored for a variety of cell cultures, predominantly insect cell cultures, microalgae and yeast with a very small number of studies into reusing mammalian cell culture media. In

general, the results have varied in terms of the utility of the spent media depending on the cell type used, however the general consensus from these studies suggests that (i) the use of partial recycled media is beneficial in secondary culture systems and (ii) inhibitory effects on cell growth using recycled media can also be mitigated by addition of supplements, or by using additional treatment steps on the media prior to use.

4.1 Recycling of Yeast Culture Media

Yeast fermentation is a major biotechnology system used to produce a wide range of products from biotherapeutic proteins to commodities at very large scales such as alcohols, oils, and amino acids. Reusing spent medium would be of distinct benefit with regards to reduction of industrial costs of media and reduced consumption of water. One study reported a wastewater reduction of 70% by medium recycle [33]. Most yeast species appeared to be largely unaffected for long periods of time in recycled media. For example, a *Saccharomyces cerevisiae* immobilised culture in a matrix, with recycled media supplemented with nutrients continuously pumped through the matrix, facilitated cell growth and alcohol production at equivalent levels for up to 40 days, followed by inhibition of the culture due to a build-up of ions and polysaccharides after this time point [34]. Media recycling in this system provides a highly interesting premise for evaluation of other bioprocesses.

Another yeast fermentation system *Apiotrichum curvatum* producing microbial lipids in a chemically defined medium, was recycled with centrifugation, supplemented with carbon and nitrogen sources, and sterilized before testing with ratios of spent medium to fresh medium of between 0.25, 0.5 or 0.75 recycled medium. The results showed that recycled medium addition did not negatively impact growth or lipid production until after the 3rd subsequent recycling step, where lipid production and cell dry weight dropped [35]. The effect of ions building up in the media was investigated using an ion exchange matrix, that successfully restored the lipid production and growth to a state similar to fresh media for a further two recycling steps. Upon testing a second media, whey permeate media, they found similar results, with a drop in productivity after the 2nd recycling step. These results appear to indicate that cellular metabolites in spent medium do not inhibit growth of yeast cultures, but that ion concentration plays a large factor. These studies provide very useful signposts for the redesign of spent media such as chemically defined media used in CHO cell bioprocess, in particular the monitoring of ion concentration levels in spent media.

4.2 Recycling of Microalgae Culture Media

Microalgae cultures are often used to produce feed for animals and is today a potential candidate for biofuel production [36]. So it is interesting to note that microalgae cultures, like yeast, appear to survive in low density cell cultures in a recycled medium. Furthermore, doing so could save up to 75% water and 62% on nutrients [37]. The area of using recycled media with regards to heterotrophic algae culture has been comprehensively reviewed [38]. One of the earliest studies with media recycling in microalgae cultures was completed using cultures of *Chorella pyrenoidosa* [39]. This study used low cell density cultures (< 3 g per litre of biomass), where the media was supplemented with urea, iron and calcium and fed back into the culture. The culture was maintained for over 72 days, with no build-up of auto-inhibitory molecules seen. However more recent studies show that cultures over 3 g per litre in density show a decrease in biomass obtained [40–41] while studies that stayed below this density were mostly uninhibited [37, 39, 42].

The study of low cell density cultures of *Chlorella vulgaris* used a media designed by the authors for cell culture reuse, substituting any free ions in the medium with ammonium instead, a chemical which microalgae assimilate for a nitrogen source [37]. Use of this medium appeared to negate much of the inhibitory effects of using a spent medium on the culture, even in high-productivity cultures, compared to their control medium. This minimal growth medium had a lower ion concentration after recycling than the control did. They estimated they could save 75% of water and 62% of nutrients in this manner. In a second study by the same team, they tested how many days they could continue the culture with no negative impact on biomass [42]. Interestingly, they actually had increases in biomass for the first 10 days of recycling, and then the levels returned to the original culture's biomass level. As this was a low cell density culture (~1.5 g per L) they were unaffected by the growth inhibition previous studies had found at high densities. They maintained the same growth and biomass production over 62 days of recycling, an impressive feat, using their specially designed media.

In summary, the culture density and associated quantities of free ions and secreted components in the spent media, were seen to impact on the benefit of reusing spent media and informs the investigative criteria for new study design with animal cell spent media recycling.

4.3 Recycling of Insect Cell Culture Media

One of the most active areas of research into the recycling of spent cell culture media is with insect cell cultures. It has long been known that using a small amount of spent media to supplement fresh media has beneficial effects on insect and mammalian cell cultures, attributed to the secretion of beneficial growth factors into the media by the culture cells. The extent to which spent media can be used to elicit the best

growth response from these cultures is of particular relevance to a broader circular bioeconomy question. One of the earliest attempts at media recycling in insect cell culture immediately identified the presence of cytotoxic substances in the media which inhibited growth in most replicates studied [43]. They showed this in two separate insect cell lines, TN-368 and CP-169, but interestingly when the spent media were crossed over, the TN-368 spent media had no cytotoxic effect on the CP-169 cultures, leading to the hypothesis that the inhibitory substances may be cell line specific. Further assessment of various ratios of spent media to fresh media to test the growth of an insect cell line showed that the growth rate declined with increasing ratio of spent medium [44]. The spent media used in this study was from an eight-day culture and supplemented with 5% FBS. A 1:16 ratio of spent media to fresh media gave a growth rate similar to the fresh media alone, while 1:8 dropped by 8%, as did 1:5, and 1:1 gave a total reduction in growth rate of 67%. The effect of spent medium on protein production from insect cell expression systems was examined using two widely used cell lines, Sf-9 and High-Five, producing beta-galactosidase from E. coli [45]. Using the insect cell medium IPL-41, they investigated which supplements were optimal to add to the spent media by multiple combination experiments, whereby the optimal recipe was determined to contain yeastolate ultrafiltrate (8 g per L), glucose (2 g per L) and glutamine (8 mmol per L) plus 15–20% fresh medium. While their overall protein yield dropped, the biomass achieved was actually higher than the fresh media. The protein decrease could have been caused by a number of things, for example, it has been shown that ammonia accumulation occurs in spent media and has a negative impact on protein production by *Trichoplusia ni* cells [46]. The production of ion metabolites by cultures in spent media is again highlighted as an area of study by these findings.

Numerous studies have been completed attempting to determine not just the detrimental constituents of the spent media, but also the growth-promoting factors that may be present. Metalloproteinases were determined to be the only factor in spent media that was responsible for any and all growth-promoting effects in *Trichoplusia ni* cell cultures [47]. They tested this with conditioned media (spent media added), and in which they had inhibited metalloproteinases. None of the beneficial growth effects were seen in this culture, but when metalloproteinases were added, the benefits were restored. Since it has been shown that metalloproteinases are secreted into the media by insect cells and break down growth factor binding proteins, it was hypothesised by this group that metalloproteinases are necessary to release the growth factors in the spent media from their binding proteins [48–50]. Binding proteins of IGFs (insulin-like growth factors) have been found in the recycled mediums of the cell lines Sf-9 and High Five insect cells, which must therefore be broken down by secreted metalloproteinases in order to release the IGFs, which can then perform their growth-promoting function [49]. Cell cycle progression has also been found to be impacted by these autocrine secreted factors, further contributing to the beneficial effects of spent medium recycle [51].

Ex-Cell medium was examined for reuse possibilities using the High-Five insect cell line producing beta-galactosidase from *E. coli* [52]. Media was filtered through a 0.22 micron filter and supplementation with 0%, 25% and 50% spent

media volumes were used to test effects on protein production. Production of beta-galactosidase increased in the 25% spent media test compared to the 0% control. Similar findings have been observed with a *Lepidopteran* Se301 cell line. Various percentages of spent media mixed with fresh media were tested, with measurement of culture growth rates and volume of baculovirus production, and it was found that these were only negatively affected when above a 60% spent media composition [53]. Most interestingly, there was actually a significant effect on baculovirus production at around 20% spent media composition, with a 90% increase in production compared to the fresh media control.

Taken together, it is obvious that using at least partial medium recycling has a beneficial effect on protein production in insect cell lines. It also does not appear to inhibit growth unless above a high ratio of spent media to fresh (>60%). These results were very instructive for this heterologous expression system, with conditioned media (recycled media) routinely added as a small percentage of the total culture volume as a result of these types of studies. These different cell systems also highlight that with further investigation into the composition of spent media there is significant potential to develop new methods to utilise the waste media streams from the bioprocessing of animal cell cultures.

4.4 Recycling of Mammalian Cell Culture Media

Mammalian cell culture is highly sensitive to extracellular metabolites and while recycling of cell culture media has been attempted, it has had very limited success. Early studies into recycling animal cell culture media witnessed a significant drop in cell growth from the first recycling attempt of 100% spent media [54]. In follow up studies, a partial medium recycle was proposed to mitigate some of the inhibitory effects on growth. Using a mouse hybridoma cell line and harvesting spent DMEM F-12 medium from cells producing an IgG like antibody, cells were grown in 80% spent medium for two subsequent recycling steps [55]. The first step did not see a significant drop in protein produced, at 29 mg/L compared to the control of 33 mg/L, but it did drop substantially for the second, at 18 mg/L. It was concluded that there is a greater negative effect of cytotoxic metabolites than positive effects from growth factors in the spent medium from mammalian cells at these high levels of spent media usage.

A later study proposed a smaller ratio of spent media of 63% to 37% fresh medium, which could possibly be used in subsequent recycling steps. Using mouse hybridoma cells over four subsequent recycling steps were tested with serum-free media with nutrient supplementation. Interestingly, these cells demonstrated a slight adaptation to the recycled medium, where the first and second had lower antibody production levels, which then recovered somewhat in the third and fourth. By the end of the recycling steps, 112 mg/L of protein yield was obtained, compared to just one round of fresh media giving 63 mg/L, highlighting a potentially useful benefit of media recycling in mammalian cells for the first time [56]. Spent media that had

been run through a Protein G affinity column, was used in the culture of mouse hybridoma cells producing an IgG like protein. A percentage of the "recovered" media was employed, whereby spent media was concentrated 12-fold and then diluted 1:8 with fresh media. This approach was beneficial, with 60% recovered media giving a 33% increase in protein production compared to the fresh media [57]. The spent medium was proposed to contain multiple beneficial factors as well, though the mammalian cells appear more sensitive to high concentrations of spent medium than the insect cells. Some of these positive factors have already been identified, such as anti-apoptotic factors that some mammalian cell types secrete [58–59] or secreted growth factors such as platelet-derived growth factor (PDGF) [60].

For spent media from animal cell culture to prove valuable as a recycled waste stream to feed new bioprocesses, it will be necessary to carefully examine the chemical and protein composition of the media when recovered from the primary bioprocess. The identification of positive and negative growth factors and the requirements for supplementation will require careful empirical experimentation. The secondary bioprocess chosen to use this spent media will also require careful biochemical assessment to build a sustainable and valuable platform to harness the spent media waste to generate new value in the form of secondary bioprocessed products such as other recombinant proteins. One such organism that represents an excellent candidate to harness spent chemically defined media from mammalian cell bioprocess is *Escherichia coli*. It has been shown that the growth of *E. coli* is eventually inhibited by the production of acetic acid in culture and it has also been shown to produce much less acetic acid when growing in a chemically defined media, where carbon source is not in excess [60]. This type of fundamental understanding of the metabolism of one of the best studied 'microbial cell factories' along with the key signposts provided by previous studies in a range of other heterologous systems represents the blueprint for the applied study of animal cell culture waste for reuse in microbial fermentation as just one example (Fig. 2).

5 Analysis of Chemically Defined Spent Media

Utilising spent media as a potentially valuable waste stream requires thorough analysis of its composition. Many of the studies mentioned previously used multiple methods of cataloguing the various known nutrients within the recycled media which may have been depleted, or even the unknown cellular components left behind in such a media.

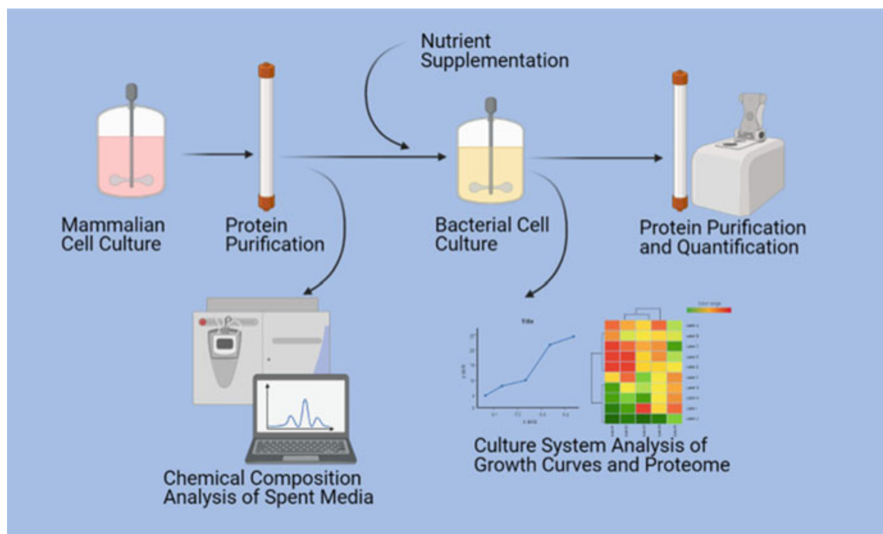

Fig. 2 Redesigning spent media from cell culture bioprocess to feed new bacterial fermentations. Harnessing a systems biology approach to analysis of spent cell culture media, fermentation conditions and protein production. Created with BioRender.com

5.1 *Metabolomics*

A benefit of using a chemically defined culture medium is that the concentration of nutrients within the media is known prior to consumption by cell cultures. It is therefore possible to analyse the remaining concentrations of these components to determine how much of each was consumed by the culture. This can be achieved through numerous analytical methods, principally Gas Chromatography (GC) and High-Performance Liquid Chromatography (HPLC). In the study of spent media effects on antibody production in mouse hybridoma cells, the free amino acids and glucose concentration in the spent media was analysed using HPLC, thereby finding the exact concentration of each which was required to be supplemented back into the media [56]. HPLC is a method best used to detect levels of a known component of the media, as you need to know its behaviour on a chromatographic peak, most usually achieved by running against known standards . While it can be used to detect the presence of impurities (unknown components), it cannot identify these components unless you know their properties in advance. Gas chromatography can be used in much the same way as HPLC to identify chemical components of the media that have known profiles [61]. When used in combination with mass spectrometry GC-MS can very sensitively identify metabolic patterns in culture media [62]. Glucose uptake can also be measured a number of ways, including using radiolabelled glucose analogues [63], or even using simple colorimetric assays [64]. The concentration of glucose, or other carbon sources, is known in defined

mediums prior to use, so it is therefore feasible to find the concentration remaining in the media for supplementation purposes. Certain known proteins of known molecular weights can be tested using simple visual methods such as SDS-PAGE for an approximation of quantity based on band intensity, as was used to test for the depletion of transferrin in the spent media, a nutrient necessary for cultures producing an antibody like IgG [57].

5.2 Proteomic Analysis of Unknown Components

In many of the studies conducted into spent media usage, there were unknown growth-promoting or inhibiting factors within the media which impacted on further cell growth. As extracellular secretions are generally proteins, it is possible to analyse the media for the presence of these unknown proteins using mass spectrometry. Analysis of spent media from iPSC-derived neuronal cells to study the cell's secretome in Parkinson's models, using just 0.5 ml of spent media facilitated identification of over 500 proteins contained in the media [6]. Testing of a variety of preparation methods found that using media containing BSA hampered the identification of proteins by a 10-fold difference when compared with using B27-free media (contains no commercially added proteins). They also found that acetone precipitation was the most effective enrichment technique, yielding the most proteins, followed by TCA (trifluoracetic acid) precipitation. Mass spectrometry has been used to analyse secretomes for the presence of proteins in other disease models as well, such as metastatic cancers [65].

Proteomic analysis with peptide identification software, e.g., MaxQuant™, after mass spectrometry analysis is a critical component of these studies [66]. From this software, a file called "proteinGroups.txt" is generated with all of the information regarding identities and quantities of proteins in the data in tab delimited format. From here, the data can be analysed using an analysis software such as Perseus [67], or a bioinformatician may design their own code to gather relevant data from the file, such as the protein pathways significantly represented. Statistical analysis such as student's t-tests is then performed to check differences in protein concentration between samples to a level of significance (P-value <0.05). For this purpose, the UniProt ID mapping tool is invaluable, as lists of identifiers can be input to return most information about the proteins as an easily parsed tab delimited file, such as each protein's molecular weight, the pathway analysis, and whether it is a metabolite with catalytic or anabolic properties. All the information can be easily expressed in graph formats using Python's Matplotlib library, or R, with many good online resources for learning how, such as on GitHub, DataCamp, Rosalind, or StackOverflow among many others. Most of these courses are free and are a good use of free time during a pandemic, particularly if statistics is something you use often in your data, not just for proteomic or genomic data analysis.

5.3 Chemical Composition Analysis of Unknown Components

Some unknown components left behind in a spent media by cell cultures are not proteins, and therefore not detectable by mass spectrometry. These are generally carbohydrates, or lipids. There are certain analyses that can be run on these components to detect functional groups. For example, if testing for presence of short chain fatty acids such as acetic acid, gas chromatography or a carboxylic acid analyser can be used to estimate if any is present, and based on what functional groups it may contain, what the identity of that acid may be. Detecting unknown carbohydrates is more difficult and will need multiple tests to identify different functional groups present that can then help narrow down the possibilities, such as Benedict's test or an Iodine test.

While it is entirely possible to find the concentrations of known components in a spent medium and to identify unknown proteins, the difficulty lies in identifying unknown carbohydrates and lipids, and generally relies on functional group tests to find possible matches. Luckily, with the current advances in genome and proteome analysis, most cell lines secretome is very well studied and spent media components can therefore mostly be identified through mass spectrometry, SDS-PAGE and HPLC/GC.

6 Future Considerations for Developing CBE Research in the Bioprocessing Sector

Heterologous expression of recombinant human proteins in animal cell cultures at all scales, from investigative projects in research institutes to the production of drug substance by the biopharmaceutical industry, poses challenges and opportunities to create new value from the spent culture media waste stream that is being produced in ever increasing quantities globally. To participate in and help grow this opportunity these stakeholders will need to be encouraged to design new research strategies and new research funding schemes at one level, and will need to be incentivised to participate in the design of new policies with the aim of stimulating new practice by the global bioprocessing industry at another. There are sufficient precedents in the scientific literature that highlight the potential of turning waste from the bioprocess of cells into new value as part of a circular approach to changing the description of single use culture media to renewable media. The application of a systems biology led approach to the study of spent culture media and its components, and to the secondary culture systems that can utilise these waste streams as feed, has exciting potential to generate new value and place the bioprocessing field onto the Circular Bioeconomy map.

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