Continuous Biomanufacturing with Microbes - Upstream Progresses and Challenges

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Abstract:

Current biomanufacturing facilities are mainly built for batch or fed-batch operations, which are subject to low productivities and do not achieve the great bioconversion potential of the rewired cells generated via modern biotechnology. Continuous biomanufacturing should be the future directions for high-yield and low-cost manufacturing of various fermentation products. This review discusses the major challenges and the strategies for continuous biomanufacturing with microbes, which include minimizing contamination risk, enhancing genetic stability over a long-term continuous operation, achieving high product titer, rate, and yield (TRY) simultaneously by decoupling cell growth from product formation, and using modeling approach to accelerate research and development of continuous biomanufacturing. New strain designs and process engineering strategies including integration with artificial intelligence are also discussed for intelligent and the next generation of continuous biomanufacturing.

Introduction

Biomanufacturing is to use a biological system that consists of enzyme(s), microorganisms, or more advanced biological cells to make fuels, chemicals, nutraceuticals, pharmaceuticals, or other value-added products from renewable resources [1]. Since 1910s, biomanufacturing has evolved through four generations, and today, biomanufacturing 4.0 focuses on new products that address the most critical challenges in energy crisis, health issues, food security, climate changes and sustainability [2]. For most biomanufacturing processes, fermentation in a bioreactor is the core unit, where the raw materials are converted into a target product with the catalysis by enzyme(s) or biological cells. Since 1950s batch or fed-batch fermentation process has been the standard design and practice for biomanufacturing of fuels, chemicals, and high-value products due to its advantages in simple bioreactor structure and design, easy scale-up, and flexibility for different products. However, too much down time between different batches and inability of keeping at optimum production rate in the entire batch or fed-batch process significantly limit the overall productivity, which leads to increases in both capital and operating costs [3]. At the same time, significant progresses have been made in synthetic biology, metabolic engineering, and protein biochemistry. Thus, conventional batch and fed-batch technologies from 70 years ago are far behind the fast pace of modern biotechnology, and more importantly, do not achieve the true conversion potential of these rewired cells. There is an urgent need for fully controlled continuous biomanufacturing processes to make biofuels, commodity chemicals, and high-value products at significantly lower manufacturing cost.

In the past a few decades, continuous fermentation has been widely studied to significantly increase the productivities and reduce the capital investment and biomanufacturing cost. A wide range of fermentation products have been studied by using continuous processes, which include (1) biofuels such as ethanol [4–6] for gasoline and lipids for biodiesel [20], (2) commodity chemicals such as acetone-butanol-ethanol (A-B-E) solvents [8, 9], lactic acid (LA) [10, 11], succinic acid [12, 13], and 1,3-propanediol (PDO) [14,15], and (3) high-value molecules such as omega-3 fatty acids [16] and recombinant proteins [17, 18]. Recently, the prospect and potential of continuous manufacturing is beginning to be embraced in the biopharmaceuticals sector, especially for production of monoclonal antibodies (mAbs) by using mammalian cells [19, 20]. However, most current fermentation products are still manufactured via batch or fed-batch processes at commercial scale. To make continuous biomanufacturing a new standard practice in near future, a series of technology breakthroughs and innovations in both strain design and process engineering are needed to address the major technical challenges (**Figure 1**).

This review focuses on upstream progresses and major challenges in continuous biomanufacturing for products derived from bacteria, yeast, and microalgae. First, an overview is given for current status of continuous biomanufacturing of several representative fermentation products from different organisms.

After that, the major challenges that limit the successful application of continuous biomanufacturing at commercial scale is analyzed, followed by a further discussion on strategies to address or overcome these challenges. Future perspectives toward high-yield and low-cost continuous biomanufacturing at large scale are also discussed.

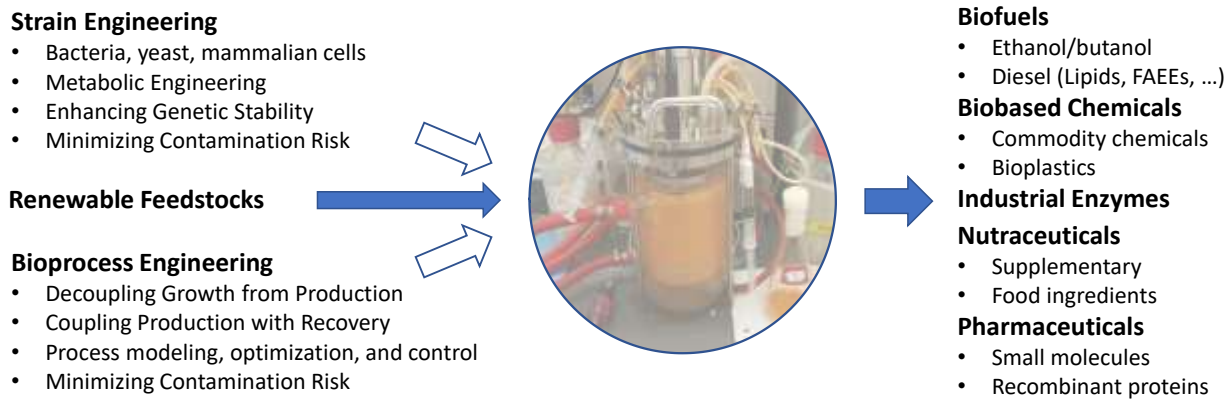

Figure 1. General strategies for designing a continuous biomanufacturing process

Current status and major challenges

Upstream continuous biomanufacturing has been studied for most categories of fermentation products such as ethanol, A-B-E solvent, lactic acid, succinic acid, 1,3-propanediol, recombinant proteins, and monoclonal antibodies (mAbs) that cover the applications in fuels, commodity chemicals, materials, specialties, and pharmaceuticals. However, continuous biomanufacturing is still not a preferred choice for current commercial production. A comparison of typical production parameters (titer, rate/productivity, and yield) for major fermentation products between batch/fed-batch and continuous fermentation processes for bacteria, yeast, and microalgae is summarized in **Table 1**.

Table 1. A comparison between the batch/fed-batch and continuous processes for biomanufacturing of several major fermentation products.

Product	Strain	Typical Outcomes (Titer, Rate, Yield) *						Reference
		Batch/Fed-Batch			Continuous			
		Titer	Rate	Yield	Titer	Rate	Yield	
Ethanol	*S. cerevisiae* and *T. italicus*	116 g/L	2.4 g/L/h	0.41 g/g	60-106 g/L	1.3-13 g/L/h	0.36-0.44 g/g	[4,21]
A-B-E: Acetone, Butanol, Ethanol	*Clostridium acetobutylicum*	18 g/L (A+B+E)	0.40 g/L/h (A+B+E)	0.27 g/g (A+B+E)	~10 g/L (A+B+E)	~0.90 g/L/h (A+B+E)	~0.23 g/g (A+B+E)	[9,22]
Lactic acid	*Bacillus coagulans* A534	88-94 g/L	1.8-2.8 g/L/h	0.85-0.90 g/g	~50 g/L	7.4-9.9 g/L/h	0.7-0.8 g/g	[10, 11]
Succinic acid	*Actinobacillus succinogenes*	64 g/L	1.1 g/L/h	0.76 g/g	33 g/L	11 g/L/h	0.90 g/g	[12]
	Escherichia coli KJ 134	50-61 g/L	0.35-0.45 g/L/h	0.94 g/g	~18 g/L	~0.87 g/L/h	0.62-0.77 g/g	[13]
1,3-Propanediol	*Klebsiella pneumoniae*	56 g/L	2.3 g/L/h	0.56 g/g	35-49 g/L	4.9-8.8 g/L/h	N/A	[14,15,23, 24]
Lipids – triacylglycerides (TAGs)	*Acutodesmus obliquus* (microalgae)	4.4-5.1 g/L	0.15-0.17 g/L/d	0.16-0.20 g/mol photon	0.56-0.68 g/L	0.54-0.64 g/L/d **	0.14-0.16 g/mol photon	[7]
Recombinant protein - PAmCherry	*E. coli* BL21(DE3)	8.4 g/L	0.12 g/L/h	0.075 g/g	1.2 g/L	0.15 g/L/h	0.022 g/g	[25]
Recombinant Inclusion Body	*E. coli* BL21(DE3)	1.5-4.0 g/L	< 0.20 g/L/h **	0.06-0.16 g/g	6.1 g/L	~0.24 g/L/h **	0.013 g/g	[26]

* "Titer" and "Rate" also refer to "concentration" and "productivity", respectively, for fermentation product in other literatures.

** Data are estimated based on the titers and actual fermentation time in the reference (for fed-batch, including the time before induction; for continuous, the total residence time in all reactors).

In general, continuous processes significantly improve the productivity or production rate, but the gain in productivity or rate is often accompanied by loss(es) in product titer, specific titer (product titer/cell density), conversion yield, or other related fermentation parameters. Specific features for the selected continuous fermentation product examples are summarized below:

Biofuel – Ethanol: Continuous fermentation improved ethanol productivity by > 50%, but decreased ethanol titer by nearly half [4,21]. A recent study conducted with 62 Brazilian distilleries (51 batch and 11 continuous) in the past nine years showed that the continuous processes failed to improve the overall ethanol productivity due to frequent contaminations [5]. Lignocellulosic biomass, corn or corn stovers, and sugar canes, syngas can all be used as the feedstock for continuous production of ethanol and other fuels [6]. Antibiotics usage could reduce or minimize the contamination frequency, but it would increase cost and cause concerns about leaking antibiotics to the environment during the application. Further, antibiotic in ethanol beverage would be a health concern.

A-B-E (acetone-butanol-ethanol) Solvent: Continuous fermentations integrated with other novel technologies are being developed for ABE production [8]. Most integrated continuous fermentation processes achieved a production of total ABE with titers between 10~20 g/L, yields of 0.2~0.4 g/g, and productivities less than 1 g/L/h. Since butanol is extremely toxic to the *Clostridia* cells, solvent extraction has been used for in-situ product removal (ISPR) to significantly relieve the toxicity of the produced butanol and enhance the total butanol and ABE production. A two-stage continuous fermentation process with cells immobilized in both stages and an ISBR unit for each stage improved the total ABE titer to 25 g/L [9]. However, the complexity of the process and the relatively low productivity still do not allow for a successful application at large scale.

Lactic acid – Monomer for biodegradable polymer polylactide (PLA): Continuous fermentation improved LA productivity by 3 folds, but with > 40% decrease in LA titer and > 10% decrease in conversion yield [10]. By increasing cell retention time using a cell recycling unit, cell density and LA productivity were further improved [11]. This increased LA productivity by > 4 folds over the batch fermentation, however, was also accompanied by decreases in LA titer by 24% and yield by 17%, respectively [12].

Succinic acid – Precursor for polyesters: Succinic acid (SA) is listed by the US DOE as one of the 12 top platform chemicals that can be produced from microbes, especially the engineered *Escherichia coli* [13]. Continuous fermentation basically doubled the SA productivity over the batch fermentation, but it also decreased the SA titer by nearly 70% and the yield by 20-30% [13].

1,3-Propanediol – Precursor for polytrimethylene terephthalate (PTT): PDO can be produced from glycerol by the bacteria *Klebsiella sp*., *Citrobacter sp*., or *Clostridium sp.* [14]. Continuous fermentation of *K. pneumoniae* has improved PDO productivity by 3 folds over the fed-batch fermentation, but it decreased PDO titer by 20-30% and the yield by up to 15%. DuPont and Genocor have successfully engineered *E. coli* to produce PDO from glucose at significantly higher titers (> 135 g/L), productivities/rates (> 3.5 g/L/h), and yields (up to 0.51 g/g) in fed-batch fermentation experiments [15]. As of yet, there is no report of using this engineered *E. coli* for continuous PDO production.

Recombinant proteins: Recombinant proteins, such as α-amylase, β-galactosidase, cutinase, and many therapeutic proteins, have a broad range of applications from biocatalysis to pharmaceutical development. Recombinant proteins are produced by genetically modified microorganisms. Often, the recombinant DNA encoding the target protein is contained in a plasmid and expressed under a consecutive or inducible promoter [17]. In general, plasmid stability, contamination risk control, premotor selection and optimization, culture environment and physiology change are the major challenges hindering adoption of continuous fermentation processes for recombinant proteins. The use of inducible promoters such as the T7 promoter in *E. coli* allows for the unique design using a two-stage or multi-stage continuous reactor system, which enables the spatially decoupling cell growth from protein production [26]. The use of the two-stage continuous fermentation has improved the recombinant protein production by 100 folds [26].

In addition to the fermentation products from microbes, pharmaceuticals such as monoclonal antibody (mAb) from continuous biomanufacturing with mammalian cells has also been well studied [19]. Though continuous cultivation of mammalian cells is not a focus in this review, similar results were observed, i.e., continuous cultivations give higher mAb rates but lower titers as compared to typical fed-batch processes [19, 20]. Furthermore, the downstream recovery and purification processer for mAb are also similar to those for recombinant proteins from microbes [20].

Based on the progress made so far, the following major challenges still remain for developing the next generation of upstream continuous biomanufacturing for products derived from microbes:

(1) Contamination risks: It is always a challenge to keep contamination-free for a long-term continuous fermentation process (up to several weeks or months).
(2) Genetic instability: In a long-term continuous fermentation, cells may evolve to grow faster but produce less product, which can be caused by plasmid loss or mutations.
(3) Decoupling product formation from cell growth: Most of the current or past continuous fermentation processes have difficulties in simultaneously maintaining high product titer, rate (i.e., productivity), and yield (TRY), as shown in Table 1. One major reason is that cell growth and product formation in a

single-stage continuous fermentation are coupled, while they actually prefer different optimal medium and process conditions.

(4) Lack of efficient research tools for continuous biomanufacturing: Continuous fermentation experiments are time-consuming and labor-intensive. It can take a long period to reach a steady state, and the culture should be still monitored daily. Efficient research methods or tools are missing to accelerate the continuous process development.

(5) Disconnection between product formation and recovery: The importance of downstream recovery has not been well addressed for continuous fermentation processes. It is desired that product recovery coupled to the continuous fermentation be designed with the capability to handle flow and concentration variations.

Strategies to overcome major challenges in upstream continuous biomanufacturing

Contamination risk control

From the very beginning, contamination risk has been one of the major factors that restricts the application of continuous biomanufacturing at large scale. Junker et al [27] have summarized their pilot-facility experience during 1989 to 2004 for various industrial fermentation products. In general, the fermentations with yeast have relatively lower contamination rates (0.93~2.2%), as compared to those with *E. coli* (7.6%), filamentous bacteria (10.2~16.9%), fungal (11~22.9%), and animal cells (16.1%) [27]. The continuous feeding of substrates and the long-term operations significantly increase the chance of contamination by other bacteria from the feeding sources, gas supplement lines, or the environment.

Alcohols such as ethanol were one class of fermentation products that have demonstrated early the feasibility of continuous production at large scale. This could be attributed to the addition of antibiotics in the fermentation culture and the lower culture pH values, which are not suitable for most bacterial growth [27]. Since the yeast strains are resistant to many antibiotics, adding antibiotics such as penicillin and tetracycline to the fermentation medium or the feed solution has become a standard practice for enhanced productivities of many alcoholic fermentation processes for non-beverage applications [28,29].

Lactic acid bacteria are the major contaminant in bioethanol fermentation, which compete with the yeast strains for sugar substrates and micronutrients and produces LA [30]. Adding antibiotics may not be able to completely stop the contamination [31]. Though LA bacteria can also tolerate certain lower pH values, developing more acid-tolerant yeast strains could be considered to control the bacterial contamination for fermentation without pH control [32]. Compared with the LA bacteria, acetic acid bacteria are less commonly seen, but the contamination of acetic acid bacteria is a much more problematic obstacle to

overcome [33]. Adding NaCl and ethanol may help control both types of bacterial contamination levels. In addition to the methods of using antibiotics or chemical reagents, new bioreactor design, such as the immersed membrane bioreactor (iMBR) to separate yeast from bacterial cells, can also be used to control or minimize the contamination risk [34].

Enhancing genetic stability of production strain

Genetic instability, indicated by gradually decreased production capability and sometimes increased cell growth rate, is another major challenge that restricts the application of a continuous fermentation at large scale. In general, the fermentation conditions during the production phase are less favorable for cell growth due to product/byproduct toxicity, which reduces growth rates. Therefore, genetic mutations may be induced to a shift of the metabolic pathways from what are designed for product formation to those in favor of cell growth under more stressful/harsh conditions. The risk factors for genotype changes include the genetic mutations in chromosomes and/or plasmid loss. A recent review summarized the genotype-directed strategies for leveraging DNA-sequencing data to guide the new strain design, which include deleting insertion sequence (IS) repeats and coupling or overlapping the product formation genes with the essential genes required for cell growth or primary metabolism [35]. In addition, a new platform with a two-vessel continuous flow system and reduced genome bacterial strains was also developed to significantly extend the continuous fermentation period with stable performance [US Patent 10,604,736B2].

Escherichia coli is one of the most commonly used microorganisms that can be engineered for various biomanufacturing products due to its fast growth rate, well-established knowledge and tools for metabolic engineering, and relatively low contamination risk. Genetic instability is the major barrier that limits the application of continuous fermentation with an *E. coli* strain [36]. Strategies such as parallelization, cascade processing, reducing the genome size [37], and population controls have been tried to address the challenge, but it is still tricky to maintain a stable productivity in a long-term continuous bioreactor [36]. Most *E. coli* strains express the target gene in transformed plasmids with antibiotic or auxotrophic selection markers, but partial or complete loss of plasmid copy numbers are seen during the propagation process, which leads to decreased productivities. If ColE1-like plasmids containing a synthetic human interferon-gamma (hIFNγ) gene under controllable promoters are used, turning-off transcription of the hIFNγ gene helped stabilize the ColE1-like plasmids [38]. Constitutive gene expression and larger plasmid size may also cause the segregational plasmid instability [39]. For the yeast *S. cerevisiae* harboring plasmid DNA for gene expression, using low-copy-number plasmids such as pGAC9 may minimize the plasmid loss issue [40].

Our recent experience showed that manipulation all gene edits in chromosome significantly improved the strain stability. For example, the oleaginous yeast *Yarrowia lipolytica* was introduced 30 copies of 9

different genes for biosynthesis of omega-3 EPA from glucose [3]. The strain was then used for steady-state production of omega-3 EPA in a 6-week continuous fermentation process [16].

Decoupling cell growth from production by multi-stage continuous fermentation

Similar to chemical manufacturing that uses catalysts to convert petrochemical feedstocks into chemical products, biomanufacturing uses microorganisms as biocatalysts via the fermentation process to convert substrates such as sugars derived from renewable feedstocks into desired products. A typical fermentation process starts with a growth stage to accumulate enough cell mass as biocatalyst, followed by a production stage to convert substrate(s) into product(s). This two-stage concept makes it necessary to develop a dynamic metabolic control (DMC) strategy, which enables the decoupling of microbial growth from product formation [41]. Cell growth and product formation are each controlled under their own optimal conditions so that competition for limiting substrates can be avoided and/or accumulation of growth-inhibiting products or intermediates is unnecessarily a concern [36]. Therefore, a maximum product titer, rate, and/or yield can be achieved. This two-stage concept has well been adopted in fed-batch fermentation processes. For example, using an aerobic growth of *Lactobacillus* and anaerobic production led to 156 g/L lactate [42]. A two-stage pH control profiles for separate growth of *Klebsiella pneumoniae* and production of 2-ketogluconic acid led to a final product titer of 186 g/L [43].

Most current continuous fermentation designs use a single-stage chemostat due to its simpler design and operation. In a single chemostat, the growth and production occur together, which results in significantly lower product titer, rate and/or yield. In our recent study on production of omega-3 eicosapentaenoic acid (C20:5, EPA) with the metabolically engineered *Y. lipolytica* [16], a two-stage continuous fermentation was developed, which was equipped with a smaller growth bioreactor (Stage 1) and a larger production bioreactor (Stage 2) to decouple cell growth and omega-3 EPA production. Compared with the fed-batch process, the continuous fermentation improved the overall productivity by 80% and the titer by 40%, while maintaining a similar conversion yield. A similar two-stage continuous fermentation system was also developed for production of ethanol from syngas, which consisted of a 1-L stirred tank reactor for cell growth and a 4-L bubble column equipped with a cell recycle module for ethanol production [44]. The cell recycle unit allowed maintain cell densities to be maintained up to 10 g DCW/L. The maximum ethanol titer of 21 g/L was achieved at a rate of 0.37 g/(L·h).

Higher productivities achieved in multistage continuous fermentation (MCF) processes can be attributed not only to the decoupling cell growth and production, but also to the privileged reaction engineering principle from MCF itself. In chemical reaction engineering, a plug flow reactor (PFR) can be approximated by multiple continuous stirred-tank reactors (CSTRs) connected in series. For any reactions where production rates are positively impacted by the concentration of reactant(s), a PFR is preferred to a CSTR.

Many fermentation processes belong to this reaction category, *i.e.*, the bioethanol production rates benefit from relatively higher substrate concentrations. Therefore, the MCF system, which approximates PFR, achieves higher product titers and yields than a single-stage CSTR (or called a chemostat). For example, the yeast *S. cerevisiae* in a five-stage continuous fermentation system was able to produce ethanol > 130 g/L when the first stage reactor was supplied with 320 g/L glucose [45].

Using modeling tools to accelerate continuous process development

For a continuous fermentation process, it takes a long period to reach a steady state (about 4 or 5 residence times) after a new condition (such as the dilution rate and/or the substrate concentration in feed) applies to the continuous fermentation system. Therefore, it is time-consuming and labor-intensive to rely only on experiments to determine the optimal conditions. Mathematical modeling can be used as a powerful tool to accelerate the process development of a continuous fermentation without the need of a large number of optimization experiments. In general, the fundamental understanding of the microorganism and metabolic pathways and the first principles of the fermentation process can be obtained from batch or fed-batch experiments. The specific kinetic parameters determined from batch/fed-batch data can then be used to model continuous fermentations. After that, only a limited number of continuous fermentation experiments are needed to validate and recalibrate the continuous fermentation model. The model can be eventually used to guide process optimization, control, and scale-up for an advanced continuous fermentation (**Figure 2**).

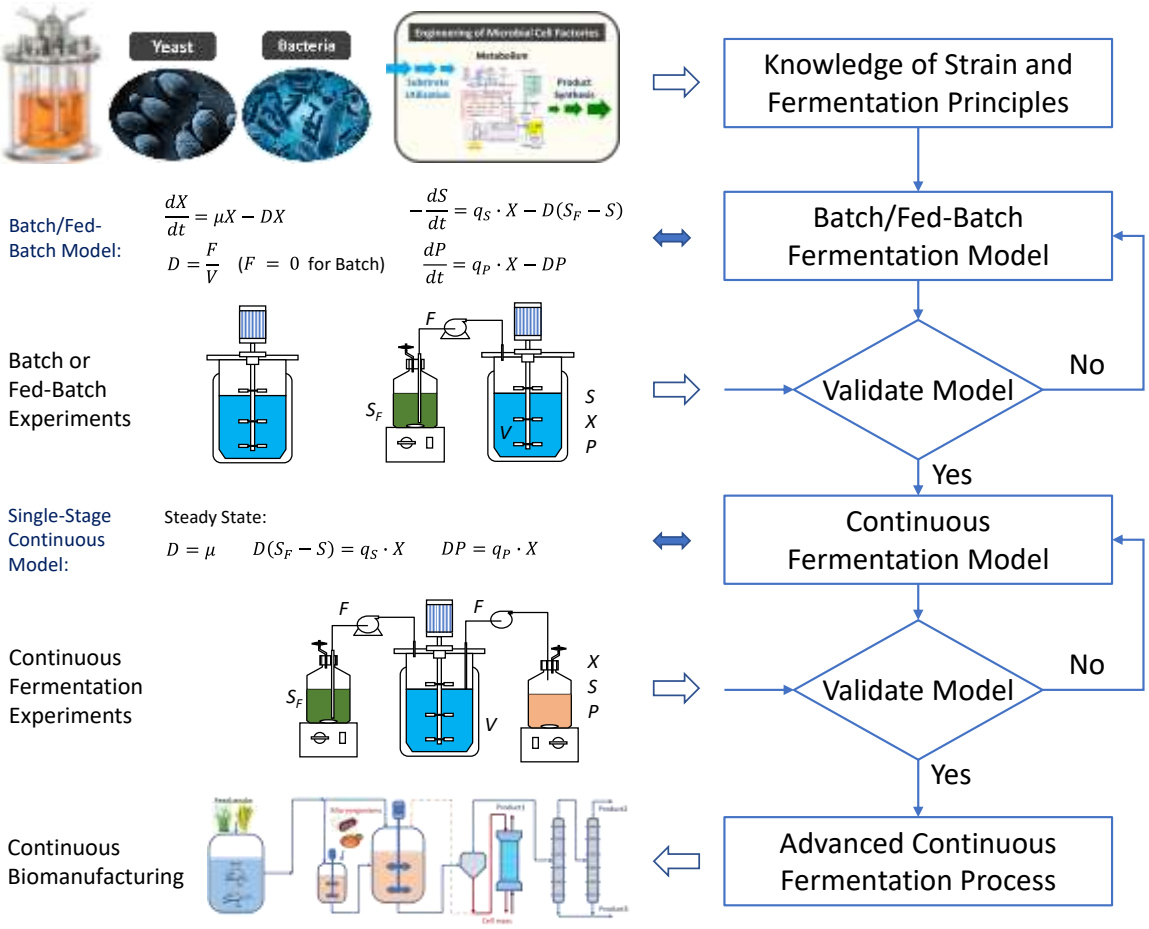

Figure 2. General strategy to use models to assist or guide continuous fermentation process development. Abbreviations: D – dilution rate, F – feed rate, S – substrate, P – product, V – reactor volume, X – cell density, μ - specific growth rate, q_S - specific substrate consumption rate, q_P - specific product formation rate.

Modeling is extremely helpful in optimizing the continuous fermentation with complexities in media and process design. Wang et al. [11] developed models for lactic acid production by continuous fermentation under conditions of different media, strain of *Enterococcus mundtii* QU 25, process with a cell recycle unit, and various cell retention time. The model successfully guided the continuous fermentation to achieve production of lactic acid at 65 g/L with a productivity of 13 g/L·h. Modeling approach has been widely adopted to develop continuous fermentations for ethanol production. For example, Esfahanian et al. [46] used a genetic algorithm to model ethanol fermentation in both a continuous conventional bioreactor (CCBR) and a continuous membrane bioreactor (CMBR), which was used to determine the range of dilution rate to have the CMBR outperform a CCBR. Herrera et al. [47] developed a mathematical model to simulate and analyze a four-stage continuous ethanol fermentation system with cell recycle unit at industrial scale. The model simulation results successfully helped develop an Infinite-Horizon Model Predictive Control (IH-MPC) strategy, which was capable of maintaining the outlet sugar concentration of the fourth reactor at a desired value by manipulating the feed flow rate, where there were fluctuations in sugar concentration in the feed stream. Ariyajaroenwong et al. [48] developed a kinetic model for ethanol production from *S. cerevisiae* immobilized on sweet sorghum stalks in a continuous fermentation system. Taking together, these model examples have demonstrated a promise of using similar models to assist and/or guide the design and optimization of continuous fermentation system for other microbial systems.

Future Perspectives

As more advanced scientific research tools and methods become available in the field of industrial biotechnology and many other science and engineering areas, continuous biomanufacturing will eventually replace the current batch or fed-batch biomanufacturing as the major method for producing fuels, specialty and commodity chemicals, novel materials, nutraceuticals, and pharmaceuticals in future. The following continuing efforts should be undertaken to realize the ambition:

***Unique strain design and engineering for continuous biomanufacturing*:** The success of continuous biomanufacturing will depend not only on innovations in bioprocesses, but also on unique design and engineering of strains that have the following features:

- **Switchable growth and production:** Decoupling cell growth and product formation might be beneficial in continuous fermentation by methods such as inducible promoters that can be switched on and off for a controllable transition between cell growth and production phases [49,50].
- **Increased genetic stability:** This can be achieved by strategies that (1) use low-copy, more stable plasmid [40], (2) manipulate gene editing in the chromosome [16], (3) delete insertion sequence (IS)

repeats [35], and/or (4) couple the product formation genes and the essential genes required for cell growth or primary metabolism [35].

- **Reduced contamination risk:** Developing strains with tolerance for organic acids [51], osmotic stress [52], and/or higher temperature could enhance the anti-contamination capability [53]. For bacterial strains, developing an anti-phage defense system would be helpful [54].

***Using economical feedstocks for continuous biomanufacturing*:** The raw material expense may contribute to more than half of the total biomanufacturing cost for products such as biofuels and commodity chemicals. New strains and processes should be developed to use more economical and/or unconventional feedstocks:

- **Efficient utilization of sugars derived from lignocellulosic biomass**: Strains capable of using C5 and C6 sugars are desired. There has been much progress in engineering strains for utilizing C5 sugars, such as xylose [55]. However, more research efforts are still needed to have these sugars metabolized at similar efficiencies and conversion yields. Also, a significant portion of sugars (7-10% of glucose and 12-20% of xylose) may be in oligomeric forms and cannot be efficiently fermented [56]. New strategies for co-expressing of gluco- and xylo-oligomers degrading enzymes should be considered in the production strain such that all sugar monomers and most oligomers can be consumed.

- **Alternative feedstocks for continuous biomanufacturing**: Many other alternative feedstocks are available for fermentation, which include waste agricultural residues, degraded waste plastics [57], waste food ingredients [58,59], or waste gases [60]. Furthermore, about half or more carbon is wasted as CO_2 during a fermentation process, it would be desired to fix the waste CO_2 into C1 or C2 chemicals such as formic acid, acetic acid and ethanol via electrochemical conversion process [61]. The strain should be engineered to use C1 and C2 chemicals so that they can be brought back to the continuous fermentation as co-substrate to minimize the carbon loss [62,63].

***Coupling production and product recovery in continuous fermentation*:** In addition to the efforts in decoupling cell growth from product formation through the design of two-stage or multi-stage continuous fermentation, an ideal continuous fermentation process should also couple product formation and product recovery. As shown in **Figure 3**, the cells produced in the last stage of the continuous fermentation could be recycled back to the fermentation or the harvested cell mass could be used for extraction of the intracellular product (Product 1, such as proteins and lipids). Extracellular products (Product 2, 3, …) should be continuously removed, recovered, and/or purified through a series of separation units. This integrated process will allow downstream purification and relieve potential product inhibition/toxicity in the fermentation with an in-situ product removal (ISPR) unit.

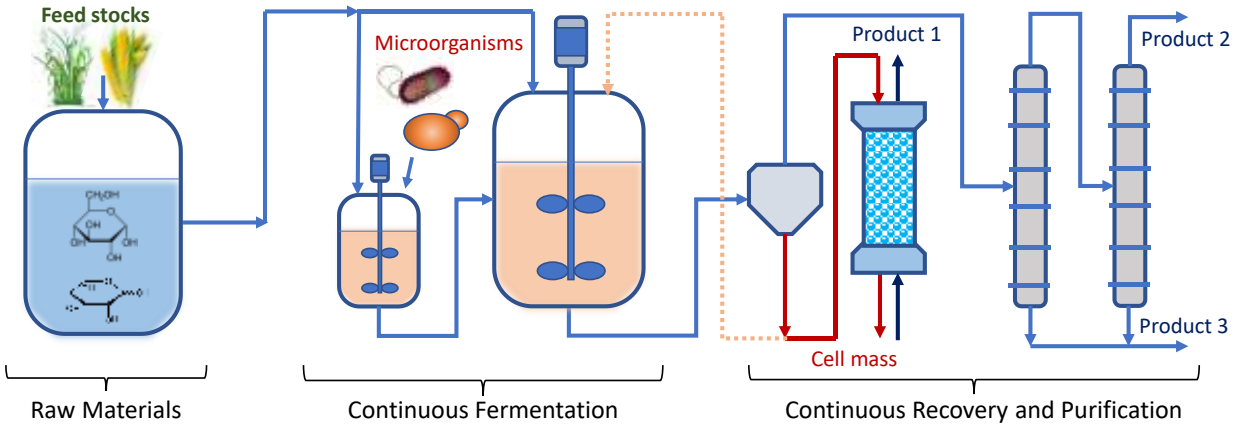

Figure 3. A conceptual continuous biomanufacturing process that decouples cell growth from product formation and couples the fermentation and product recovery.

***Intelligent Continuous Biomanufacturing*:** Artificial intelligence (AI) and machine learning have recently been applied to biomanufacturing [64]. Integration of AI into continuous fermentation process will allow (1) identification of critical factors impacting the fermentation performance, (2) application of real-time feedback control, and (3) maintaining process reliability with timely adjustments and controls for variations or fluctuations from strain growth characteristics, medium/feed ingredients, and process operations. AI modeling techniques and tools such as interpretable modeling [65], representation learning and Graph Attention Networks [66], reinforcement learning (RL) [67], and deep reinforcement learning (deep RL) [68] can be used to increase efficiency, robustness, reliability, and yield with significantly lower manufacturing cost. Continued research is still needed to determine the most appropriate AI tool(s) for continuous biomanufacturing.

Conclusion

Continuous biomanufacturing has been widely investigated for various fermentation products, but has never become a major manufacturing option in industrial biotechnology. Most of current biomanufacturing facilities are built as batch or fed-batch mode, which is not able to achieve the true bioconversion potential of the rewired biological cells via modern biotechnology. Often, continuous biomanufacturing is able to significantly enhance a fermentation product's volumetric productivity, but fails to maintain cell's specific productivity and yield. The major upstream barriers or technical challenges for applying continuous biomanufacturing include (1) contamination risk in long-term continuous operation, (2) genetic instability/mutation in continuous culture, (3) incapable of simultaneously maintaining optimal conditions for both cell growth and product formation, (4) lack of efficient tools and methods for research and

development on continuous biomanufacturing. To address these challenges, unique strategies for strain design and fermentation engineering should be developed to minimize contamination risk, increase genetic stability, and maintain high titer, rate, and yield (TRY) simultaneously. Modeling approach could be a powerful tool to accelerate research and development of continuous biomanufacturing. In addition to novel strains, processes capable of using alternative economical feedstocks should be explored. Finally, the integration of AI and computer model have the potential to aid continuous biomanufacturing towards more sustainable, robust, reliable, and intelligent processes in future.

Conflict of interest statement

Nothing declared.

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Reference

* reference of special interest

** reference of outstanding interest

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