ABS

Review

Continuous Bioprocessing for Recombinant Protein Production in *Bacillus subtilis*: Opportunities and Challenges

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Abstract

The Gram-positive bacterium *Bacillus subtilis* has long been used for industrial protein production owing to its well-characterised genetic background, capacity for high-density fermentation, and effective protein secretion. With the growing global demand for recombinant proteins across various sectors, there is an increasing need for more efficient and sustainable protein production methods. This review explores the application of continuous bioprocessing for recombinant protein production using *B. subtilis*, critically evaluating current technologies and discussing their potential for integration into streamlined, integrated biomanufacturing framework. We primarily focus on transgenic *B. subtilis*, however the solutions for continuous fermentation and protein isolation/purification are the same for both endogenous and heterologous (i.e., recombinant) proteins.

Keywords

Continuous Bioprocessing; *Bacillus subtilis*; Upstream Processing; Downstream Processing; Recombinant Protein Production

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Kontinuirno bioprocesništvo za proizvodnjo rekombinantnih proteinov v *Bacillus subtilis*: priložnosti in izzivi

Izvleček

Po Gramu pozitivno bakterijo *Bacillus subtilis* že dolgo uporabljamo v industrijski proizvodnji proteinov. K temu prispevajo njena dobra raziskana genetska zasnova, možnost doseganja visoke biomase med fermentacijo in učinkovito izločanje proteinov. Zaradi naraščajočega svetovnega povpraševanja po rekombinantnih proteinih v različnih sektorjih, se povečuje tudi potreba po učinkovitejših in trajnostnih metodah proizvodnje proteinov. V članku predstavljamo potencial kontinuirnega bioprocesništva pri proizvodnji rekombinantnih proteinov z uporabo *B. subtilis*. Ponujamo pregled obstoječih tehnologij in njihovega potenciala za vključitev v integrirani koncept učinkovitejše bioproizvodnje rekombinantnih proteinov. Čeprav se prispevek primarno posveča transgenim bakterijam *B. subtilis*, so rešitve kontinuirne fermentacije in izolacije/čiščenja proteinov enake za endogene in heterologne (tj. rekombinantne) proteine.

Ključne besede

Kontinuirno bioprocesiranje; *Bacillus subtilis*; pripravljalni procesi; zaključni postopki; proizvodnja rekombinantnih proteinov

Introduction

The demand for recombinant proteins is rapidly increasing, driven by their expanding applications in healthcare, agricultural and material sector. While the expanding biopharmaceutical market remains a key driver, the need for recombinant enzymes, bio-pesticides, bio-stimulants and bio-based materials is also steadily rising, encouraging the development of efficient and scalable production strategies (Evens and Pharm, 2022; Kergaravat et al., 2025; Marrone, 2024). To meet this growing demand, the bioindustry is actively exploring strategies for process intensification. Continuous processing has already demonstrated its ability to boost productivity and reduce turnaround times across various industries. Accordingly, the bioindustry is shifting towards continuous manufacturing to improve productivity and ensure consistent product quality, while also reducing environmental impact (Jungbauer et al., 2024; Peebo and Neubauer, 2018). Importantly, the motivation for adopting continuous biomanufacturing is not solely economic. Regulatory bodies have recognized several advantages of continuous processing that contribute to improved product quality. Maintaining a steady-state operation is crucial for ensuring consistent quality, while the elimination of interruptions between process steps enhances both the reliability and safety of manufacturing (Godawat et al., 2015; Subramanian, 2017; Walther et al., 2015). In the bioindustry, the cultivation of microorganisms is a critical step in protein production (Yang et al., 2021). In this context, *Bacillus* species represent an attractive platform due to their high secretion capacity and ability to produce a range of recombinant proteins at high titers, supporting the needs of the expanding market. This paper focuses on *Bacillus subtilis*, a microorganism long employed for producing diverse proteins, enzymes, and various chemicals (Lopez et al., 2009). The aim is to review approaches in upstream and downstream processing for recombinant protein production using *B. subtilis* and to explore the potential of continuous biomanufacturing as a solution for process intensification.

Bacillus subtilis as a Host for Recombinant Protein Production

Escherichia coli remains the most widely used prokaryotic host for recombinant protein production. However, *B. subtilis* is widely used in industrial homologous protein production and its popularity for recombinant protein expression is increasing (Errington and van der Aa, 2020). Classified as Generally Recognised as Safe (GRAS), *B. subtilis* offers several advantages: a well-characterised genetic

framework, suitability for high-density fermentation, lack of lipopolysaccharides, and an inherent ability to secrete proteins (Yang et al., 2020, 2021). Numerous strategies, including selection of parental strains and strain engineering, have been used to enhance recombinant protein production in *B. subtilis*. (Zhang et al., 2020a). The initial step is to select a parental strain that shows promising characteristics in terms of its genetic background. This is followed by strain engineering, a process that is essential for meeting the demands of modern recombinant protein production (Zhang et al., 2018). One of the major challenges is that heterologous proteins are generally more susceptible to proteolytic degradation than native proteins. This vulnerability arises primarily from slower folding kinetics and an increased tendency for misfolding after membrane translocation (Li et al., 2004). Two main strategies have been proposed to mitigate these issues. The first focuses on improving the secretory protein-folding environment, while the second aims to reduce extracellular proteolytic activity (Zhang et al., 2020a). Regarding the first approach, *B. subtilis* naturally produces intracellular chaperones that assist in the folding of nascent polypeptides and prevent the formation of intracellular protein aggregates – processes that have been identified as potential bottlenecks in recombinant protein production. Advances in genome editing technologies have enabled the generation of multiple *B. subtilis* variants in which secretory pathways and signal peptides have been optimized, and key intracellular chaperones have been characterized and overexpressed (Zhang et al., 2020b). The second approach targets extracellular proteolysis. *B. subtilis* secretes seven extracellular proteases, each capable of degrading proteins at different cleavage sites, often contributing to nutrient recycling. By precisely modulating protease expression levels and activities, researchers have significantly improved the yield of extracellular recombinant proteins (Zhang et al., 2018).

Upstream Processing

Upstream processing represents a critical phase in each biomanufacturing operation and has specific characteristic when using *B. subtilis* as a host. Unlike *E. coli*, *B. subtilis* naturally secrets many proteins into the medium, avoiding the need for cell lysis (Chen et al., 2024). Generally, three primary modes of upstream processing are distinguished: batch, fed-batch, and continuous (Subramanian, 2017) with various sub-variants, such as repetitive fed-batch processes developed to bridge the gap between traditional fed-batch and fully continuous upstream processing through semi-continuous strategies (Kopp et al., 2020). All these approaches are applicable to *B. subtilis*. However, their implementation must consider organism-specific challenges, including oxygen transfer rates due to the aerobic nature of *B. subtilis*, as well as the control of sporulation and protease activity during cultivation (Chen et al., 2024). Despite differences in operation, all three modes share a common initiation process. Each begins with a working cell bank (WCB) aliquots revival carried out in shake flasks, followed by seed expansion in a bioreactor and, ultimately, inoculation of the production bioreactor. The major distinctions between batch, fed-batch, and continuous processes emerge during the production phase itself (Subramanian, 2017). A brief comparison of these three operational modes is provided in the following section.

Batch mode

In batch processing, the bioreactor is filled with growth medium up to the final working volume, inoculated, and processed until completion. The process is monitored online using integrated sensors, and regular sampling is performed for in-process analysis (Subramanian, 2017). For *B. subtilis*, batch production typically spans 1-2 days (Ji et al., 2015). When the bioprocess is complete, the culture is transferred to downstream processing. Manufacturing is carried out through successive batch operations, with the product accumulated at the end of each cycle.

Fed-batch mode

In fed-batch processing, the bioreactor is initially filled with growth medium to approximately 65-90% of the final working volume, followed by inoculation. Feeding with nutrient supplements is then initiated based on predefined criteria and continues until the final working volume is reached. As with batch mode, the process is monitored online through integrated sensors, and samples are collected for in-process analysis, including product yield, cell density and biochemical profiling (Subramanian, 2017). For *B. subtilis*, fed-batch production typically lasts between one and four days, depending on the feeding strategy employed (Klausmann et al., 2021). After the bioprocess concludes, the culture is transferred to the downstream processing.

Like batch mode, production in fed-batch mode is achieved through successive, repeated cycles.

Continuous mode

In continuous processing, the bioreactor is initially filled to 40-50% of the final working volume and inoculated. Once the target cell density is reached, a continuous dilution process is initiated, involving the steady feeding of fresh medium while simultaneously harvesting culture at the same flow rate (Subramanian, 2017). The system is maintained at a steady state, ensuring a constant cell density and a constant working volume throughout the operation. For *B. subtilis*, continuous bioprocesses typically run for five to ten days (Kittler et al., 2025). Culture containing the desired product is continuously harvested and sent directly to downstream processing, running in parallel with upstream operations.

Continuous Upstream Processing

Historically, conventional fed-batch bioprocesses have been the industrial standard for *B. subtilis*, primarily due to their operational robustness and high space-time product yields (Zhang et al., 2020b). However, technological advances now support continuous processing, and model-based studies suggest that continuous cultivation of microorganisms can achieve similarly high, and sometimes superior production rates. The primary advantage of a continuous bioprocessing lies in the ability to maintain cells in a steady state, enabling sustainable and consistent protein production (Kittler et al., 2025; Subramanian, 2017). For example, continuous cultures of *B. subtilis* have demonstrated the capacity to produce recombinant proteins at stable, high levels over extended periods. One study reported that continuous cultivation-maintained chloramphenicol acetyltransferase production at relatively high level, with stable protein expression observed at a dilution rate of 0.2 h^{-1} (Vierheller et al., 1995). In contrast, batch and fed-batch processes often experience fluctuations in nutrient concentrations and perturbation of cellular metabolic states, leading to variability in both protein yield and quality. While fed-batch processes allow for controlled nutrient addition, they still face challenges such as accumulation of inhibitory by-products and undesirable metabolism shifts. Despite optimization, fed-batch processes for *B. subtilis* continues to encounter issues related to oxygen limitation and by-product accumulation (Öztürk et al., 2016).

Nonetheless, continuous bioprocessing is not without challenges. Prolonged cultivation times increase the risk of contamination, and maintaining genetic stability over extended periods is critical. Loss of plasmids, spontaneous mutations, and genetic drift can lead to significant reductions in productivity (Csörgo et al., 2012; Croughan et al., 2015). Thus, the development of a robust expression system is imperative for successful continuous protein production. Recent studies have further nuanced this discussion. For instance, a study with *Bacillus lichenioformis* reported that an optimized fed-batch process outperformed a continuous process in terms of productivity (Kittler et al., 2025). Therefore, while continuous upstream processing can enhance sustainability and process consistency, it typically demands more sophisticated control systems and may result in lower per-cycle productivity compared to highly optimized fed-batch operations.

Downstream Processing

Downstream processing (DSP) aims to eliminate impurities and product variants while preserving the integrity of the final product (Jungbauer et al., 2024). When using *B. subtilis* for recombinant protein production, DSP benefits from the organism's natural capacity to secrete proteins into the extracellular environment, which avoids the need for cell lysis in many cases and simplifies the clarification step (Chen et al., 2024). However, *B. subtilis*-specific challenges include the removal of secreted host-cell proteins, notably extracellular proteases that can degrade the product. Therefore, the implementation of protease-deficient strains could be critical to preserve product integrity (Zhang et al., 2018). A streamlined biomanufacturing workflow relies on the integration of various technologies, including cell lysis, filtration, chromatography, refolding, precipitation, and extraction (Jungbauer, 2013; Jungbauer et al., 2024). The following section provides a brief general overview of key the technologies used in the context of continuous downstream protein processing while assessing their relevance for *B. subtilis*-based recombinant protein production.

Cell lysis

When the product is expressed intracellularly, the first step of DSP is typically cell lysis, which ruptures the cell membrane to release the intracellular components. High-pressure

homogenisation is the most widely used industrial method due to its high efficiency and robustness. In this method, a piston pump generates high pressures, leading to slit cavitation around the valve area. The sudden release of the pressure causes effective cell breakage. Modern high-pressure homogenisers are capable of operating in continuous mode, enabling integration into continuous biomanufacturing workflows (Subramanian, 2017). In the production of recombinant proteins with *B. subtilis*, the product is most often found extracellularly. Therefore, cell lysis is generally bypassed in *B. subtilis*-based processes unless targeting specific intracellular product (Yang et al., 2021).

Centrifugation

Centrifugation separates solids from liquids and is a crucial step in DSP. In *B. subtilis*-based recombinant protein production, this operation primarily used for clarification of the fermentation broth by separating the biomass from the extracellularly secreted target proteins (Patel et al., 2019). The most common centrifuge designs are the tubular, chamber and disk-stack centrifuge. Among these, the disk-stack centrifuge is particularly suitable for pseudo-continuous operation, allowing the transient removal of liquid and solid phases during processing (Jungbauer, 2013).

Filtration

Filtration plays a vital in DSP, supporting tasks such as cell-liquid separation, protein concentration, and buffer exchange. Filtration processes are classified into microfiltration, ultrafiltration and nanofiltration, distinguished by their pore sizes. Microfiltration, with the largest pore size, is used for cell and cell debris separation, whereas ultrafiltration and nanofiltration are commonly employed for protein concentration and buffer exchange. For *Bacillus* cultures, the high tendency for foaming and the presence of DNA fragments can increase the viscosity of the broth, requiring adapted filtration strategies or precipitation approaches to ensure cell debris and DNA removal, respectively, during continuous processing (Prabakaran and Hoti, 2008). Continuous filtration is an established technique in biomanufacturing and can be achieved by connecting multiple filtration units in series, where the permeate from one unit feeds into the next enabling uninterrupted flow. Engineering strategies for implementing continuous filtration have been comprehensively reviewed (Jungbauer, 2013).

Chromatography

Chromatography is often an indispensable step in DSP. Conventional packed-column chromatography operates in batch mode, with sequential loading, washing, and elution steps. However, significant research efforts have focused on developing continuous chromatography methods to improve productivity, purity, cost efficiency, and equipment footprint (Subramanian, 2017; Vogel et al., 2002). Depending on the desired purity of the final product, multiple chromatographic stages (i.e. purification train) may be implemented, utilising various physical and chemical differences between proteins to achieve a higher level of purity (Liu et al., 2010). The simplest form of continuous chromatography involves operating multiple columns in parallel, where columns alternate between loading, washing, elution, regeneration, and re-equilibration phases. This rapid cycling approach is already widely applied in the bioindustry. Additionally, alternative technologies such as rotating chromatography devices or counter-current chromatography have been developed to minimise equipment needs and streamline continuous operations (Jungbauer, 2013). When applying these strategies to *B. subtilis*, careful optimisation of chromatographic conditions becomes essential due to its high secretion of endogenous proteases and other host proteins, which can co-elute with the target recombinant proteins, requiring tailored purification strategies to achieve the desired purity levels (Shih et al., 2013).

Extraction

Liquid–liquid extraction (LLE) has emerged as a promising alternative to chromatography-based purification and has been extensively studied over recent decades (Subramanian, 2017). LLE relies on the differential partitioning of target products between the two immiscible aqueous phases. For example, phase-separating mixtures of polyethylene glycol (PEG) or dextran with appropriate salts can be used to achieve selective protein separation. Several continuous LLE methods have been developed and successfully applied for protein separation (Kee et al., 2021; Muniasamy et al., 2022). Since *B. subtilis* translocate recombinant proteins in the extracellular space, LLE can be applied directly to fermentation broth, enabling in situ or integrated extraction approaches (Kee et al., 2021).

Integration of Upstream and Downstream Processing

Current trends in the bioindustry are increasingly shifting toward integrated continuous bioprocessing. Efforts are focused on developing solutions at every stage of production to realise the fully integrated continuous bioprocessing concept (Jungbauer et al., 2024), as illustrated in Figure 1. However, literature regarding the continuous production of recombinant proteins using *B. subtilis* remains limited. Nevertheless, several studies have explored integrative approaches with *B. subtilis* for enzyme production, providing useful insights into potential strategies for continuous recombinant protein manufacturing. The following paragraph presents various applications of integrative approaches for protein production using *B. subtilis*. Kee et all (2021) implemented an in situ extractive approach for the production and recovery of *B. subtilis* xylanase. In their study, crude fermentation broth was directly fed to a biphasic (alcohol/salt) flotation unit during the bioprocess. The authors demonstrated that gas bubbles facilitated the partitioning process, with xylanase being retained in the upper alcohol phase. Muniasamy et all (2022) combined *B. subtilis* fermentation with in situ liquid-liquid extraction of a secreted fibrinolytic protease (FLP). In this process, shrimp waste hydrolysate served as a sustainable, low-cost substrate, while the FLP enzyme was concurrently extracted in a micellar two-phase system. Additionally, gel chromatography was used to quantify the amount of recovered enzyme, followed by final purification through anion exchange chromatography to achieve maximum purity and enzyme activity. Ng et all (2018) reported the direct extraction of xylanase enzyme from fermentation broth with alcohol/salt aqueous systems. Two earlier studies also highlight that interest in continuous biomanufacturing has existed for several decades. Stredanský et all (1993) demonstrated the simultaneous production and purification of *B. subtilis* α-amylase. Their bioreactor contained an aqueous two-phase system composed of PEG and dextran. During fermentation, culture medium was transferred to an external settler; following phase separation, the dextran-rich bottom phase, containing cells, was returned to the bioreactor, while the PEG-rich upper phase was pumped to an affinity column where the amylase was bound. The PEG flowthrough from the column was then recycled back into the bioreactor. Cooper et all (1981) developed a large-scale continuous bioprocess for the production of surfactin, a cyclic lipopeptide surfactant. In their system, surfactin accumulated in the foamed broth within the bioreactor, and the foam was collected in an external separator for product extraction via acid precipitation.

Challenges and Future prospects

In the bioindustry, continuous processing is the dominant approach for several applications, including wastewater treatment, composting, and specific bioenergy processes such as biogas and bioethanol production (Brethauer and Wyman, 2010; Matassa et al., 2016; Subramanian, 2017).

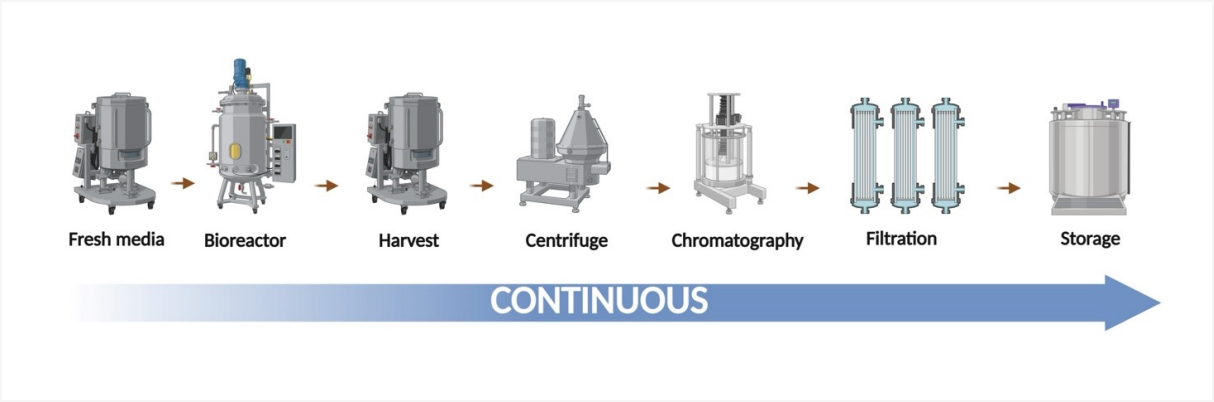

Figure 1. Fully integrated continuously manufacturing platform concept. Created in BioRender.

Slika 1. Koncept popolnoma integrirane platforme za neprekinjeno proizvodnjo. Ustvarjeno v BioRender.

Nevertheless, most industrial bioprocesses still operate in batch or fed-batch modes, with fed-batch currently being the predominant production strategy. Despite the potential advantages of continuous processing, several organism-specific challenges must be addressed for recombinant protein production in *B. subtilis* including control of sporulation and protease activity during prolonged cultivations, maintenance of genetic stability, and adaptation of oxygen transfer systems due to the aerobic nature of the microorganism (Chen et al., 2024). Strategies such as the use of sporulation-deficient strains, targeted protease knockouts, and optimized feeding strategies could help mitigate these limitations while supporting steady-state productivity (K. Zhang et al., 2018, 2020a). Furthermore, the integration of synthetic biology tools for strain stabilization, combined with advanced process monitoring under Quality-by-Design framework, may facilitate the consistent quality and yield of recombinant proteins during continuous production. In DSP, continuous strategies such as counter-current chromatography, continuous filtration, and integrated LLE systems are emerging to complement continuous upstream operations. These approaches can reduce buffer consumption, equipment footprint and overall processing times while increasing productivity and cost efficiency. However, implementing continuous DSP for *B. subtilis* remains challenging due to the complexity of fermentation broth and the secretion of host proteins that may co-elute with target products, requiring careful optimisation, as well as real-time monitoring and control. While challenges remain, ongoing advances in synthetic biology and process optimisation hold significant promise for facilitating the transition of the bioindustry towards continuous biomanufacturing.

Author Contribution

Conceptualization, A.I. and T.B.; investigation, A.I.; writing—original draft preparation, A.I.; writing—review and editing, A.I. and T.B.; supervision, T.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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